Abstract

Chiral ligand exchange chromatography (CLEC) separates enantiomers of alkaloids, amino and carboxylic acids, barbiturates, β-blockers and other adrenergic drugs. It relies on subtle energetic differences between ternary homo- and hetero-chiral complexes formed between a ligand capable of chelating a divalent transition-metal ion and an enantiomer. CLEC separation efficiency is strongly dependent on column operating conditions, including pH, temperature, mobile-phase composition, and feed composition. Each enantiomer participates in a large number of solution and stationary-phase complexes within the column. As a result, the mechanism of separation is complex and poorly understood, making it difficult to identify optimal column operating conditions using conventional empirical strategies.

A new model for CLEC-based separations is presented that provides a molecular understanding of the separation process. It combines the non-ideal equilibrium dispersion model of chromatography with multiple chemical equilibria theory to accurately predict enantiomer transport and partitioning, elution band profiles, and separation efficiency over a wide range of permissible column operating conditions. Mass transport parameters are determined by moment analysis and used to show that solute mass transfer and binding is limited by pore diffusion during separation of α-amino acid racemates on a Nucleosil Chiral-1 column (bearing a L-hydroxyproline as the chiral selector) or of dopa enantiomers on a Chirex 3126 column (bearing a derivative of D-penicillamine as the chiral selector). As a result, the local equilibrium approximation can be applied at all standard column operating conditions. Stoichiometries and formation constants for all equilibrium complexes formed in the column are taken from standard thermodynamic databases or independent potentiometric titration experiments.
Model performance is assessed through comparison with chromatograms for hydrophobic amino-acid racemates loaded on a Nucleosil Chiral-1 CLEC column. The model is then applied to a medically relevant separation: the resolution of dopa enantiomers on a Chirex 3126 CLEC column. In both cases, the model is shown to provide an accurate and detailed picture of the separation process useful for elucidating the mechanism of separation and the associated influence of key column operating variables on speciation profiles. Finally, the model is successfully applied to a restricted optimization of column operating conditions for the separation of D,L-valine, indicating that it may provide a rapid and comprehensive path to process optimization.
Table of Contents

Abstract ................................................................................................................. ii
Table of Contents ............................................................................................... iv
List of Tables ......................................................................................................... vii
List of Figures ....................................................................................................... ix
Nomenclature ...................................................................................................... xiv
Dimensionless Numbers ...................................................................................... xv
Greek Letters ...................................................................................................... xvi
Acknowledgments .............................................................................................. xvii
Dedication ............................................................................................................ xviii

1 Introduction, Background and Thesis Objectives .............................................. 1
  1.1 Thesis Overview .......................................................................................... 1
  1.2 Routes to Pure Enantiomer Production ....................................................... 6
    1.2.1 Preferential and Diastereomeric Crystallization .................................... 7
    1.2.2 Chiral Chromatography ........................................................................ 8
    1.2.2.1 Chiral Cavity Phases ...................................................................... 10
    1.2.2.2 Antibiotic Phases ......................................................................... 12
    1.2.2.3 Protein Phases ............................................................................. 12
    1.2.2.4 Polymer Phases ........................................................................... 13
    1.2.2.5 Charge Transfer (Pirkle) Phases .................................................... 15
    1.2.2.6 Ligand Exchange Phases .............................................................. 15
  1.3 Chiral Ligand Exchange Chromatography .................................................. 17
    1.3.1 Chiral Stationary Phases ................................................................. 17
    1.3.1.1 Covalently Grafted CSPs ............................................................ 18
    1.3.1.2 Physically Immobilized CSPs ....................................................... 19
    1.3.2 Chiral Mobile Phase Additives .......................................................... 20
    1.3.3 Medical Applications of CLEC ......................................................... 21
  1.4 Characterization and Modeling of CLEC ..................................................... 21
  1.5 Research Objectives .................................................................................. 26
  1.6 Tables .......................................................................................................... 29
  1.7 Figures ......................................................................................................... 30
  1.8 References ................................................................................................. 34

2 Formation Constants and Coordination Thermodynamics for Binary and Ternary Complexes of Copper (II), L-Hydroxyproline and an Amino-Acid Enantiomer ........................................ 49
  2.1 Introduction ............................................................................................... 49
  2.2 Materials and Methods ............................................................................. 52
    2.2.1 Materials ......................................................................................... 52
    2.2.2 Potentiometric Titrations ................................................................. 52
    2.2.3 Data Regression .............................................................................. 54
  2.3 Results and Discussion .............................................................................. 57
    2.3.1 Protonation Constants ..................................................................... 57
    2.3.2 Concentration Formation Constants for Binary Complexes .......... 59
<table>
<thead>
<tr>
<th>2.3.3</th>
<th>Concentration Formation Constants and Thermodynamics for Ternary Complexes</th>
<th>64</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4</td>
<td>Conclusions</td>
<td>69</td>
</tr>
<tr>
<td>2.5</td>
<td>Tables</td>
<td>70</td>
</tr>
<tr>
<td>2.6</td>
<td>Figures</td>
<td>75</td>
</tr>
<tr>
<td>2.7</td>
<td>References</td>
<td>81</td>
</tr>
</tbody>
</table>

3 Interpreting the Effects of Temperature and Solvent Composition on Separation of Amino-Acid Racemates by Chiral Ligand-Exchange Chromatography .............................................................................. 86

| 3.1   | Introduction                                                               | 86 |
| 3.2   | Experimental                                                              | 90 |
| 3.2.1 | Chiral Ligand Exchange Chromatography Experiments                         | 91 |
| 3.2.2 | Potentiometric Titration Experiments                                      | 92 |
| 3.2.3 | Determination of Chemical Equilibria                                      | 93 |
| 3.3   | Results and Discussion                                                    | 94 |
| 3.3.1 | Mass Transfer Limitations                                                  | 94 |
| 3.3.2 | Influence of Temperature on Chemical Equilibria                            | 98 |
| 3.3.3 | Influence of Solvent Composition on Chemical Equilibria                   | 100 |
| 3.4   | Conclusions                                                               | 102 |
| 3.5   | Tables                                                                    | 104 |
| 3.6   | Figures                                                                   | 107 |
| 3.7   | References                                                                | 115 |

4 A Multiple Chemical Equilibria Approach to Modeling and Interpreting the Separation of Amino Acid Enantiomers by Chiral Ligand-Exchange Chromatography .............................................................................. 118

| 4.1   | Introduction                                                               | 118 |
| 4.2   | Materials and Methods                                                     | 122 |
| 4.3   | Theory                                                                    | 123 |
| 4.3.1 | Column Model Development                                                  | 123 |
| 4.3.2 | Boundary Conditions and Solution Algorithm                                | 131 |
| 4.4   | Results and Discussion                                                    | 133 |
| 4.4.1 | Column Properties and Solute Transport Characteristics                    | 133 |
| 4.4.2 | Model Evaluation                                                          | 136 |
| 4.4.2.1 | Copper Concentration Effects                                             | 137 |
| 4.4.2.2 | pH Effects                                                              | 138 |
| 4.4.2.3 | Sample Loading Effects                                                   | 140 |
| 4.4.2.4 | Temperature Effects                                                      | 141 |
| 4.5   | Optimization of CLEC Operating Conditions                                | 142 |
| 4.6   | Conclusions                                                               | 144 |
| 4.7   | Tables                                                                    | 146 |
| 4.8   | Figures                                                                   | 148 |
| 4.9   | References                                                                | 160 |

5 Modeling Profiles and Separation of Dopa-Enantiomer Elution Bands During Chiral Ligand Exchange Chromatography .............................................................................. 165

<p>| 5.1   | Introduction                                                               | 165 |
| 5.2   | Theory                                                                    | 167 |</p>
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.2.1</td>
<td>Model Development</td>
<td>167</td>
</tr>
<tr>
<td>5.2.2</td>
<td>Determination of Chemical Equilibria</td>
<td>169</td>
</tr>
<tr>
<td>5.3</td>
<td>Experimental</td>
<td>171</td>
</tr>
<tr>
<td>5.3.1</td>
<td>Materials and Methods</td>
<td>171</td>
</tr>
<tr>
<td>5.3.2</td>
<td>Potentiometric Titrations</td>
<td>171</td>
</tr>
<tr>
<td>5.3.3</td>
<td>Chromatography Experiments</td>
<td>172</td>
</tr>
<tr>
<td>5.4</td>
<td>Results and Discussion</td>
<td>173</td>
</tr>
<tr>
<td>5.4.1</td>
<td>Column Properties and Solute Transport Characteristics</td>
<td>173</td>
</tr>
<tr>
<td>5.4.2</td>
<td>Equilibrium Formation Constants for the Cu²⁺-Dopa-p-MBD-Penicillamine System</td>
<td>173</td>
</tr>
<tr>
<td>5.4.2.1</td>
<td>Protonation Constants</td>
<td>174</td>
</tr>
<tr>
<td>5.4.2.2</td>
<td>Equilibrium Formation Constants for Binary and Ternary Complexes</td>
<td>176</td>
</tr>
<tr>
<td>5.4.2.3</td>
<td>Species Distribution Within the Chirex CLEC Column</td>
<td>177</td>
</tr>
<tr>
<td>5.4.3</td>
<td>Modeling the Separation of Dopa Enantiomers</td>
<td>178</td>
</tr>
<tr>
<td>5.4.3.1</td>
<td>Copper Concentration Effects</td>
<td>180</td>
</tr>
<tr>
<td>5.4.3.2</td>
<td>pH Effects</td>
<td>181</td>
</tr>
<tr>
<td>5.5</td>
<td>Conclusions</td>
<td>183</td>
</tr>
<tr>
<td>5.6</td>
<td>Tables</td>
<td>184</td>
</tr>
<tr>
<td>5.7</td>
<td>Figures</td>
<td>188</td>
</tr>
<tr>
<td>5.8</td>
<td>References</td>
<td>198</td>
</tr>
<tr>
<td>6</td>
<td>Conclusions and Suggestions for Future Work</td>
<td>201</td>
</tr>
<tr>
<td>6.1</td>
<td>References</td>
<td>206</td>
</tr>
</tbody>
</table>

Appendix I: Measurement of the Ligand Density by Breakthrough Analysis 208

Appendix II: CLEC Model Solution Method 215
- Fully Implicit Discretization Method Applied to the Column Continuity Equation 215
- Column Boundary Conditions 222
- Discretization of the Bead Continuity Equation 226

Description of CLEC Simulation and Related Programs 231
- Numerical Algorithm 231
- Input File <Valine.txt> 234
- Output File 234
- Compiling and Running the Program 234
- Comments on the Input File 235
- Example of the <Valine.txt> Input File 237
- Main Source Code Program <CLECj90> 238

References 270
List of Tables

Table 1.1 Worldwide sales of single-stereocentre enantiomer drugs...........................................29

Table 2.1 Amino-acid concentration-based protonation constants \( (I_c = 0.1 \text{ M KNO}_3) \)........70

Table 2.2 Enthalpy and entropy changes for amino-acid protonation over the temperature range \( T = 298.15 \) to \( 333.15 \text{ K} \) \( (I_c = 0.1 \text{ M KNO}_3) \).................................................................71

Table 2.3 Binary amino acid/copper concentration formation constants \( (I_c = 0.1 \text{ M KNO}_3) \) ........................................................................................................71

Table 2.4 Enthalpy and entropy changes for mono- and bis-binary complex formation over the temperature range \( T = 298.15 \) to \( 333.15 \text{ K} \) \( (I_c = 0.1 \text{ M KNO}_3) \)...................................................72

Table 2.5 Thermodynamic properties for the conversion reaction \( \text{Cu}^{2+} + \text{CuL}_2 \rightleftharpoons 2\text{CuL}^+ \) at \( T^\circ = 298.15 \text{ K} \) \( (I_c = 0.1 \text{ M KNO}_3) \)...........................................73

Table 2.6 Ternary \( \text{Cu}(D' \text{ or } L')(L-\text{HyPro}) \) concentration formation constants \( (I_c = 0.1 \text{ M KNO}_3) \) ........................................................................................................73

Table 2.7 Enthalpy and entropy changes for stepwise formation of ternary \( \text{Cu}(D' \text{ or } L')(L-\text{HyPro}) \) complexes at \( 298.15 \text{ K} \) \( (I_c = 0.1 \text{ M KNO}_3) \)...................................................74

Table 2.8 Thermodynamic properties for the conversion reaction \( \frac{1}{2}\text{CuA}_2 + \frac{1}{2}\text{CuB}_2 \rightleftharpoons \text{CuAB} \) at \( T^\circ = 298.15 \text{ K} \) \( (I_c = 0.1 \text{ M KNO}_3) \).......................74

Table 3.1 Properties of the Nucelosil Chiral 1 column.................................................................104

Table 3.2 Temperature dependence of mass transfer coefficients for \( L \)-proline injected onto a Nucelosil Chiral 1 column as a 20-\( \mu \text{L} \) pulse at concentration of 1 mM and a flow rate of 1 ml min\(^{-1}\). The mobile phase contained 0.5 mM CuSO\(_4\) at pH 5.4. ........................................................................................................104

Table 3.3 Temperature dependence of dimensionless parameters characterizing the transport of \( L \)-proline injected onto a Nucelosil Chiral 1 column as a 20-\( \mu \text{L} \) pulse at concentration of 1 mM and a flow rate of 1 ml min\(^{-1}\). The mobile phase contained 0.5 mM CuSO\(_4\) at pH 5.4.................................105

Table 3.4 Protonation and binary formation constants for proline and \( L \)-hydroxyproline in aqueous solution \( (I_c = 0.1 \text{ M KNO}_3) \) over the temperature range 298.15 to 333.15 K.................................................................105

Table 3.5 Ternary \( \text{Cu}(D' \text{ or } L \text{-proline})(L-\text{HyPro}) \) formation constants \( (I_c = 0.1 \text{ M KNO}_3) \) for the temperature range 298.15 to 333.15 K. .................................................................106
Table 3.6 Protonation and equilibrium formation constants as a function of methanol concentration (298.15 K, \(I_c = 0.1 \text{ M KNO}_3\))

Table 4.1 Column characteristics, mass transfer coefficients and some dimensionless parameters characterizing the transport of L-valine injected onto the Nucleosil Chiral-1 column as a 10 \(\mu\text{L}\) pulse at a flow rate of 1 \(\text{mL min}^{-1}\) at 298.15 K.

Table 4.2 Ternary equilibrium formation constants determined from potentiometric titration measurements (\(I_c = 0.1\text{ M KNO}_3, T = 298.15 \text{ K}\)) and the on-column correction factor \(k_I\) accounting for effects of chiral selector immobilization for various amino-acid\(\text{Cu(II)}\)\(\text{L-hydroxyproline complexes}\).

Table 4.3 Ternary equilibrium formation constants determined from potentiometric titration measurements and \(k_I\) values for the \(L\)-valine\(\text{Cu(II)}\)\(\text{L-hydroxyproline complex over the temperature range 298.15 to 333.15 K}\).

Table 5.1 Column characteristics, mass transfer coefficients and some dimensionless parameters characterizing the transport of L-dopa injected onto the Chirex 3126 column as a 10 \(\mu\text{L}\) pulse at a flow rate of 1 \(\text{mL min}^{-1}\) at 298.15 K.

Table 5.2 Protonation constants for dopa and \(p\)-MBD-penicillamine determined from potentiometric titration experiments (\(T = 298.15 \text{ K}, 0.1 \text{ M KNO}_3\)) in two solvent systems: water and an aqueous solution containing 10% isopropanol.

Table 5.3 Equilibrium formation constants for binary \(\text{Cu}^{2+} \cdot (\text{H}^+) \cdot L\)-dopa and \(\text{Cu}^{2+} \cdot p\)-MBD-penicillamine complexes determined from potentiometric titration data (\(T = 298.15 \text{ K}, 0.1 \text{ M KNO}_3\)) in two solvent systems: water and an aqueous solution containing 10% isopropanol. The last column indicates the ratio of \([L\text{-dopa}]\) to \([\text{Cu(II)}]\) in the titration experiment used to regress the corresponding \(\beta_{\text{abcd}}\) value. The pH range over which the titration was carried out was 3 to 6.5 when the \(L\)-dopa to \(\text{Cu}^{2+}\) ratio was 1, and was 6.5 - 11.5 when the \(L\)-dopa to \(\text{Cu}^{2+}\) ratio was 2.

Table 5.4 Ternary equilibrium formation constants for \(\text{Cu}^{2+} \cdot L\)-dopa\(\cdot p\)-MBD-penicillamine and \(\text{Cu}^{2+} \cdot D\)-dopa\(\cdot p\)-MBD-penicillamine complexes determined from potentiometric titration measurements (\(T = 298.15 \text{ K}, 0.1\text{ M KNO}_3\)) in two solvent systems: water and an aqueous solution containing 10% isopropanol. The on-column correction factor \(k_I\) accounting for the effects of chiral selector immobilization within the Chirex 3126 column is also reported.
List of Figures

Figure 1.1 Example of chirality: ritalin enantiomers

Figure 1.2 Thalidomide enantiomers

Figure 1.3 Structure of the ternary complexes formed between the ligand selector (L-hydroxyproline), metal ion and the two enantiomers (Top: L-proline•Cu•L-Hydroxyproline and Bottom: D-proline•Cu•L-Hydroxyproline) with water molecule binding to the axial coordination sites of the metal

Figure 1.4 Fully protonated and zwitterionic forms of an amino acid bearing an uncharged side chain

Figure 1.5 Mono-binary (left) and bis-binary (right) complexes formed in a CLEC system containing the Cu(II) ion

Figure 1.6 Species distribution plot for a ternary mixture of L-proline (10 mM), Cu(II) (10 mM), the chiral selector L-hydroxyproline (10 mM) in aqueous solution at 298.15 K containing 0.1 M KNO₃. Species abedef indicates the molecules of Cu²⁺ (a), protons (b), L-proline (c), D-proline (d), and L-hydroxyproline (e) in the complex: filled triangles - species 01100, filled squares - species 02100, filled stars - species 10100, filled circles - species 10200, open triangles - species 01001, open squares - species 02001, open stars - species 10001; open circles - species 10002, open inverse triangle - species 10101

Figure 2.1 van’t Hoff plots for stepwise protonation of proline: (a) K₁, (b) K₂

Figure 2.2 van’t Hoff plots for stepwise formation of mono- and bis-binary Cu²⁺ complexes containing proline: (a) K₁, (b) K₂

Figure 2.3 van’t Hoff plots for stepwise formation of ternary complex: Cu(L-HyPro)⁺ + D⁻ or L⁻ \xrightarrow{K_t} Cu(L-HyPro)(D'or L') for which the constant is Kₜ. (a) leucine, (b) valine, (c) proline, (d) phenylalanine (filled squares: L-amino acid, filled circles: D-amino acid)

Figure 3.1 Structure of the immobilized L-hydroxyproline ligand bound to the stationary phase of the Nucleosil Chiral 1 Column

Figure 3.2 Temperature dependence of the elution chromatogram for a 10-μL pulse injection of 1 mM D,L-valine onto the Nucleosil Chiral-1 column at 1 mL min⁻¹. The mobile phase contained 0.5 mM CuSO₄ at pH=5.4

Figure 3.3 Temperature dependence of the elution chromatogram for a 10-μL pulse injection of 1 mM D,L-proline onto the Nucleosil Chiral-1 column at 1 mL min⁻¹. The mobile phase contained 0.5 mM CuSO₄ at pH=5.4
Figure 3.4 Comparison between experimental (filled circles) and predicted (filled inverse triangles) retention times for 0.5 mM D-proline injected as a 10-µL pulse onto the Nucleosil Chiral-1 column. The mobile-phase (0.5 mM CuSO₄, pH 5.4) flow rate was 1 ml min⁻¹.

Figure 3.5 Temperature dependence of Cu(II)-containing complexes at equilibrium in an aqueous solution (pH 5.4) containing 10 mM L-hydroxyproline, 10 mM L-proline and 10 mM Cu(II). Dominant complexes present at equilibrium include \( \text{Cu}(\text{II})\text{L}_2 \) (open squares), \( \text{Cu}(\text{II})\text{L}_3 \) (open diamonds), \( \text{Cu}(\text{II})\text{L}_1 \) (filled squares), \( \text{Cu}(\text{II})\text{L}_2 \) (filled diamonds), and the ternary complex \( \text{Cu}(\text{II})\text{L}_1 \) (filled stars).

Figure 3.6 The dependence on mobile-phase methanol content of the elution chromatogram for a 10-µL pulse injection of 1 mM \( D,L \)-valine onto the Nucleosil Chiral-1 column at a flow rate of 0.6 mL min⁻¹. The mobile phase contained 0.5 mM CuSO₄ at pH 5.4 and varying amounts of methanol: 20% (v/v) MeOH – continuous line, 0% (v/v) MeOH – dashed line. The column temperature was 298.15 K.

Figure 3.7 The dependence on mobile-phase methanol content of the elution chromatogram for a 10-µL pulse injection of 1 mM \( D,L \)-proline onto the Nucleosil Chiral-1 column at a flow rate of 1 mL min⁻¹. The mobile phase contained 0.5 mM CuSO₄ at pH 5.4 and varying amounts of methanol: 40% (v/v) MeOH – continuous line, 20% (v/v) MeOH – dashed-dotted line, 0% (v/v) MeOH – dashed line. The column temperature was 298.15 K.

Figure 3.8 The dependence of formation constants for binary and ternary complexes on solvent composition. Formation constants include \( \beta_{1010} \) (squares), \( \beta_{1020} \) (circles), \( \beta_{1001} \) (triangles), \( \beta_{1002} \) (stars), and \( \beta_{1011} \) (hexagons). Superscript ‘aq’ refers to the cosolvent-free system.

Figure 4.1 General description of an elution chromatography column containing spherical, porous sorbent particles as the stationary phase.

Figure 4.2 Measured first central moment (\( \mu_1 \)) as a function of superficial velocity \( u \) of the mobile phase for 10 µlu pulses of 10 mg m⁻¹ Dextran T110 over the superficial velocity range \( u = 2.65 \times 10^{-4} \) to \( 1.33 \times 10^{-3} \) (m s⁻¹). The mobile phase contained 0.5 mM CuSO₄ (pH =5.4, T= 298.15 K).

Figure 4.3 Height equivalent of a theoretical plate (HETP) values for 10 µlD pulses of 1 mM \( D \)-glucose (filled squares) and 1 mM \( L \)-valine (filled triangles) loaded onto a Nucleosil Chiral-1 column at interstitial velocities \( u_0 \) ranging from \( 2.06 \times 10^{-3} \) to \( 3.83 \times 10^{-3} \) (m s⁻¹). The mobile phase contained 0.5 mM CuSO₄ (pH = 5.4, T = 298.15 K).

Figure 4.4 Comparison between experimental (dashed line) and model predicted (solid line) elution profiles for a 10 µL pulse injection of 2 mM \( D,L \)-valine onto the Nucleosil Chiral-1 column at a flow rate of 1 m lin⁻¹. The mobile phase...
contained 1 mM CuSO$_4$ at pH 5.4. The column was maintained at a temperature of 298.15 K and contained 310 mM immobilized L-hydroxyproline.

Figure 4.5 Comparison between experimental (dashed line) and model predicted (solid line) elution profiles for a 10 μL pulse injection of 2 mM D, L-phenylalanine onto the Nucleosil Chiral-1 column at a flow rate of 1 mL min$^{-1}$. The mobile phase contained 2 mM CuSO$_4$ at pH 5.4. The column was maintained at a temperature of 298.15 K and contained 510 mM immobilized L-hydroxyproline.

Figure 4.6 Comparison as a function of mobile-phase Cu(II) concentration between experimental (filled squares: L-valine, filled stars: D-valine) and predicted retention times (solid lines) for valine enantiomers (2 mM D,L-valine) injected as a 10 μL pulse onto the Nucleosil Chiral-1 column at 1 mL min$^{-1}$. The column was maintained at a temperature of 298.15 K and contained 310 mM immobilized L-hydroxyproline. The mobile phase contained CuSO$_4$ at pH 5.4.

Figure 4.7 The pH dependence of equilibrium Cu(II)-containing complexes in an aqueous solution containing 310 mM L-hydroxyproline and 2 mM L-valine at 298.15 K. The copper concentration in the system was set at 184.47 mM, which corresponds to the total concentration of Cu(II) when the mobile phase Cu(II) concentration is 0.5 mM, or at 223.29 mM, the total Cu(II) concentration when the mobile phase Cu(II) concentration is 3 mM. Subscript 'abcde' on concentration $C_{abcde}$ of complex abcde indicates the molecules of Cu$^{2+}$ (a), protons (b), L-amino-acid enantiomer (c), D-amino-acid enantiomer (d), and chiral selector (e) in the complex.

Figure 4.8 The dependence of enantiomer retention times on pH. Comparison between experimental (D-valine: open stars and L-valine: open squares) and predicted (line) retention times for 2 mM D,L-valine injected as a 10 μL pulse onto the Nucleosil Chiral-1 column. The column was at 298.15 K and contained 310 mM immobilized L-hydroxyproline. The mobile phase contained 0.5 mM CuSO$_4$ at pH 5.4 and was flowing at 1 ml min$^{-1}$.

Figure 4.9 The dependence of the elution chromatogram on sample loading concentration for a 10 μL injection of (a) 10 mM D,L-valine, (b) 5 mM D,L-valine and (c) 2 mM D,L-valine onto the Nucleosil Chiral-1 column. Both experimental (dashed lines) and predicted (solid lines) elution profiles are shown. The mobile phase flow rate was maintained at 1 mL min$^{-1}$, and the mobile phase contained 1.5 mM CuSO$_4$. The column contained 310 mM immobilized L-hydroxyproline and was maintained at 298.15 K.

Figure 4.10 Temperature dependence of the retention time for a 10 μL pulse of 0.5 mM L-valine injected onto the Nucleosil-Chiral-1 column at a flow rate of 1 mL min$^{-1}$. The mobile phase contained 0.5 mM CuSO$_4$ at pH 5.4 and the stationary phase contained 510 mM L-hydroxyproline. Both experimental (filled squares) and predicted (solid line) retention times are shown.
Figure 4.11 Response surface plot of the predicted resolution ($R_s$) of valine enantiomers on a Nucleosil Chiral-1 column. All simulations were for a 10 μL pulse injection of 1 mM $D,L$-valine onto the column and the results were validated with 16 experimental chromatograms randomly distributed across the response surface (data not shown to avoid graphical clutter). The column was 0.1 m in length and contained a ligand density of 310 mM $L$-hydroxyproline. The column temperature and mobile-phase flowrate were maintained at 298.15 K and 1 ml min$^{-1}$, respectively. The resolution $R_s$ was calculated as $(t_{e1} - t_{e2})[0.5(W_1 + W_2)]$, where $t_{e1}$ is the retention time of enantiomer 1 and $W_1$ is the corresponding peak width at baseline.

Figure 5.1 Structure of 3-(3',4'-dihydroxyphenyl)-$L$-alanine ($L$-dopa) ........................................... 188

Figure 5.2 Structure of the chiral selector (N-octyl-3-octylthio-$D$-valine) immobilized through hydrophobic bonding with the C18 reversed-phase chemistry of the Chirex 3126 column ........................................................................................................ 188

Figure 5.3 The pH dependence of major complexes formed in an aqueous solution containing 10% isopropanol, 2 mM CuSO$_4$ and 10 mM $L$-dopa injected as a 10 μL pulse onto the Chirex 3126 column at 1 ml min$^{-1}$. The column was maintained at 298.15 K and contained 50 mM immobilized N-octyl-3-octylthio-$D$-valine. .................................................................................................................. 189

Figure 5.4 Comparison between experimental (dashed line) and model (solid line) elution profiles for a 10 μL pulse injection of 10 mM $D,L$-dopa onto the Chirex 3126 column. The aqueous mobile phase (1 ml min$^{-1}$) contained 10% isopropanol and 3 mM CuSO$_4$ at pH 5.4. The column was maintained at 298.15 K and contained 50 mM immobilized N-octyl-3-octylthio-$D$-valine .................................................. 191

Figure 5.5 Comparison as a function of mobile-phase Cu(II) concentration between experimental (filled squares: $L$-dopa, filled triangles: $D$-dopa) and predicted (solid lines) retention times for dopa enantiomers (10 mM $D,L$-dopa) injected as a 10 μL pulse onto the Chirex 3126 column at 1 ml min$^{-1}$. The column was maintained at 298.15 K and contained 50 mM immobilized N-octyl-3-octylthio-$D$-valine. The mobile phase contained 10% isopropanol at pH 5.4 ....................... 192

Figure 5.6 The pH dependence of the dominant equilibrium complexes in an aqueous mixture at 298.15 K containing 10 mM $L$-dopa, 50 mM N-octyl-3-octylthio-$D$-valine and 10% isopropanol. The copper concentration in the system was set at 47.24 mM, corresponding to the total concentration of Cu(II) when the mobile phase Cu(II) concentration is 1 mM, or at 51.68 mM, the total Cu(II) when the mobile phase Cu(II) concentration is 3 mM. The open and filled symbols represent complexes when [Cu(II)] is 1 mM and 3 mM, respectively, in the mobile phase ............................................................................................................ 193

Figure 5.7 Comparison between the experimental (filled circles) and predicted (solid lines) resolution of dopa enantiomers as a function of mobile phase Cu(II)
concentration. The data apply to a 10 μL pulse of 10 mM L,D-dopa injected onto the Chirex 3126 column at a flow rate of 1 mL min⁻¹. The column was maintained at 298.15 K and contained 50 mM immobilized N-octyl-3-octylthio-D-valine. The mobile-phase contained 10% isopropanol at pH 5.4.............195

Figure 5.8 The dependence of retention time on pH. Comparison between experimental (L-dopa: filled stars and D-dopa: filled circles) and predicted (line) retention times for injection of 10 mM D,L-dopa as a 10 μL pulse onto the Chirex 3126 column operated at 298.15 K. The column contained 50 mM N-octyl-3-octylthio-D-valine. The aqueous mobile phase contained 10% isopropanol and 2 mM CuSO₄. The flow rate was 1 ml min⁻¹..........................196

Figure 5.9 Comparison between experimental (filled squares) and model (solid line) results for the resolution $R_s$ of dopa enantiomers on the Chirex 3126 column. A 10 μL pulse of 10 mM L,D-dopa was injected onto the column (298.15 K) at a flow rate of 1 ml min⁻¹. The aqueous mobile phase contained 10% isopropanol and 2 mM CuSO₄..........................................................197
Nomenclature

c_i: total concentration of component i in the mobile phase (mol/m^3 mobile phase)
c_i^* total equilibrium concentration of component i in the intraparticle fluid (mol/m^3 pore fluid)
c_i^{feed} total concentration of the component i in the injected sample (mol/m^3)
c_i^0 total concentration of the component i in the column prior to injection (mol/m^3 mobile phase)
c_i^m total concentration of the component i in the mobile phase buffer solution (mol/m^3 mobile phase)

D_L: axial dispersion coefficient (m^2/s)
D_m: molecular diffusion (m^2/s)
D_p: intraparticle diffusivity (m^2/s)
E^0: standard electrode potential (mV)
E^{obs}: observed electrode potential (mV)
E^{calc}: calculated electrode potential (mV)

HETP: Height equivalent to a theoretical plate (m)

I_c: ionic strength (M)
k_f: forward adsorption rate constant (m^3/mole s)
k_r: reverse adsorption rate constant (s^{-1})
k_{ads}: intrinsic rate constant (s^{-1})
k_f: film mass transfer coefficient (m/s)
k_l: correction factor for mixed ternary complex equilibrium formation constant

K_a: stepwise equilibrium binding constant (M^{-1})
K_i: stepwise concentration equilibrium constant (M^{-1})
K_L: Langmuir equilibrium binding constant (M^{-1})
K_m: overall mass transfer coefficient (s^{-1})
K_p: effective partition coefficient of the enantiomer

L: column length (m)
N_0: flux of solute into the particle (mol/m^2 s)
NTU: number of theoretical units (plates)
\( Q \) volumetric flow rate \((m^3 s^{-1})\)

\( q_i^* \) equilibrium concentration of the solute adsorbed to the sorbent surface \((mol/m^3 sorbent)\)

\( q_{\max} \) maximum binding capacity of the sorbent \((mol/m^3 sorbent)\)

\( R \) gas constant \((J/mol. K)\)

\( R_c \) column radius \((m)\)

\( R_p \) particle radius \((m)\)

\( s_i \) average total concentration of a given component i within stationary phase \((mol/m^3 sorbent)\)

\( T \) temperature \((K)\)

\( T_i \) total concentration of component i \((M)\)

\( t \) time \((s)\)

\( t_{feed} \) injection time \((s)\)

\( t_0 \) system dead time \((s)\)

\( t_R \) retention time \((s)\)

\( U \) sum of squared residuals between observed and calculated electrode potential

\( u \) superficial liquid velocity \((m/s)\)

\( u_0 \) interstitial velocity \((m/s)\)

\( V_p \) volume of the stationary particle \((m^3)\)

\( V_e \) elution volume \((m^3)\)

\( V_{feed} \) sample volume \((m^3)\)

\( W \) weighting factor

\( z \) axial coordinate \((m)\)

**Dimensionless Numbers**

\( Bi \) Biot Number

\( Da \) Damkohler Number

\( Re \) Reynolds Number

\( Sc \) Schmidt Number

\( Sh \) Sherwood Number

\( Pe \) Peclet Number

\( u_{\text{red}} \) Reduced Velocity
### Greek Letters

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>σ</td>
<td>total estimated error in electrode reading</td>
<td>(mV)</td>
</tr>
<tr>
<td>σ_E</td>
<td>error in electrode potential</td>
<td>(mV)</td>
</tr>
<tr>
<td>σ_v</td>
<td>error in titrant volume readings</td>
<td>(mV)</td>
</tr>
<tr>
<td>σ_c</td>
<td>error in reagent concentration</td>
<td>(mV)</td>
</tr>
<tr>
<td>σ</td>
<td>standard deviation</td>
<td></td>
</tr>
<tr>
<td>ε</td>
<td>column void fraction</td>
<td></td>
</tr>
<tr>
<td>ε_p</td>
<td>sorbent porosity</td>
<td></td>
</tr>
<tr>
<td>μ₁</td>
<td>first central moment of an eluent peak</td>
<td>(s)</td>
</tr>
<tr>
<td>μ₂</td>
<td>second central moment of an elution peak</td>
<td>(s²)</td>
</tr>
<tr>
<td>Δ_H_c</td>
<td>molar enthalpy change due to reaction</td>
<td>(kJ mol⁻¹)</td>
</tr>
<tr>
<td>Δ_G_c</td>
<td>molar Gibbs energy change due to reaction</td>
<td>(kJ mol⁻¹)</td>
</tr>
<tr>
<td>Δ_S_c</td>
<td>molar entropy change due to reaction</td>
<td>(J mol⁻¹ K⁻¹)</td>
</tr>
<tr>
<td>β</td>
<td>concentration equilibrium formation constant of a complex</td>
<td></td>
</tr>
<tr>
<td>β*</td>
<td>equilibrium formation constant of a ternary complex regressed on column</td>
<td></td>
</tr>
<tr>
<td>νο</td>
<td>kinematic viscosity</td>
<td>(m²/sec)</td>
</tr>
<tr>
<td>ρ</td>
<td>density</td>
<td>(kg/m³)</td>
</tr>
</tbody>
</table>
Acknowledgments

I would like to first express my sincere appreciation to my research supervisor Dr. Charles Haynes, not only for his invaluable guidance during the course of my thesis, but also for making this amazing experience possible and for bringing all patience and efforts to success. I am particularly thankful for all his contributions to my development as a scientist, for appreciating my research strengths, for his confidence in my abilities and the handful of fresh new opportunities that he had offered me aside from my thesis.

I would like to thank all my fellow members of the Michael Smith Laboratories and especially my colleagues at the Haynes lab for providing a friendly environment and the most enriching experience of my life. I am particularly grateful to Dr. Louise Creagh, who trained me upon my arrival to the lab and for constantly keeping the lab organized during all these years.

Finally, I am very grateful to my parents whose love and unwavering support brought me comfort and warmth. I have no doubt that without their faith in me I would never have pursued this challenging research career.

Last but not least my heartfelt thanks goes to my patient and loving husband, who has been a great source of strength through the completion of this work.
Dedication

To my parents Nasrin and Mohammad Ali Sanaie

&

My grandmother Akram Shahabadi (Maman Goli)
1 Introduction, Background and Thesis Objectives

1.1 Thesis Overview

Chiral (from the Greek word 'cheir', meaning hand) chemistry is based on the phenomenon of stereoisomerism, a property of organic compounds known since the pioneering work of Louis Pasteur in the 19th century [1]. Atoms that generate non-planar molecular structures through covalent bonds may create chirality. Whether or not a molecule is chiral is determined by its symmetry. A molecule is achiral (i.e., non-chiral) if it possesses a plane of symmetry, a center of symmetry, or any n-fold (where n is an even number) alternating axis of symmetry; that is, if an n-fold rotation (a rotation by 360°/n) followed by a reflection in the plane perpendicular to this axis maps the molecule onto itself. Chiral molecules lack such planes and axes of symmetry and are therefore called dissymmetric. They are not necessarily asymmetric (i.e. without symmetry) (Figure 1.1) because they can possess other types of symmetry. However, all amino acids (except glycine) and many sugars are both asymmetric and dissymmetric.

Fundamental research on optically active compounds started without knowledge of the true arrangement of atoms leading to chirality. As a result, enantiomers were distinguished through the sign of their optical rotation. In 1891, Fisher [2] arbitrarily assigned the D configuration (corresponding to dextrorotation of polarized light) to molecules that are configurationally related to (+)-glyceraldehyde, and the L configuration (laevorotation) to those configurationally related to (-)-glyceraldehyde. The D, L nomenclature system is still very much used to define the chirality of many biological analytes, including carbohydrates, hydroxy-acids, and amino acids. However, the method is not general. In 1956, Cahn, Ingold and Prelog [3] (CIP) therefore developed a new nomenclature applicable to any chiral molecule
that is based on rigorous assignment of atoms bonded to a stereocentre or alkene bond carbon. In the CIP system, each group attached to the stereocentre is assigned a priority, 1, 2, 3 or 4. Assignment is given in order of atomic number of the atom that is directly bonded to the stereocentre; priority 1 is given to that atom possessing the largest atomic number and priority 4 to that possessing the smallest. If two atoms attached to the stereocenter have the same atomic number, then the atomic numbers of the atoms directly bonded to the two priority atoms are compared, and so on. After the atoms bonded to a stereocenter have been assigned, the molecule can be oriented in space so that the bonded atom with the lowest priority points away from the observer. The sense of rotation of a route passing through 1, to 2 and then 3 distinguishes the stereoisomers. A stereocentre with a clockwise sense of rotation is termed an R or rectus center, and a stereocentre with an anticlockwise sense of rotation is an S or sinister center.

Nearly all biological polymers (proteins, DNA, RNA, etc.) are homochiral. Amino acids in proteins are left-handed (L enantiomers), while all sugars in DNA, RNA and within the metabolic pathways are right-handed. Many vitamins, flavors, fragrances, herbicides, pesticides, and more than half of all small molecule synthetic drugs are also chiral [4]. Often, only one of the enantiomers is responsible for the desired response while the other is either ineffective or toxic [5]. For example, the S-enantiomer of Ibuprofen (a non-steroidal anti-inflammatory drug) is at least 160 times more potent than the R-enantiomer in reducing arthritis pain, minor inflammation, and prostaglandin synthesis[6-8].

More striking is the story of thalidomide (Figure 1.2), a tranquilizer widely prescribed in the 1960's to treat morning sickness during the early stages of pregnancy. The R-enantiomer
of thalidomide was initially thought to provide the desired therapeutic effect, while the $S$-enantiomer was determined to be toxic, causing serious malformations in fetus development [9]. The large number of cases of aborted or medically compromised fetuses related to thalidomide were therefore linked to administration of the racemate. Later, it was found that it is not possible to unambiguously determine which optical isomer is teratogenic. This is because whatever form is administrated, the drug undergoes rapid *in vivo* inversion of configuration to reach a steady state $R/S$ ratio of 1.07 [10]. The Food and Drug Administration (FDA) has therefore developed increasingly stricter guidelines for defining the stability, inter-conversion, pharmacokinetic properties, and dosage of chiral drugs. Pharmaceutical companies have responded by developing strategies to eliminate the presence and subsequent uptake of the unwanted enantiomer in new chiral drug formulations, even in cases where that enantiomer is nontoxic or serves as a less active antipode in the racemic mixture [11]. An important example is provided by the $2$ billion per annum drug ritalin. Between 5 and 10% of the school-age population has been diagnosed with attention deficit disorder and is currently under treatment. The standard therapy uses a racemic form (i.e. a mixture containing near equimolar amounts of the $R$ and $S$ isomers) of ritalin. Clinical trials monitored by the FDA revealed that the $R$ enantiomer of ritalin is the therapeutically active agent, and its therapeutic effect is more potent and long lasting in the absence of the $S$ enantiomer. Thus, the $S$ enantiomer is not an innocuous bystander. Not only is the $S$ form therapeutically inactive, it actually inhibits the therapeutic action of the $R$ isomer. Celgene Pharmaceuticals has therefore recently completed consignment of a 130 million dollar facility for the production of $R$-ritalin, the second-generation form of its biggest selling drug. Similarly, Forest Laboratories Inc. is currently working on a more potent version of its antidepressant celexa (citalopram), which is currently marketed as a racemate. The $R$ isomer of celexa is inactive. Forest Laboratories Inc. has
therefore applied for approval of escitalopram, the pure $S$-enantiomer form of their antidepressant [12,13].

Enantiopure drugs are now a multi-billion dollar per annum industry (Table 1.1), representing *ca.* one third of the synthetic drug market [4]; in 2001, $139.3$ billion (36\%) of the $368$ billion in worldwide sales of synthetic pharmaceutical products were single-stereocentre chiral drugs (Table 1.1). This market share is rising [14]. With combined sales of almost $14$ billion in 2002, lipitor and zocor are the two top selling drugs worldwide; both contain a single stereocentre. This shift towards enantiomerically pure therapeutics is driven by an increased awareness in pharmacology and medicine of the importance of chirality in the human biosystem [6]. As a result, there is an increasing need for effective, inexpensive methods for both quantitative analysis and large-scale resolution of enantiomeric mixtures. Similarly, there is a growing need for new technologies to purify the chiral precursors (*e.g.* amino acids) required for direct asymmetric synthesis of complex chiral therapeutics.

Synthesis of chiral compounds from achiral reagents always yields the racemic mixture. This is a consequence of the laws of thermodynamics and the fact that the $R$- and $S$-enantiomers of a chiral compound have identical Gibbs free energy. They also share the same molecular weight, molecular size, melting point, boiling point, solubilities, etc. The nearly identical physical properties of enantiomeric isomers make chiral molecule purification one of the most challenging problems in the separation sciences. Chemists are addressing this challenge through novel asymmetric chemical and enzymatic synthesis protocols [15,16]. However, achiral synthesis remains more efficient for many small molecule therapeutics,
necessitating efficient purification technology to recover the desired enantiomer. Current methods for separating chiral enantiomers include preferential and diastereomeric crystallization, and chiral chromatography. Preferential crystallization, although highly cost effective, is of limited practical utility. Enantiomers that naturally form separable pure crystals are rare, the most famous being the sodium ammonium tartrate system, whose unusual crystallization behavior was studied by Pasteur in his pioneering work on chirality [17].

The most general routes to separating enantiomers to high resolution are diastereomeric crystallization and chiral chromatography, the latter of which is the focus of this thesis and includes both Pirkle-type chromatography and chiral ligand exchange chromatography (CLEC). Owing to the subtle differences in the analytes being separated, the efficiency and economics of chiral chromatography systems are known to be highly sensitive to the selection of the stationary-phase chemistry and column operation conditions (i.e. temperature, flowrate, pH, solvent composition, etc.) [18-20]. Application of chiral chromatography would therefore benefit from an experimental or theoretical protocol to identify optimal column chemistries and operating conditions for a given chiral separation.

My Ph.D. thesis research focuses on CLEC and the associated advantages of establishing a general method for 1) identifying a suitable CLEC ligand for separating a target racemate, and 2) optimizing the operating conditions of a CLEC-based separation to maximize product purity, yield and throughput while minimizing cost. The second objective is the main goal of my thesis research, which aims to derive and validate a general model describing transport and separation of a racemic mixture within a CLEC column. The model uses molecular thermodynamic concepts to predict column performance as a function of key operating
parameters such as temperature and pH, and thereby permits one to optimize process conditions. When a more complete thermodynamic database is in place, the model should also make it possible to quickly screen a range of possible ligands to identify the optimal ligand for a given separation.

1.2 Routes to Pure Enantiomer Production

Current methods for production of an enantiomerically pure molecule include (chemical or enzymatic) asymmetric synthesis or conversion, preferential and diastereomeric crystallization, and chiral chromatography [21-25]. Although not the focus of this work, asymmetric synthesis processes have become increasingly popular in the production of pharmaceutical compounds. The reasons include the growing prevalence of more complicated molecular architectures featuring one or several stereocentres in pharmaceutical compounds and the desire to reduce wastage resulting from the production of unwanted enantiomers. The approach requires the use of enantiomerically pure starting reagents, and several companies have been established in recent years to produce these chiral materials. Among the largest is Avecia Inc., which reported sales of chiral reaction precursors of $858 million in 2002. Avecia produces and purifies a range of C3, C4, and C5 chiral compounds, including (S)-hydroxy-7-butyrolactone, a key building block for cholesterol-lowering drugs such as Lipitor [26].

Direct synthesis of complex chiral drugs can be difficult and expensive. One of the most effective protease inhibitors now in general use for AIDS therapy, Crixivan (indinavir sulfate), manufactured by Merck Pharmaceuticals Inc., contains five stereocentres. Its synthesis involves 15 major reaction steps and most of the reactants are lost during this process due to racemization and other reaction inefficiencies. Amino indanol, a key Crixivan precursor that
provides two of the five stereocentres, is now a primary target for improving the overall efficiency of the synthesis. For example, O'Brien et al. [27] recently reported using three oxygenases from *Rhodococcus* to more efficiently catalyze the stereoselective production of the amino indanol enantiomer.

Although asymmetric synthesis of chiral therapeutics is a growing area of interest, this thesis is concerned with the purification of chiral enantiomers. I therefore review in more detail the two major approaches to resolving racemic mixtures: crystallization and chiral chromatography.

### 1.2.1 Preferential and Diastereomeric Crystallization

Racemic solutes may be crystallized from solution either as racemic crystals (both enantiomers present in the same crystal) or, in certain cases, as crystal conglomerates (a mixture of two types of crystal where each crystal type is composed of a single enantiomer). Preferential crystallization exploits this latter phenomenon to crystallize a desired enantiomer out of a solution of the racemate by supersaturating the solution with only that enantiomer [28,29]. Although highly cost effective, it is limited to crystal conglomerates where the heats of formation of the two enantiomeric crystals are sufficiently different to overcome the large entropic penalty of demixing. One such system of industrial significance is *D,L*-aspartic acid, which forms a stable crystal conglomerate at ambient conditions [30]. A chiral reactant for production of *α*-methyl-dopa, used in the treatment of Parkinson's disease, is also purified by preferential crystallization [31,32].

Of significantly broader application, diastereomeric crystallization separates the enantiomers of a racemate by coupling each to an enantiomerically pure complexant to form
either a salt complex or a covalent compound. The two diastereomers thus formed are then separated by selective crystallization or another traditional method since diastereoisomers, unlike enantiomers, can have different physical and chemical properties (such as solubility or melting point). Many chiral drugs are therefore produced through pre-column covalent derivatization of the enantiomers with chiral complexants to form diastereomers that are subsequently separated in the normal or reversed phase mode of chromatography [33-35].

Diastereomeric crystallization is used to purify a number of fine chemicals and intermediates. Engineering of the crystallization process is highly product specific, requiring a complete understanding of the physical chemistry of the system, including phase diagrams, solubility curves and crystallization kinetics. Merck Pharmaceuticals Inc. currently applies the method to purify L-glutamic acid on a scale of several hundred tons per annum [30].

1.2.2 Chiral Chromatography

Chiral chromatography remains the most general method for separating enantiomers to high resolution [36-38]. Like diastereomeric crystallization, chiral chromatography discriminates between the enantiomers of a racemate through binding of a complexant, also called a chiral selector, that has been either added to the solution (mobile) phase or, much more frequently, immobilized to the surface of the stationary phase (usually porous silica gel). The appeal of chiral chromatography lies in the inherent advantages of any chromatographic separation, including the speed, reproducibility and flexibility of the separation, and the possibility to analyze or purify enantiomers in complex mixtures. Moreover, successful separations at the analytical scale can be scaled-up to achieve preparative separations without significant change in performance.
A recent review by Göbitz and Schmid [39] that builds on classic reviews by Göbitz [40] and Taylor and Maher [41] describes the principles underlying chiral discrimination by the various modes of chiral chromatography. The conventional classification of types of chiral stationary phases first proposed by Göbitz [40] is used here: (1) chiral cavity phases (cyclodextrins, crown ethers and imprinted polymers), (2) macrocyclic antibiotic phases (e.g., immobilized vancomycin, thiostrepton, or rifamycin B), (3) protein phases (e.g., serum albumin, α1-acid glycoprotein, ovomucoid and chymotrypsin), (4) helical chiral-polymer phases (surface derivatized/immobilized or free polysaccharides such as cellulose or amylose in solution), (5) π-donor/π-acceptor type chiral aromatic amide phases (i.e. Pirkle type columns), and (6) chiral ligand-exchange phases (copper ions complexed with chiral moieties).

If the chiral selector (ligand) is immobilized on the stationary phase, the chromatography mode is called chiral stationary phase (CSP) chromatography, and if the selector is continuously added to the mobile phase it is called chiral mobile phase (CMP) chromatography.

To be effective, a chiral selector must be sufficiently stable to withstand immobilization and packing procedures, as well as normal HPLC operating conditions (e.g., appropriate solvents, temperature, pressure, pHs, ionic strengths, etc.). Complexation with the chiral selector is generally engineered to be reversible, so that the separation is dictated by the difference in the stabilities of the diastereomeric complexes (measured by the difference in the free energy $\Delta(\Delta G^\circ)$ of the two transient complexes). In general, $\Delta(\Delta G^\circ)$ is quite small due to the chemical similarity of the two enantiomers. Therefore a successful enantioseparation by chiral chromatography often requires a large number of equilibrium stages.
In the following sections, I review the chemistry and separation mechanisms of the most common chiral chromatography phases. Particular attention is given to chiral ligand exchange chromatography and its areas of application.

1.2.2.1. Chiral Cavity Phases

Chiral cavity phases, which for the purposes of this discussion can be divided into cyclodextrin and crown-ether type phases, are widely used for stereochemical analysis by chromatography [42-45]. Cyclodextrins are cyclic oligosaccharides containing six to twelve glucose units which are bonded through α-(1,4) glucopyranoside linkages. The three smallest homologs are alpha (α), beta (β) and gamma (γ) cyclodextrin, made up of six, seven and eight D-glucopyranose units, respectively. All are available commercially. The mechanism for the observed difference in complexation energies with a cyclodextrin presented at the stationary phase or as a mobile phase additive is not fully understood. However, dipole-dipole and Van der Waals interactions, the hydrogen bonding potential of the enantiomeric guest molecules, and solvent interactions are known to contribute to enantioselectivity, as does the cavity size of the cyclodextrin host relative to the size of the guest. Ameyibor and Stewart [46] investigated separation of selected anti-inflammatory drugs on a reverse phase column in which derivatives (hydroxypropyl, methyl or sulfonated) of β-cyclodextrin served as chiral mobile additives. They found that both the resolution and the capacity factor decreased with increasing organic modifier (acetonitrile), presumably because it lowers the strength of the inclusion complex formed between the analyte and cyclodextrin. Beeson and Vigh [47] achieved a complete separation of Ibuprofen and other profen enantiomers on a silica stationary phase bearing β-cyclodextrin. They noted that the pH of the mobile phase and the pKₐ of the acid group on the profen had a major effect on the separation quality.
Crown ethers having macrocyclic polyether rings form stable inclusion complexes with alkali metals, ammonium cations, and protonated amines [48]. Discovered by Pedersen [49], crown ethers have been intensely studied, and a wide range of achiral and chiral crown ethers have been synthesized, most notably through the work of Cram [50] and Lehn [51]. They are primarily used as phase transfer catalysts in organic synthesis [52,53] and as crude enzyme models in biochemical studies [54,55]. However, Cram [56] also recognized the potential of chiral crown ethers as complexants for chromatographic separation of enantiomers. The chiral crown ether he originally synthesized for this purpose contained binaphthyl substituents in a staggered arrangement. Commercially available phases for chiral chromatography now include binapthyl, tartaric acid, and sugar substituents [57,58] which have been used to separate a wide range of racemates, including aromatic amino acids, catecholamines, androgens, aminophenanthrenes, β-blockers, fluoroquinolone, aminogluthethimide and aminodecaline [59-62].

Most crown ether phases for chiral chromatography utilize substituted (+)-18-crown-6-ethers. The 6 oxygen atoms of the ring are directed towards the centre of the cavity and roughly define a plane. The primary amine of a chiral analyte may therefore bind inside the cavity by hydrogen bonding with the ring oxygens. This alone does not provide chiral recognition. Additional interactions between the ring substituents and the ligand are necessary for the discrimination of enantiomers. By controlling the size and the spatial orientation of these substituents, diastereomeric complexes with different formation constants (stabilities) may be formed. As a result, numerous applications of crown ethers in chiral chromatography have been reported [63,64].
1.2.2.2. Antibiotic Phases

Introduced by Armstrong [65], macrocyclic antibiotics have been shown to be very effective chiral selectors. Useful chiral HPLC phases have been prepared from macrocyclic glycopeptides such as vancomycin [66], ristocetin A [67], teicoplanin [68,69] and avoparcin [70], as well as from the ansamycin rifamycin B [65,71] and the polypeptide thiostrepton [72]. Macrocyclic antibiotics have multiple stereocentres and substituent functional groups that allow multiple interactions with chiral analytes, making them quite general chiral phases. For example, macrocyclic glycopeptides consist of a fused ring that forms a characteristic basket shape and a set of carbohydrate substituents that act as pendant arms. Hydrophobic parts of the analyte partition into the hydrophobic basket, allowing chiral recognition through hydrogen bonds, dipole-stacking, ionic, π–π and steric interactions formed with the pendant substituents. Immobilized vancomycin [73] has therefore found application in the chiral separation of barbiturates, hydantoins, piperidine-2,6-dione and cyclic amides, dihydropyrimidinones, pyridone derivatives, warfarin, α-methyl-α-phenyl succinimide, and semi-synthetic ergot alkaloids [74-76]. A CSP based on vancomycin derivatized with 3,5-dimethylphenylisocyanate [65] has shown different chiral recognition properties, permitting resolution of hydroxyzine and althiazide racemates. Finally, Péter and coworkers [77] applied a ristocetin-A bearing stationary phase to separate enantiomers of tryptophan and a range of aromatic, normal and alkyl amino acids.

1.2.2.3. Protein Phases

Due to their L-amino acid chemistry and chiral glycans, proteins are known to bind stereoselectively. Purified proteins can therefore serve as effective chiral selectors for separating a broad spectrum of chiral compounds containing aromatic and polar groups, including amino acids, non-steroidal anti-inflammatory drugs (NSAIDs), benzodiazepines,
arylpropionic acid derivatives, benzoin and warfarin [78-80]. Introduced by Allenmark et al. [81], immobilized human serum albumin was the first and remains the most popular CSP. However, α₁-acid glycoprotein, bovine serum albumin, ovomucoid and α-chymotrypsin are also popular for bio-analytical applications because they provide CSPs that are compatible with a wide range of aqueous mobile phases [82].

Commercial and prototype protein phases show good stability and enantioselectivity for many important pharmaceuticals, including beta-adrenergic receptor blocking agents such as timolol (glaucoma treatment) and propranolol (hypertension treatment) [83,84]. For example, Hermansson et al. [85,86] applied an α₁-acid glycoprotein column to efficiently separate racemates of benzodiasepines (anti-anxiety treatment) and beta-adrenergic receptor blocking agents through careful selection of cosolvents such as acetonitrile and isopropanol. While the resolving power of these chiral phases is not particularly good, they have been successfully used to purify enantiomers of various primary amines and β-alkyl amino acids.

1.2.2.4. Polymer Phases

A number of polysaccharide-based stationary phases composed of cellulose or amylose esters and carbamates is now commercially available for purifying low molecular weight therapeutic enantiomers from the corresponding racemate [e.g., see Daicel Chromatography Inc., Japan]. Although the range of applicability of polysaccharide CSPs is fairly broad, the resolving power of these resins is generally lower than observed with other CSPs. Hesse and Hagal [87] have shown that a microcrystalline cellulose triacetate CSP forms chiral cavities upon swelling that are able to accept aromatic enantiomers stereoselectively. In contrast, CSPs based on cellulose trisphenylcarbamate adsorbed to silica [88,89] provide chiral recognition
through the left-handed 3/2 helical conformation of the polymer that positions the polar carbamate groups inside a helical groove and hydrophobic aromatic groups of the polymer outside the groove. Polar enantiomers may therefore insert into the groove to interact with the carbamate residues via hydrogen bonds. Specific $\pi-\pi$ and related interactions between aromatic groups on the enantiomer and the phenyl pendant group of the polysaccharide then provide the necessary chiral recognition elements. Through this basic chiral recognition mechanism, CSPs composed of cellulose tris (3,5-dimethyl-phenylcarbamate) or cellulose tricinnamate are currently used to resolve profen enantiomers after amidation or esterfication of the carboxylic group of the profen [90].

Synthetic polymers are also used as CSPs. While the field is too large to review here, an important example is the work of Blaschke et al. [91], who synthesized polyacrylamides with chiral side chains and successfully resolved racemates of a number of barbiturates, hydantoins and benzodiazepines. Chemical bonding of the polymers to a porous silica gel resulted in a line of stable and versatile CSPs that have been commercialized by EM Merck Inc. (Darmstadt, Germany).

Molecular imprinting technology (template polymerization) has also been used to create novel CSPs. The approach is based on polymerizing a monomer with a cross-linking agent in the presence of a target enantiomer that serves as a chiral template. After extraction of the template, an imprinted cavity remains that exhibits selectivity for the template. The principle was pioneered by Wulff [92] and has been advanced by Mosbach [93] and others [94,95]. However, although these adsorbents have the potential to deliver high specificity for the target molecule, they have not found significant commercial application.
1.2.2.5. Charge Transfer (Pirkle) Phases

Named in honor of their inventor, Pirkle (charge transfer) CSPs are characterized by a chiral recognition mechanism that emphasizes subtle differences in the formation of \( \pi-\pi \) interactions, dipole-stacking interactions and hydrogen bonds between the selector and each enantiomer [96]. Pirkle type ligands are generally divided into \( \pi \)-acidic type selectors, which establish favorable \( \pi-\pi \) interactions with a complementary \( \pi \) electron donor group on the enantiomer to form a charge transfer complex, and \( \pi \)-basic type selectors, which contain a \( \pi \)-electron donor group complementary to \( \pi \)-acidic groups on the enantiomer. In either case, the shared electron stacking and hydrophobic interactions between the enantiomer and the chiral selector then result in chiral discrimination. The first charge-transfer phase developed by the Pirkle group utilized \((R)\)-N-(3,5-dinitrobenzoyl)phenylglycine as the chiral selector. Since then a large number of \( \pi \)-acidic and \( \pi \)-basic phases have been developed by the Pirkle group [97-99], and others [100-102], including several types of dinitrobenzoyl amino acid based CSPs, as well as \( N \)-aryl amino acid, phthalide, naproxen, and 2-arylamidoalkane derived CSPs. Pirkle CSPs have been successfully applied to a broad spectrum of compounds, and a number of charge-transfer type chiral chromatography resins are commercially available [103,104]. For example, Welch and co-workers [105] reported separation of therapeutic enantiomers including anticonvulsants, mephenytoin, nirvanol and a number of NSAIDs on the recently commercialized \( \pi \)-acidic Welk-O1 stationary phase.

1.2.2.6. Ligand Exchange Phases

Chiral ligand exchange chromatography, one of the most efficient chiral separation methods, is based on the formation of labile complexes between a ligand (chiral selector) and an enantiomer, centered around a transition metal ion. CLEC therefore differs from other
modes of chiral chromatography in that the interaction between the chiral selector and the enantiomers does not occur through direct contact. Rather, it is mediated by a metal ion that is capable of coordinating simultaneously to the chiral selector and either of the enantiomers to be separated to form a ternary mixed-ligand complex. The formation of these ternary complexes at the stationary phase generally requires a concurrent proton displacement reaction at participating amino (and possibly carboxy) groups on the chiral selector and the enantiomer. Most transition metals used in ligand exchange reactions are characterized by having unfilled inner-shell \( d \)-orbitals. A ternary complex can therefore form between a selector and an enantiomer such that both can donate electrons to the transition metal. The stability of the complex is stereoselective, so that the \( \Delta A G^\circ \) for the two ternary complexes can be used to achieve separation of a racemic mixture. Solvent molecules (\textit{i.e.}, water) can stabilize the complex by binding to the axial coordination sites of the metal (Figure 1.3). In this way, solvent molecules can also affect chiral selectivity.

Although the overall chemical equilibrium within a CLEC column is quite complex, the general nature of the ligand-exchange reaction is captured by the following reaction:

\[
CS \cdot M \cdot (H_2O)_{m-n} + EH \leftrightarrow CS \cdot M \cdot E \cdot (H_2O)_{m-n-2} + 2H_2O + H^+ \tag{1.1}
\]

where \( CS \) is the bidentate chiral selector, \( M \) is the transition metal ion having \( m \) coordination sites, \( E \) is the enantiomer, and \( n \) is the total number of coordination sites not occupied by water. The solvent, water, therefore participates in the reaction since diastereomeric mixed-ligand complexes generally form by a ligand displacement or exchange mechanism that requires deprotonation of one or more chelating groups on the associating enantiomer. As suggested by
Equation (1.1), chiral selectors used in CLEC CSPs are typically bidentate; that is, they are designed to offer two functional groups for chelation with two of the planar coordination sites of the metal ion. The enantiomers to be separated are also bidentate to achieve chiral recognition. As a result, CLEC columns are particularly well suited to separating enantiomers of amino acids, hydroxy carboxylic acids, β-amino alcohols, etc. [18,106,107].

In the next section, a more comprehensive review of CLEC chemistry and technology is provided.

1.3 Chiral Ligand Exchange Chromatography

The pioneering work in chiral ligand exchange chromatography (CLEC) was carried out by Davankov and Rogozhin [108]. They immobilized L-proline on polystyrene beads and, through formation of bidentate ternary complexes in the presence of copper (II) ions, separated a number of amino-acid enantiomers. Since then the field of chiral ligand-exchange chromatography has expanded to include a wide range of chiral selectors and column configurations [109-111]. These methods can be generally classified into two broad categories: (1) chiral stationary phases (CSPs), and (2) achiral stationary phase systems containing a chiral mobile phase (CMP) additive.

1.3.1 Chiral Stationary Phases

CLEC CSPs exploit an immobilized chiral selector capable of complexing with a transition metal ion to create an activated ligand that provides chiral recognition. Numerous CSPs have been designed and synthesized. They can be divided into two general classes:
1.3.1.1. Covalently Grafted CSPs

In most cases, porous microparticle silica is used as the support matrix onto which the chiral selector is covalently grafted. However, Davankov and co-workers [112] synthesized and characterized a wide range of polystyrene-based stationary phases specifically designed to separate racemates of amino acids. Their results revealed that the most promising ligand exchangers contain cyclic amino acids, such as proline and hydroxyproline, and utilize the copper (II) ion as the complexing agent. Gübitz and coworkers [106,113] established robust chemistry to commercialize this technology, utilizing silica-bonded stationary phases to present derivatized cyclic amino-acid ligands, the most extensively used and characterized being L-proline. These columns efficiently resolve most α-amino acids (enantioselectivities (α) up to 3.0), as well as dansyl amino acids, dipeptides and many barbiturates.

CLEC CSP technology has since matured to permit application to a much broader range of compounds. Galli and coworkers [114] covalently bonded (S) or (R)-phenylalanineamide on silica to achieve selectivities up to 3 for an impressive range of amino acid derivatives. Hyun et al. [115] developed a new ligand exchange chiral stationary phase by covalently bonding R-N,N-carboxymethyl undecyl phenylglycinol mono-sodium salt to silica gel that can be used to resolve most α and β-amino acid racemates [110,115]. These and several other CLEC CSPs [116,117] are commercially available: for example, the Chiral ProCu (immobilized proline) and Chiral ValCu (immobilized valine) columns from Serva, and the Nucleosil Chiral-1 (immobilized hydroxyproline) column by Macherey-Nagel [118].
1.3.1.2. Physically Immobilized CSPs

Providing a much simpler route to CLEC CSP formation, physical immobilization of a chiral selector to an achiral chromatography matrix has been widely utilized [20,119-121], including work by Davankov [122], who modified a Lichrosorb RP-18 column (a C18 reverse-phase column) by coating it with n-alkyl derivatives of L-histidine. The alkyl tail (where the alkyl group is typically n-C7H15, n-C10H21 or C16H31) provides for irreversible adsorption of the chiral selector on the hydrophobic interface layer of the reversed phase packing material, presumably by integrating with the C18H37 chains of the achiral resin. This immobilization strategy directs the hydrophilic amino acid head group of the chiral selector towards the polar mobile phase to allow for formation of ternary mixed-ligand complexes. Using this approach, Öi et al. [123] were able to separate various carboxylic acids and amines on C18 columns presenting physisorbed N-S-dioctyl-D-penicillamine. Nikolić and coworkers [124] used the same principle to separate diastereomers of exametazime (a drug used for the diagnosis of various brain disorders such as stroke or vascular disease). They achieved complete resolution of all isomers using N-N-dimethyl-L-phenylalanine as chiral selector adsorbed on a C-18 reversed phase column. Remelli et al. [119] applied an octadecyl silica (ODS) reversed phase column coated with N'-n-decyl-L-histidine as a chiral selector to separate most amino-acid racemates. Important experimental parameters such as flowrate, temperature and mobile phase concentration were investigated, allowing them to achieve very efficient separations. Śliwka et al. [125] used S-phenylglycinol derivatives loaded onto an octadecyl silica (ODS) column to study the influence of alkyl tail chemistry on CLEC column performance. Wan and coworkers [120] performed similar studies on porous graphite carbon with a series of N-substituted L-proline chiral selectors. In both studies, an increase in the chain length of the alkyl N-substituents resulted in improved enantioselectivity.
1.3.2 Chiral Mobile Phase Additives

Although sometimes used at the analytical scale, chiral mobile phase (CMP) additives are rarely used at the preparative scale. The presence of the chiral selector in the eluent tends to compromise product detection and its removal requires an extra purification step. I therefore provide only a brief overview of this technology.

Lepage et al. [126] introduced the CMP method by using chiral triamines (L-2-ethyl and L-2-isopropyl-4-octyldiethylenetriamine) as mobile phase additives in the presence of Zn(II) to resolve a series of dansyl amino acids. The determining factor for enantiomer recognition is the lipophilic interaction of the diastereomeric mixed ternary complex with the achiral column. The concept has since been advanced through a variety of studies [127,128], including work by Galaverna and coworkers [129], who achieved chiral discrimination of amino acids, with enantioselectivities ranging between 1.13 and 2.3, using copper (II) complexes of L-amino acid amides added to the eluent. Similarly, the use of L-phenylalanine as a mobile phase additive has been shown to provide excellent resolution of dopa racemate on a LiChrosorb (C-18) column [130].

Non-amino acid CMAs have found numerous applications [131,132]. Lepage et al. [126] studied complexation of triamine additives with zinc and other transition metal ions for the separation of dansyl-derivatives of amino acids on reversed phase columns. Aminophosphonic acids, which are phosphorous analogs of amino acids, have also been used as CMAs. For example, Belov and coworkers [133] successfully used aqueous solutions of the monoethyl ester N-(1-hydroxymethyl) propyl-α-aminobenzyl-phosphonic acid for the enantiomeric resolution of amino acids on an ODS column.
1.3.3 Medical Applications of CLEC

CLEC is widely used for separating enantiomers of barbiturates, pyridone carboxylic acids (e.g., the chemotherapeutic agent ofloxacin), α-amino acids, dansyl amino acids, hydroxy acids, peptides, amino alcohols, and many potent alkaloid, β-blocker and other adrenergic drugs such as ephedrine, norephedrine, and epinephrine [40,106,114,118,134]. For example, after derivatization with bromoacetic acid, β-blocking agents create strong ternary mixed-ligand complexes with amino acids and their derivatives [107]. Enantioselectivities as high as 2.7 have been reported for different amino alcohols such as ephedrine and adrenaline separated on covalent CSP columns packed with Si-L-proline or Si-L-hydroxyproline using a mobile phase of 80% potassium dihydrogen phosphate containing 0.1 mM Cu(II) and 20% methanol. Similarly, thyroid hormones, including thyroxine, triiodothyronine and diiodothyronine are efficiently separated on CLEC CSP columns using L-proline chemically bonded to porous silica [135]. Finally, the Nucleosil chiral-1 column, which presents chemically bonded L-hydroxyproline Cu$^{2+}$ complexes on silica, is used to separate enantiomers of an anti-HIV nucleoside (a chiral nucleoside consisting of a guanine nucleus attached to a pseudo-sugar) [136].

1.4 Characterization and Modeling of CLEC

The use of CLEC in the purification of amino acid enantiomers and chiral therapeutics has motivated intense investigation of the fundamental principles of CLEC and the mechanism of chiral recognition. As a result, although the separation mechanism is generally simpler in other modes of chiral chromatography, CLEC remains the best investigated technique from a theoretical point of view. Theories of chiral recognition in CLEC columns began with the
work of Davankov and Rogozhin [137], who proposed a model for predicting the elution order of α-amino acids. Their model screens different ligand orientations to predict the relative stabilities of the two ternary mixed-ligand complexes. Steric-packing effects, hydrophobic interactions, and interactions with the underlying achiral stationary phase are included in this prediction, indicating that Davankov recognized that retention times and the order of elution can be influenced by weak intermolecular interactions with the support material. Although only qualitative in nature, the selection rules proposed by Davankov and Rogozhin correctly predict the elution order of α-amino acid enantiomers on many CLEC columns [138-140]. However, exceptions to these rules are known to exist [23,125,141].

Chilmonczyk et al. [142] used quantum mechanical semiempirical and density functional theories to address limitations to Davankov and Rogozhin's selection rules which are based on a molecular model of the ternary complex that accounts for the first coordination sphere of Cu(II) cation but not for all steric interactions involving the side chains of the chiral ligand and the selector. As a result, Chilmonczyk et al. argue that the original model is too crude to interpret enantiomer elution order and propose improvements to better represent the influence of the stationary phase, the solvent, and the ligand and selector side chains. While this more advanced model certainly improves prediction of elution order in CLEC separations, it is not designed to provide a prediction of elution chromatograms, including retention times, band profiles, and resolution. Such predictions require a model that accounts for momentum and mass transfer, chemical equilibria, and stationary phase architecture within the column.

Building on the classic plate-theory model of chromatography proposed by Martin and Synge [143,144] and the rate theory of chromatography pioneered by van Deemter [145],
Giddings [146] and Acrivos [147] established the complete set of transport and reaction criteria required to quantitatively connect elution band profiles to the underlying mass-transfer resistances. They each showed that solute mass transport within the interstitial volume of a column can be modeled with the now widely accepted one-dimensional continuity equation of chromatography

\[
\frac{\partial c_i}{\partial t} = D_L \frac{\partial^2 c_i}{\partial z^2} - \frac{u_c}{\varepsilon} \frac{\partial c_i}{\partial z} - \frac{1 - \varepsilon}{\varepsilon} \frac{\partial s_i}{\partial t}
\]  

(1.2)

where \( c_i \) and \( s_i \) are the concentration of solute \( i \) in the mobile and stationary phase, respectively, \( D_L \) is the axial dispersion coefficient, \( u \) is the mobile phase velocity, \( \varepsilon \) is the column void fraction, and \( z \) and \( t \) are axial position and time. The column is assumed to be uniformly packed, and due to the large column aspect ratio \( (L/R_c > 100) \) and high flow resistance, solvent velocities and solute concentration gradients in the radial direction can be neglected, allowing the column to be modeled as a one-dimensional system with a uniform axial velocity profile. In Chapter 4 of this thesis, I will show that differential mass balance equations may also be written to describe solute transport though the stagnant fluid film at the surface of each stationary phase particle, as well as within the stationary phase particle itself [148,149]. If the solute binds to one or more ligands presented on the surface of the stationary phase, the solution of Equation (1.2) requires an expression quantifying the rate of binding. Often, Langmuir-type reaction kinetics are assumed

\[
\frac{\partial q_i}{\partial t} = k_1 (q_{\text{max}} - q_i) c_i - k_{-1} q_i
\]  

(1.3)
where $q_t$ and $q_{\text{max}}$ are the current and maximum concentration of the adsorbed solute, and $k_1$ and $k_{-1}$ are the forward and reverse rate constants for adsorption, respectively. In many column chromatography processes, the rates of solute adsorption and desorption are fast compared to the rate of solute transport to the stationary phase surface. The local equilibrium approximation (i.e., $\frac{dq_t}{dt} = 0$) can be invoked, reducing Equation (1.3) to the well-known single-solute Langmuir adsorption isotherm equation

$$q_t = \frac{K_L q_{\text{max}} c_i}{1 + K_L c_i} \quad (1.4)$$

where $K_L(=k_1/k_{-1})$ is the Langmuir adsorption equilibrium constant. In certain chromatography applications a more complex isotherm relation is required to properly quantify adsorption equilibria [150-155]. For example, although its theoretical basis is questionable, the two-site Langmuir isotherm is often used to account for nonspecific interactions of the solute with the stationary phase [156,157]. Extension of the Langmuir equation to treat multicomponent adsorption is straightforward [158,159], allowing one to model both the nonlinear behavior of the isotherm at high solute concentrations and the competition between different solute molecules for binding sites on the stationary phase [160-163].

A comprehensive model of CLEC based on solution of Equation (1.2) has not been proposed. However, a few models have been proposed for other modes of chiral chromatography [164,165]. Jacobson et al. [166], for example, combined Equation (1.2) with a competitive two-site Langmuir-type isotherm to model elution bands of benzoylalanine
enantiomers separated on an immobilized bovine serum albumin column. More recently, Mihlbachler et al. [167] proposed a general quadratic equilibrium isotherm which accounts for both competitive adsorption of the enantiomers and nonspecific interactions with the stationary phase to model the separation of Trögers base on a Chiralpak AD column that utilizes amylose tri-(3,5-dimethylphenyl carbamate) as the CSP selector. Finally, Cavazzini et al. [165] used the binary two-site Langmuir isotherm to model elution band profiles for enantiomers of 1-phenyl-1 propanol on a Chiracel OB column (cellulose tribenzoate coated on silica support) under overload conditions.

The separation of enantiomers by CLEC is known to depend strongly on column operating conditions [168,169]. In the absence of an appropriate fundamental model, characterization of and fundamental insights into the CLEC separation process have largely been achieved through experiment [111,170]. Temperature, solvent composition and pH, metal-ion concentration, and the concentrations of enantiomers affect the separation [18,171]. The general dependence of retention time and resolution on each of these operating variables is reasonably well understood, thereby laying a firm foundation for model development [20,110].

CLEC distinguishes itself from other modes of chromatography through the unusually complex chemical equilibria within the column. Consider, for example, the separation of enantiomers of an α-amino acid bearing an uncharged side chain. The amino acid therefore possesses two protonatable groups and can exist in three unique charge states in aqueous solution (Figure 1.4). In addition, each enantiomer may form a mono-binary and two unique bis-binary complexes with the transition metal ion, usually Cu(II) (Figure 1.5). The immobilized chiral selector also exists in at least two different charge states and can form
*mono*- and *bis*-binary complexes with the Cu(II) ion. Formation of the stereoselective ternary mixed-ligand complex at the stationary phase therefore requires the dissociation or displacement of existing equilibrium complexes (Figure 1.6).

A model for CLEC, particularly one capable of predicting the dependence of separation performance on column operating variables, must therefore consider the influence of the complex chemical equilibria governing the ligand exchange environment, as well as momentum and mass transfer within the column. The development of such a model is the goal of this thesis.

1.5 Research Objectives

The objective of this thesis is to interpret and model chiral-ligand exchange chromatography by combining the theory of multiple chemical equilibria with the one-dimensional reaction-diffusion theory of column chromatography. Accounting for all reaction equilibria within a CLEC column necessarily requires the equilibrium constant and reaction stoichiometry for each complex formed in the system. In general, the chromatography experiment does not provide accurate enough data to regress these parameters. As a result, chromatography models rarely include a comprehensive description of all reaction equilibria within both the mobile and stationary phases of the column. Ignoring chemical equilibria within CLEC columns would undoubtedly simplify model development and parameter estimation while permitting prediction of the effect of sample load on column performance [167,172,173]. However, it would restrict model application to a particular set of column operating conditions, and would thereby limit insights into the separation mechanism and its dependence on metal-ion concentration, mobile-phase pH, etc.
In this thesis, I exploit the fact that the parameters required to specify chemical equilibria within CLEC columns can be taken from standard thermodynamic databases such as the NIST database [174] and the SC-database [175]. The number of entries in each of these databases is growing rapidly, yet little attention has been given to using these fundamental equilibrium data to interpret and model chromatographic separations. Moreover, simple, inexpensive and reliable techniques exist for measuring required thermodynamic parameters, making it possible to rapidly determine any formation constants not currently available in the standard tables.

This model development strategy is first applied to the separation of α-amino acids on a CSP bearing a derivative of L-hydroxyproline as the chiral selector. The model is then applied to a medically relevant separation: the resolution of dopa enantiomers on the Chirex 3126 column in which immobilized N-octyl-3-octythio-D-valine, a derivative of D-penicillamine, serves as the chiral selector. Chapter 2, entitled “Formation constants and coordination thermodynamics for binary and ternary complexes of copper (II), L-hydroxyproline and an amino acid enantiomer” demonstrates how the potentiometric titration experiment, when coupled with an appropriate chemometric model, can be used to rapidly and accurately measure equilibrium formation constants for complexes formed in CLEC separations. Chapter 3, entitled “Interpreting the effects of temperature and solvent composition on the separation of amino-acid racemates by chiral ligand exchange chromatography”, defines how these fundamental thermodynamic data can be combined with multiple chemical equilibria theory to model and interpret thermal and solvent-composition effects in CLEC separations. The comprehensive model of CLEC that combines multiple chemical equilibria theory with the reaction diffusion theory of chromatography is presented in chapter 4, entitled “A multiple
chemical equilibria approach to modeling and interpreting the separation of amino acid enantiomers by chiral ligand-exchange chromatography”. The application of the model to a medically relevant separation is reported in chapter 5, entitled “Modeling profiles and separation of dopa-enantiomer elution bands during chiral ligand exchange chromatography”. The thesis then concludes with chapter 6, where the anticipated impact and future areas of application of this work are discussed.
1.6 Tables

Table 1.1 Worldwide sales of single-stereocentre enantiomer drugs

<table>
<thead>
<tr>
<th>YEAR</th>
<th>2001</th>
<th>2002</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Single</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Enantiomer</td>
<td></td>
</tr>
<tr>
<td>$ Billions</td>
<td>Total Market</td>
<td>Sales</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>$33.0</td>
<td>$26.9</td>
</tr>
<tr>
<td>Hematology</td>
<td>16.5</td>
<td>13.5</td>
</tr>
<tr>
<td>Cancer</td>
<td>17.0</td>
<td>13.3</td>
</tr>
<tr>
<td>Hormones</td>
<td>26.0</td>
<td>18.5</td>
</tr>
<tr>
<td>Cardiovascular</td>
<td>50.0</td>
<td>30.2</td>
</tr>
<tr>
<td>Vaccines</td>
<td>8.5</td>
<td>4.3</td>
</tr>
<tr>
<td>Antiviral</td>
<td>20.0</td>
<td>6.1</td>
</tr>
<tr>
<td>Ophthalmic</td>
<td>8.0</td>
<td>2.4</td>
</tr>
<tr>
<td>Nervous System</td>
<td>55.0</td>
<td>10.5</td>
</tr>
<tr>
<td>Respiratory</td>
<td>42.0</td>
<td>7.9</td>
</tr>
<tr>
<td>Gastrointestinal</td>
<td>50.0</td>
<td>5.4</td>
</tr>
<tr>
<td>Dermatology</td>
<td>18.5</td>
<td>1.2</td>
</tr>
<tr>
<td>Analgesics</td>
<td>23.5</td>
<td>1.2</td>
</tr>
<tr>
<td>Others</td>
<td>42.0</td>
<td>6.8</td>
</tr>
<tr>
<td>TOTAL</td>
<td>$368.0</td>
<td>$139.3</td>
</tr>
</tbody>
</table>
1.7 Figures

Figure 1.1  Example of chirality: ritalin enantiomers

\[
\begin{align*}
&\text{R-Enantiomer} \\
&\text{S-Enantiomer}
\end{align*}
\]

Figure 1.2  Thalidomide enantiomers

\[
\begin{align*}
&(S)-\text{N-phthalyglutamic acid amide} \\
&(R)-\text{N-phthalyglutamic acid amide}
\end{align*}
\]
Figure 1.3 Structure of the ternary complexes formed between the ligand selector (L-hydroxyproline), metal ion and the two enantiomers (Top: L-proline•Cu•L-Hydroxyproline and Bottom: D-proline•Cu•L-Hydroxyproline) with water molecule binding to the axial coordination sites of the metal.
Figure 1.4  Fully protonated and zwitterionic forms of an amino acid bearing an uncharged side chain.

Figure 1.5  *Mono*-binary (left) and *bis*-binary (right) complexes formed in a CLEC system containing the Cu(II) ion.
Figure 1.6 Species distribution plot for a ternary mixture of L-proline (10 mM), Cu(II) (10 mM), the chiral selector L-hydroxyproline (10 mM) in aqueous solution at 298.15 K containing 0.1 M KNO₃. Species abcde indicates the molecules of Cu²⁺ (a), protons (b), L-proline (c), D-proline (d), and L-hydroxyproline (e) in the complex: filled triangles - species 01100, filled squares - species 02100, filled stars - species 10100, filled circles - species 10200, open triangles - species 01001, open squares - species 02001, open stars - species 10001; open circles - species 10002, open inverse triangle - species 10101.
1.8 References


2 Formation Constants and Coordination Thermodynamics for Binary and Ternary Complexes of Copper (II), L-Hydroxyproline and an Amino-Acid Enantiomer*

2.1 Introduction

Amino acids are important low molecular weight ligands in humans [1-4] and other biosystems [5,6]. Their involvement in Cu(II) transport and metabolism is well documented [7,8]. In addition, the reactivity of amino acids and other small organic molecules is often modified when they are coordinated to metal ions. Nature exploits this effect through the construction of metal-ion binding cavities within the active sites of many enzymes (e.g., pyruvate kinase, superoxide dismutase) that serve to accelerate reactions that would otherwise proceed too slowly to be useful in a living system. For example, the extraordinarily high rates of cleavage observed for the enzymatic hydrolysis of carboxylic esters and amides can often be accounted for through the effect of a bound metal ion acting either as a Lewis acid catalyst by coordinating to a carbonyl oxygen atom and thereby polarizing the carbonyl group, or as a source of a nucleophile through coordination to a hydroxide ion [9].

Transition-metal-ion chelate complexes are also exploited by industry in the large-scale purification of α-amino acids and a wide range of drugs and drug precursors containing an aminocarboxylic acid moiety [10,11]. Chiral ligand-exchange chromatography, which utilizes stereoselective binding to an immobilized chiral ligand (selector), is widely used in industry for the separation of racemic mixtures of amino acids and their derivatives [12,13]. Newer

technologies for scalable continuous separation of chiral racemates present the selector either directly in solution [14] or on the surfaces of stable micelles [15,16]. These industrial applications are often best carried out at temperatures far from ambient or physiological conditions. Efficient design and optimization of these technologies therefore require knowledge of chemical equilibria within the system and its dependence on temperature.

In principle, acidic, polar and basic amino acids can bind metal ions through a mixture of available donor groups that include the $\alpha$-carboxylate and $\alpha$-amino groups, as well as appropriate groups on the side chains of the $\alpha$-aminocarboxylic acid: for example, the $\beta$-hydroxy group on the side chains of threonine and serine, the $\beta$-thiol on cysteine, and the $\beta$- and $\gamma$-amides on asparagine and glutamine, respectively. While electron donors on the amino-acid side chain can be effective ligands, glycine-like bidentate coordination through the $\alpha$-carboxylate and $\alpha$-amino groups is often favored thermodynamically among amino acids at neutral and acidic pH.

Potentiometry experiments have provided reliable protonation constants and formation constants for many binary metal-ion(amine acid) complexes in aqueous solutions of 0.1 M or 0.15 M (physiological) ionic strength at 293.15 K, 298.15 K or 310.15 K [17,18]. KNO$_3$ or NaClO$_4$ is most often employed as the background electrolyte. In certain cases, coordination enthalpies and entropies are reported at either 298.15 K or 310.15 K [19-22]. The decomposition of a formation constant into its enthalpic and entropic contributions is of fundamental importance to understanding the factors that influence the coordination reaction and the stability of the complex. These factors may include solvation, steric and electronic
effects. Although they can be measured more accurately and directly by calorimetry, coordination enthalpies $\Delta_r H_c$ at a given temperature are most often obtained through measurement of formation constants ($K_i$) at surrounding temperatures and application of the van’t Hoff equation,

$$\frac{d(\ln K_i)}{d(1/T)} = -\frac{\Delta_r H_c}{R}$$

(2.1)

This approach provides a good understanding of the thermodynamics of the complexation reaction at the given temperature, but extension to other temperatures is limited by the fact that coordination enthalpies (and entropies) are temperature dependent, and the $\Delta C_P$ data required to determine coordination thermodynamics at other temperatures through application of the Kirchoff equation are generally not available.

We report concentration (i.e. concentration-based) protonation constants and formation constants for Cu(II)(amino-acid) complexes from 288.15 to 333.15 K for leucine, valine, proline, phenylalanine, and hydroxyproline in aqueous solutions containing 0.1 M KNO₃. These data are obtained by potentiometry and used to evaluate nonlinearities in the van’t Hoff plot and to estimate $\Delta C_P$ values for each complex formed. Coordination enthalpies and entropies are then computed as a function of temperature to evaluate the factors that influence the stability of a given complex and the relative dependence of these factors on temperature. Statistical effects in the formation of binary and ternary complexes are also considered and discussed. Particular attention is paid to complexes containing L-hydroxyproline, as this ligand is often used as the immobilized selector in chiral ligand-exchange chromatography.
2.2 Materials and Methods

2.2.1 Materials

Aminocarboxylic acid enantiomers, nitric acid (0.0983 M HNO₃ standard), potassium nitrate (KNO₃), and copper nitrate (Cu(NO₃)₂) were purchased from Sigma-Aldrich Chemicals Canada Ltd. (Oakville, ON). These reagents have reported purities of greater than 99% and were used without further purification. For the preparation of all aqueous solutions, water was first double distilled and then treated with a NANOpure® II ultrafiltration system (Barnstead; Dubuque, IW). KOH standard (0.1 M) was prepared by diluting KOH Titrisol ampoules (Merck) according to the manufacturer’s instructions. All reagents and solutions were prepared immediately prior to use in an experiment.

2.2.2 Potentiometric Titrations

All potentiometric titrations were carried out in an electrically insulated Schott Titronic T110 automatic titrator (Schott Instruments, Germany). Premixed samples were introduced into the 3 mL or 10 mL water jacketed titration vessel and maintained at the desired reaction temperature by an external circulating water bath (Julabo-UC circulator, Germany) and a Teflon-coated magnetically driven stir-bar. The titration cell was maintained under nitrogen atmosphere, and aliquots of the base titrant solution (5-20 μL) were introduced into the reaction cell using a Schott TA01 automatic burette. The solution pH was measured continuously with a Metrohm micro-combination pH glass electrode (Brinkmann Instruments Ltd., Mississauga, ON).
The standard-electrode potential \( (E^\circ) \) was determined immediately prior to each titration. A standard stock solution of 10 mM HNO\(_3\) solution was prepared by adding KNO\(_3\) as a background electrolyte to create a degassed 0.1 M solution (p[H\(^+\)] = 2). The electrode was then calibrated according to the classic procedure of Carpeni et al. [23] by titrating 5.0 mL of this HNO\(_3\) solution with a 0.1 M KOH standard for which the concentration of KOH was verified potentiometrically by titration against a 10 mM potassium hydrogen phthalate (KHP) primary standard \((I_c = 0.1 \text{ M})\). The resulting titration data were analyzed using the non-linear least-squares program CHEMEQ [24] which regresses \( E^\circ \) as well as the initial total proton concentration in the vessel.

Amino-acid protonation constants were determined by titrating solutions of 0.1 M KNO\(_3\) (ca. p[H\(^+\)] = 2) containing 10 mM of the pure amino acid. Similarly, determination of concentration formation constants for binary complexes was based on a 0.1 M KNO\(_3\) solution containing 10 mM amino acid and 5 mM Cu(NO\(_3\))\(_2\); solutions for determination of ternary concentration formation constants contained equimolar (ca. 10 mM) concentrations of each aminocarboxylic acid ligand and Cu(NO\(_3\))\(_2\). The concentration of Cu\(^{2+}\) in stock solutions of Cu(NO\(_3\))\(_2\) was determined by titration with a standard EDTA solution using 1-(2-pyridylazo)-2-naphthol (PAN) indicator dye. A defined volume of copper stock solution, diluted in 5.0 mL de-ionized water, 200 \( \mu \text{L} \) of 0.1 M HNO\(_3\), and 2-3 drops of PAN (3% solution in methanol), was titrated by drop-wise addition of EDTA stock solution to equivalence, which was indicated by a color change from a light purple to light gray. In all titration experiments, a premixed 0.1 M KOH standard solution (Merck) served as the titrant and was sequentially added in aliquots of either 5 or 10 \( \mu \text{L} \) to the titration vessel by volumetric pipette. After equilibrium was reached, the electrode potential and the amount of the base added to the system were recorded.
2.2.3 Data Regression

Both standard formation constants (also known as stability constants), denoted by the symbol $\beta$, and stepwise stability constants, denoted by the symbol $K$, are reported in this work. To fix ideas, consider the binary metal ($M$) - ligand ($L$) complex $ML_n$. The reaction

$$M + nL \overset{\beta_{10n0}}{\rightleftharpoons} ML_n$$

may be used to define the standard formation constant $\beta$ for the complex

$$\beta_{10n0} = \frac{[ML_n]}{[M][L]^n} \quad (2.2)$$

which is defined in terms of equilibrium concentrations of the reactants. We therefore designate $\beta_{0n01}$ and all others reported in this work as concentration formation constants to clearly differentiate them from standard (state) formation constants based on the activities of the reactants. The subscript $10n0$ on the $\beta$ defined in Equation (2.2) indicates the molecules of Cu$^{2+}$ (1), protons (0), amino-acid enantiomer (n) and L-hydroxyproline (0), respectively, present in the complex. A negative number in the second subscript register indicates the presence and number of hydroxyl ions in the complex.

The stepwise (concentration) formation constant for the same binary $ML_n$ complex may be defined from the reaction
\[ ML_{n-1} + L \rightleftharpoons K_n ML_n \]  \hspace{1cm} \text{(Constant } T \text{ and } P) \\

as

\[ K_n = \frac{[ML_n]}{[ML_{n-1}][L]} \]  \hspace{1cm} (2.3)

From the above definitions it is evident that

\[ \beta_{10n0} = K_1 K_2 \ldots K_n = \prod_{i=1}^{n} K_i \]  \hspace{1cm} (2.4)

Protonation constants and concentration formation constants were regressed from potentiometric data using the program \textit{CHEMEQ} previously developed by our group [24]. \textit{CHEMEQ} minimizes the weighted sum \( U \) of the squared residuals between observed \( E_i^{obs} \) and calculated \( E_i^{calc} \) electrode potentials

\[ U = \sum_{i=1}^{m} \left( \frac{E_i^{obs} - E_i^{calc}}{\sigma_i} \right)^2 = \sum_{i=1}^{m} W_i \left( E_i^{obs} - E_i^{calc} \right)^2 \]  \hspace{1cm} (2.5)
The weighting factor ($W_i$) is defined as the reciprocal of the square of the total estimated error ($\sigma_i$) due to errors in the electrode potential ($\sigma_E = 0.1$ mV), reagent concentrations ($\sigma_C = 0.01$ mM), and the titrant volume readings ($\sigma_v = 0.002$ cm$^3$) [25].

$$\sigma_i^2 = \sigma_E^2 + \left( \frac{\partial E_i^{\text{obs}}}{\partial C} \right)^2 \sigma_C^2 + \left( \frac{\partial E_i^{\text{obs}}}{\partial V} \right)^2 \sigma_V^2$$

(2.6)

Since the electrode potential is not an explicit function of the equilibrium formation constants, the implicit differentiation path of Nagypál and Páka [26] was applied in a manner similar to that employed by Gans et al. [27] in their multi-parameter regression program SUPERQUAD, which includes a Gauss-Newton algorithm and the Levenburg-Marquardt technique to improve convergence.

The program CHEMEQ improves upon the implicit differentiation routine of Gans et al. [27] by establishing methods to greatly reduce computational time when certain components are not present in a given complex, to reduce round-off errors due to redundant matrix elements, and to improve parameter regression statistics. A detailed description of these improvements is provided elsewhere [24]. Each reported parameter (protonation constant, concentration formation constant) was regressed from the combined titration data for 10-15 experimental runs, with the standard deviation computed and reported in each case.
2.3 Results and Discussion

2.3.1 Protonation Constants

The side chains of all amino acids studied in this work are charge neutral. Each amino acid \( A^- \) therefore contains two protonation sites characterized by the following stepwise equilibria

\[
\begin{align*}
H^+ + A^- & \rightleftharpoons HA & \text{designated by } K_1, \Delta_r H^c(K_1), \text{ and } \Delta_r S^c(K_1) \\
HA + H^+ & \rightleftharpoons H_2A^+ & \text{designated by } K_2, \Delta_r H^c(K_2), \text{ and } \Delta_r S^c(K_2)
\end{align*}
\]

where \( K_1 \) refers to protonation of the \( \alpha \)-amino group and \( K_2 \) to that of the \( \alpha \)-carboxylate group.

Table 2.1 reports the \( \log_{10} \) value of the standard protonation constants (\( \beta_{0110}, \beta_{0210} \)) for leucine, valine, proline, phenylalanine, and hydroxyproline as functions of solution temperature. The standard deviation is provided next to each protonation constant. Martell and Smith [28] report protonation constants for all of these amino acids at 298.15 K and \( I_c = 0.1 \) M, and our results are in good agreement with their values at that solution temperature. For example, the values of \( \beta_{0210} \) for the \( H_2 \)-valine complex reported in this work and by Martell and Smith [28] are 11.77 ± 0.01 and 11.75 ± 0.02, respectively. Comparative data at other temperatures are available for certain amino acids (proline and hydroxyproline [29,30]) and again are in good agreement with our results.

The more familiar stepwise dissociation constants (\( pK_a \) values) can be calculated from the measured \( \beta \) values through the relation
\[ pK_{al} = \log_{10} K_2 = \log_{10} \beta_{0210} - \log_{10} \beta_{0110} \]  
\[ pK_{a2} = \log_{10} K_1 = \log_{10} \beta_{0110} \]  

(2.7)  

(2.8)

The Gibbs energy change \( \Delta_r G_c \) for each protonation reaction is related to the respective stepwise protonation constant as follows:

\[ \Delta_r G_c(T) = -RT \ln(K_i) = \Delta_r H_c(K_i) - T \Delta_r S_c(K_i) \]  

(2.9)

where the molar reaction enthalpy \( \Delta_r H_c \) and entropy \( \Delta_r S_c \) at system temperature \( T \) are computed from \( K_i(T) \) data using Equation (2.1) and Equation (2.9), respectively. Figure 2.1 plots \( K_1(T) \) and \( K_2(T) \) data for proline according to the van't Hoff equation (Equation (2.1)). A linear relation is observed, indicating a negligible change in heat capacity for each protonation reaction over the temperature range 288.15 to 333.15 K. As a result, \( \Delta_r H_c \) and \( \Delta_r S_c \) are constant over this temperature range for both protonation reactions. Linear dependencies of \( \ln K_1 \) and \(\ln K_2\) on inverse temperature were also observed for all other amino acids studied.

Table 2.2 reports regressed \( \Delta_r H_c \) and \( \Delta_r S_c \) values for each amino acid studied. Protonation thermodynamics measured by calorimetry have previously been reported at 298.15 K, and our results are in reasonably good agreement with those earlier studies. However, our \( \Delta_r S_c \) values are slightly higher (on average ca. 4.2 to 8.4 J/mol K) than previously measured by calorimetry, possibly due to the intrinsically higher level of error associated with indirectly determining reaction enthalpies from potentiometry data. As our results indicate that \( \Delta_r H_c \) and \( \Delta_r S_c \) remain constant from 288.15 to 333.15 K, the value of \( K_1 \) or \( K_2 \) at any temperature over
this range can be computed from the data at 298.15 K provided in Tables 2.1 and 2.2 using the relation

$$\log_{10} K_i(T) = \log_{10} K_i(T°) + \Delta \text{r}_c \frac{(T - T°)}{2.303RT°}$$

(2.10)

where $T°$ is the reference temperature (298.15 K) and $\Delta \text{r}_c$ is the enthalpy change for the protonation reaction at that temperature. For each amino acid studied, protonation of the $\alpha$-amino group is exothermic, with a $\Delta \text{r}_c(K_1)$ near $-40$ kJ mol$^{-1}$. In contrast, protonation of the $\alpha$-carboxylate group is found to be nearly athermal for each amino acid. Both results are in good agreement with previous results obtained from the calorimetric method [31,32]. Due to the observed exothermic $\alpha$-amino protonation reaction, we find that the concentrations of protonated states of the amino acids will decrease with increasing temperature.

### 2.3.2 Concentration Formation Constants for Binary Complexes

All of the amino acids investigated in this study exhibit glycine-like coordination chemistry with the Cu$^{2+}$ ion, but differences in the electron-withdrawing strengths of the side chains alter binary Cu$^{2+}$(amino-acid) complex stabilities relative to those observed in the Cu$^{2+}$(glycine) complex. Standard binary formation constants for Cu$^{2+}$ - amino acid (A) complexes are defined in this study by the following reaction equilibria:

$$\text{Cu}^{2+} + A^- \rightleftharpoons \beta_{1010} \text{CuA}^+$$

$$\text{Cu}^{2+} + 2A^- \rightleftharpoons \beta_{1020} \text{CuA}_2$$
except in the case of L-hydroxyproline (hereafter referred to as L-HyPro), where the formation constants for the equivalent binary complexes are $\beta_{1001}$ and $\beta_{1002}$, respectively. Standard formation constants at 298.15 K for all binary Cu$^{2+}$ - amino acid complexes are reported in Table 2.3 along with their standard deviations. Sóvágo et al. [33] previously measured formation constants for these binary complexes at 298.15 K, but did not study their temperature dependence. The results published here at 298.15 K are in good agreement with their data.

Given their nonpolar and neutral side chains, the amino acids included in this study are not expected to associate strongly with either the inner or the outer coordination sphere of Cu(II). Not surprisingly then, the stabilities of the two binary homo-chiral complexes (Cu$^{2+}$(L)$_2$ and Cu$^{2+}$(D)$_2$, where L and D represent the Fischer-based chirality of the amino acid) and the binary hetero-chiral complex (Cu$^{2+}$(L)(D)) were found to be equal within our experimental error. For example, the formation constant for the hetero-chiral Cu$^{2+}$(L)(D) bis-binary complex formed at 298.15 K with D-phenylalanine (LPhe-Cu-DPhe) was determined in this study to be $\log \beta_{1020} = 14.42 \pm 0.02$, and that for D-valine (LVal-Cu-DVal), was $\log \beta_{1020} = 14.79 \pm 0.02$ measured in this work. Comparison with the corresponding data for the homo-chiral bis-binary complexes shown in Table 2.3 reveals no statistically relevant difference in stability. However, this is not the case with all binary metal-ion (amino acid) complexes [34]. For example, calorimetry studies by Sharrock and coworkers [35] show that metal coordination of serine is stereoselective due to participation of the hydroxy group in the complexation of Cu(II) through hydrogen bonding to an axially coordinated water molecule.

Stepwise formation constants were also calculated and plotted versus inverse temperature to estimate the enthalpy and entropy change for formation of each binary complex
and to assess the magnitude of any heat capacity change that accompanies the reaction. As with the protonation reactions, the van't Hoff plots were linear for each binary Cu$^{2+}$-amino acid complex studied, indicating the absence of a measurable change in heat capacity (e.g., Figure 2.2). The thermodynamic data reported in Table 2.4 therefore can be assumed valid from 288.15 to 333.15 K. From these data it is evident for each amino acid that formation of the mono- and bis-binary complexes is both enthalpically and entropically favored. As $\Delta_r H^o(K_1)$ and $\Delta_r H^o(K_2)$ are similar for each amino acid (e.g. $\Delta_r H^o(K_1) = -21.8$ kJ/mol and $\Delta_r H^o(K_2) = -22.6$ kJ/mol for proline), differences in the stabilities of the mono- and bis-binary complexes are primarily due to differences in formation entropies. The relatively low values of $\Delta_r H^o(K_1)$ and $\Delta_r H^o(K_2)$ for leucine are surprising, and although great care was taken during our experiments, an error in potentiometric data acquisition for that system cannot be ruled out.

The stepwise thermodynamic data in Table 2.4 indicate that formation of the mono-binary complex is favoured over the bis-binary complex. We verified this observation and the role of entropy in the two binary complexation reactions by considering an appropriate energy cycle for converting the bis-binary complex into two mono-binary complexes

$$\text{Cu}^{2+} + \text{CuL}_2 \leftrightarrow K_B \rightarrow 2\text{CuL}^-$$

where $K_B$, the equilibrium constant for the bis-complex conversion reaction, is given by the ratio $K_1/K_2$ in which $K_1$ and $K_2$ are the concentration formation constants for the stepwise complexation reactions

$$\text{Cu}^{2+} + \text{L}^- \leftrightarrow K_1 \rightarrow \text{CuL}^+$$
\[
L^- + CuL^+ \xrightarrow{K_2} CuL_2
\]

Thermodynamic changes for the conversion of the *bis*-binary complex to the *mono*-binary complex are therefore given by

\[ \Delta_r G_B = \Delta_r G_c (K_1) - \Delta_r G_c (K_2) = -RT \ln K_B \] (2.11)

\[ \Delta_r H_B = \Delta_r H_c (K_1) - \Delta_r H_c (K_2) \] (2.12)

\[ \Delta_r S_B = \frac{\Delta_r H_B - \Delta_r G_B}{T} \] (2.13)

This analysis confirms that the *mono*-binary complex is energetically favored \((\Delta_r G_B < 0)\) due to a large and favorable \(\Delta_r S_B\) (Table 2.5). The origin of the higher entropy of the *mono*-binary complex can be understood, at least in part, by considering statistical effects [36]. Bjerrum [37] was the first to study the factors that influence consecutive complex formation by dividing the energetics of the *bis*- to *mono*-binary conversion reaction into contributions made by 'statistical effects' and 'residual ligand effects' (i.e. non-ideal effects). This division of energetic contributions recognizes that when the coordination sites of the central metal ion are completely equivalent and remain so during the formation of successive complexes, the ratio of the stepwise formation constants can be determined solely from reaction statistics. The Gibbs energy change for the conversion reaction is then given by
\[ \Delta r G_B = \Delta G_{stat} + \Delta G_r = -RT \ln K_{stat} + \Delta G_r \] (2.14)

where \(K_{stat}\) represents the enhancement in the stability constant of the *mono*-binary complex due to statistical (ideal) effects, and \(\Delta G_r\) is the contribution to the Gibbs energy change due to residual (non-ideal) ligand effects. Statistically, the tendency of complex \(CuL_n\) to split off a ligand is proportional to the number of ways in which the ligand may be removed. Similarly, the tendency to add a ligand to form \(CuL_n\) is proportional to the number of ways in which the ligand may be inserted into the available \(Cu^{2+}\) coordination sites. Using these rules, Bjerrum [37] and Beck [38] have shown for bidentate-ligand binding to \(Cu^{2+}\) that \(K_{stat} = K_{1,stat}/K_{2,stat} = 8\), so that \(\Delta G_{stat} = -5.16 \text{ kJ/mol}\) and \(\Delta S_{stat} = R \ln(K_{stat}) = 17.30 \text{ J/mol K}\). Statistical and residual ligand-effect contributions to \(\Delta_r G_B\) and \(\Delta_r S_B\) are reported in Table 2.5. Although statistical effects explain much of the higher stability of the *mono*-binary complex, non-ideal entropy effects \((\Delta_r S_B)\) are seen to provide an additional stabilizing effect. This can be explained, at least in part, by the negative hydration entropy \((\Delta S_{hyd} = -455.1 \text{ J/mol K})\) for the \(Cu^{2+}\) ion [39], which is known to be a strong kosmotrope (*i.e.*, structure maker). Solvent entropy is therefore increased during formation of a *mono*-binary complex from a *bis*-binary complex as the reaction involves removal of the hyperstructured coordination shell of water molecules solvating the free copper (II) ion [40].
2.3.3 Concentration Formation Constants and Thermodynamics for Ternary Complexes

Stereoselectivity is observed in ternary complexes containing an L- or D-amino acid, Cu$^{2+}$, and the chiral selector L-HyPro (Table 2.6). To fix ideas, we focus on the energetics of the Cu$^{2+}$(Phe)(L-HyPro) complex. In aqueous solution, Cu(II) is tetragonally coordinated by four water molecules, and by two additional water molecules positioned axially and further away from the copper. Replacement of the tetragonally coordinated water molecules with two chemically dissimilar bidentate amino-acid ligands is known to lock the backbone of each ligand into the Cu$^{2+}$ coordination plane. For the solid Cu(Phe)(L-HyPro) ternary complex, Chen et al. [41] have reported that the rigid planar structure of the coordinated backbone of D-Phe results in an unfavorable steric interaction between the phenyl ring and the $\alpha$-carboxylate group; from this it may be inferred that there is a decrease in stability of the hetero-ternary complex relative to the homo-ternary complex. Subtle differences in the spatial geometry of the homo- and hetero-ternary complexes can therefore lead to differences in the respective enthalpy and entropy of complex formation [42]. As shown in Table 2.6, the difference between the concentration equilibrium ternary formation constants for the homo- and hetero-complexes is typically small. Nevertheless, Davankov [43,44] and others [45-47] have shown that the resulting Gibbs energy difference ($\delta\Delta_r G_c = \Delta_r G_{Cu(L')(L-HyPro)} - \Delta_r G_{Cu(D')(L-HyPro)}$) can be used to separate amino-acid racemates by chiral ligand-exchange chromatography (CLEC) or by various electrophoretic modes of chiral chromatography. Our results indicate that in general, the preference of the L-HyPro ligand for either the L- or D-enantiomer of a given amino acid is maintained with a change in solution temperature. However, for leucine, the stereoselectivity of the L-HyPro ligand is seen to invert with increasing temperature,
suggesting that the distribution and energetics of accessible conformational states for each complex have unique dependencies on temperature.

Stepwise ternary formation constants for each enantiomer are defined by the following complexation reactions:

\[
\text{Cu(L - HyPro)}^+ + \text{D}^- \text{ or L}^- \leftrightarrow K_t \rightarrow \text{Cu(L - HyPro)(D' or L')}
\]

Both stepwise ternary formation constants can be computed from the standard formation constant data reported in Tables 2.3 and 2.6. For example, the overall reaction equilibria defining complexation of the L' enantiomer with the Cu(L-HyPro) complex is given by

\[
\text{Cu}^{2+} + \text{L'}^- + \text{L - HyPro}^- \leftrightarrow \beta_{001} \rightarrow \text{Cu(L')(L - HyPro)}
\]

\[
\text{Cu}^{2+} + \text{L - HyPro}^- \leftrightarrow \beta_{001} \rightarrow \text{Cu(L - HyPro)}^+
\]

The stepwise ternary formation constant \(K_t\) is therefore given by

\[
K_t = \frac{\beta_{011}}{\beta_{001}} \tag{2.15}
\]

Stepwise ternary formation constants calculated using Equation (2.15) were analyzed according to the van’t Hoff equation (Eq. (2.1)) to determine complexation thermodynamics.
As shown in Figure 2.3, curvature is observed in the resulting van’t Hoff plots, indicating a non-zero heat capacity change $\Delta_{r}C_{p}$ for complexation of each amino-acid enantiomer with the Cu(L-HyPro)$^+$ complex. The Kirchoff equation provides the temperature dependence of the Gibbs energy change for any such reaction, i.e.,

$$\frac{\Delta_{r}G_{c}(T)}{RT} = -\ln K = \frac{\Delta_{r}H_{c}^{o}}{RT} + \frac{\Delta_{r}C_{p}^{o}}{RT}(T - T^{o}) - \frac{\Delta_{r}S_{c}^{o}}{R} - \frac{\Delta_{r}C_{p}^{o}}{R} \ln \left( \frac{T}{T^{o}} \right) \quad (2.16)$$

Nonlinear fitting of Equation (2.16) to each data set shown in Figure 2.3 therefore provides an estimate of $\Delta_{r}H_{c}^{o}$ and $\Delta_{r}S_{c}^{o}$ from the tangent to the slope at 298.15 K ($T^{o}$), and an estimate of $\Delta_{r}C_{p}^{o}$ from the local curvature. The resulting thermodynamic data for stepwise formation of each ternary complex are reported in Table 2.7. To our knowledge, these are the first enthalpy and entropy data reported for ternary Cu$^{2+}$ - mixed amino acid complexes. Although the uncertainties in taking the second derivative of our formation constant data are too large to report quantitative estimates of $\Delta_{r}C_{p}^{o}$, the concave shapes of all van’t Hoff plots in Figure 2.3 indicate that addition of an unlike amino acid to the Cu(L-HyPro)$^+$ complex results in a negative change in heat capacity. Heat capacity changes for binding of uncharged analytes in aqueous solution are thought to mainly arise from solvent effects [48]. Nonpolar dehydration generally results in a decrease in heat capacity, while an increase in $C_{p}$ accompanies polar dehydration [49,50]. Thus, dehydration of the relatively polar zwitterionic backbone (i.e., dehydration of the $\alpha$-amino and carboxylate groups) of the amino acid complexing to Cu(L-HyPro) would be expected to increase $C_{p}$. In the formation of mono- and bis-binary complexes, this effect is balanced by an expected decrease in heat capacity due to desolvation.
of the kosmotropic \( \text{Cu}^{2+} \) ion. As a result, formation of these complexes is seen to result in no change in heat capacity. The observed net decrease in \( C_p \) during formation of all stepwise mixed chelate complexes suggests that additional effects contribute to \( \Delta_r C_p^0 \) when unlike amino acids chelate Cu(II), but the source of these additional contributions is unknown.

Comparison of the stability of the (mixed) ternary complex to those of the parent bis-binary complexes is facilitated by defining an equilibrium constant \( K_m \) for the conversion reaction [34]

\[
\frac{1}{2} \text{CuA}_2 + \frac{1}{2} \text{CuB}_2 \rightleftharpoons K_m \rightarrow \text{Cu(A)(B)}
\]

\( K_m \) is then given by

\[
K_m = \frac{[\text{Cu(A)(B)}]}{\sqrt{[\text{Cu(A)}_2] [\text{Cu(B)}_2]}} = \frac{\beta_{1011}}{\sqrt{\beta_{\text{Cu(A)}_2} \beta_{\text{Cu(B)}_2}}}
\]

and the enthalpy change for the reaction is given by

\[
\Delta H_m = \Delta H_{\text{Cu(A)(B)}} - \frac{1}{2} \Delta H_{\text{Cu(A)}_2} - \frac{1}{2} \Delta H_{\text{Cu(B)}_2}
\]

Dissection of \( \Delta_r G_m \) into its enthalpic and entropic contributions, including the associated statistical (s) and residual (r) effects, is shown in Table 2.8 for one aliphatic (valine), one cyclic
(proline) and one aromatic (phenylalanine) amino acid. The data reveal that the mixed ternary chelate complex is always more stable than either of the parent bis-binary complexes. However, the thermodynamics of this hyperstabilizing effect are found to be quite different for the three different classes of amino acid investigated. For valine, conversion of the parent bis-binary complexes into the mixed Cu(Val)(L-HyPro) complex is enthalpically unfavourable. A net increase in entropy therefore drives the conversion reaction. A portion of this gain in entropy hyperstabilizing the mixed chelate complex arises through statistical effects. $K_{\text{stat}}$ is equal to 2 for the conversion reaction and therefore stabilizes the mixed chelate complex by ca. 410 cal/mol.

In contrast to aliphatic amino acids, formation of a mixed chelate complex containing two cyclic amino acids via the conversion of the parent Cu(Pro)$_2$ complex is a nearly athermal process, pointing to a more favorable energy of interaction between complexed proline and L-HyPro. In this case, ideal mixing (i.e., statistical) effects provide the main contribution to the hyperstabilization of the mixed complex. Similar complexation thermodynamics are observed for the conversion reaction to form the ternary Cu(Phe)(L-HyPro) complex, where $\Delta_r H_m$ is near zero and $\Delta_\text{r} S_m$ again provides the main thermodynamic driving force for the reaction. The origin of the favorable residual complexation entropy is not clear, but may be due to a backcoordination effect where electrons of the $t_{2g}$ orbitals of Cu(II) fill vacant $\pi$ orbitals of the phenyl ring, allowing close coordination with the aliphatic portion of the cyclic ring of L-HyPro to achieve limited dehydration [51].
2.4 Conclusions

We report the first data characterizing the enthalpy and entropy changes accompanying the formation of ternary Cu\(^{2+}\)(D'-L')(L-HyPro) complexes. Our data show that the energetics and entropy of binary and ternary complexation reactions depend on both solution temperature and the side-chain properties of the participating amino acids. The formation of binary and ternary complexes from the free components is found to be favored both enthalpically, due to bidendate ligand coordination to the planar coordination sites of Cu(II), and entropically, due to dehydration of the strongly kosmotropic Cu\(^{2+}\) ion. When L-HyPro serves as one of the complexing agents, the stability of the mixed ternary complex is greater than either of the parent bis-binary complexes, irrespective of the solution temperature. In general, a gain in entropy drives the conversion reaction. A portion of this entropy gain is due to statistical effects, but significant entropy gains due to non-ideal ligand effects are also observed, and their magnitude depends strongly on the side chain of the second amino acid.

We also report the first data defining the temperature dependence of stepwise and concentration equilibrium formation constants for binary and ternary complexes containing Cu\(^{2+}\) and L-HyPro. Nonlinear van't Hoff plots are observed for stepwise formation of all ternary complexes, indicating a negative \(\Delta C_P\) for addition of an unlike amino acid to the Cu(L-HyPro)\(^+\) complex. Quantitative values for \(\Delta C_P\) could not be obtained due to the uncertainty associated with interpreting second derivatives of primary data, pointing to the need for calorimetric studies of mixed chelate complex formation. However, for the L-HyPro ligand, the nonlinear dependence of ternary complex stability on inverse temperature has obvious implications on the optimization of racemate separations by CLEC.
2.5 Tables

Table 2.1 Amino-acid concentration-based protonation constants ($I_c = 0.1$ M KNO$_3$)$^{(a)}$.

<table>
<thead>
<tr>
<th>System</th>
<th>Log$_{10}$</th>
<th>T/K</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>288.15</td>
<td>298.15</td>
<td>310.15</td>
<td>318.15</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>$\beta_{0101}$</td>
<td>9.7 ± 0.1</td>
<td>9.46 ± 0.01</td>
<td>9.15 ± 0.03</td>
<td>9.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>$\beta_{0201}$</td>
<td>11.5 ± 0.2</td>
<td>11.31 ± 0.02</td>
<td>10.9 ± 0.1</td>
<td>10.83 ± 0.02</td>
</tr>
<tr>
<td>Proline</td>
<td>$\beta_{0110}$</td>
<td>10.66 ± 0.1</td>
<td>10.50 ± 0.01</td>
<td>10.17 ± 0.01</td>
<td>9.92 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>$\beta_{0210}$</td>
<td>12.6 ± 0.1</td>
<td>12.46 ± 0.02</td>
<td>12.10 ± 0.02</td>
<td>11.81 ± 0.04</td>
</tr>
<tr>
<td>Valine</td>
<td>$\beta_{0110}$</td>
<td>9.74 ± 0.03</td>
<td>9.48 ± 0.01</td>
<td>9.23 ± 0.02</td>
<td>8.98 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>$\beta_{0210}$</td>
<td>12.03 ± 0.03</td>
<td>11.77 ± 0.01</td>
<td>11.53 ± 0.03</td>
<td>11.28 ± 0.03</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>$\beta_{0110}$</td>
<td>9.32 ± 0.02</td>
<td>9.07 ± 0.02</td>
<td>8.76 ± 0.02</td>
<td>8.62 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>$\beta_{0210}$</td>
<td>11.52 ± 0.02</td>
<td>11.27 ± 0.04</td>
<td>10.94 ± 0.03</td>
<td>10.8 ± 0.1</td>
</tr>
<tr>
<td>Leucine</td>
<td>$\beta_{0110}$</td>
<td>9.81 ± 0.03</td>
<td>9.52 ± 0.02</td>
<td>9.29 ± 0.01</td>
<td>9.11 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>$\beta_{0210}$</td>
<td>12.2 ± 0.05</td>
<td>11.86 ± 0.02</td>
<td>11.62 ± 0.01</td>
<td>11.44 ± 0.02</td>
</tr>
</tbody>
</table>

$^{(a)}$ Errors reported as standard deviation from the mean for 10-15 independently regressed data sets.
Table 2.2 Enthalpy and entropy changes for amino-acid protonation over the temperature range $T = 298.15$ to $333.15$ K ($I_c = 0.1$ M KNO$_3$).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Equilibrium</th>
<th>$\Delta S_c$ (J.mol$^{-1}$.K$^{-1}$)</th>
<th>$\Delta H_c$ (kJ.mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine</td>
<td>[HL]/[H][L]</td>
<td>49.8 ± 0.8</td>
<td>-39.7 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>[H$_2$L]/[HL][H]</td>
<td>47.3 ± 1.3</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td>Valine</td>
<td>[HL]/[H][L]</td>
<td>46.5 ± 0.8</td>
<td>-40.2 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>[H$_2$L]/[HL][H]</td>
<td>54.4 ± 0.8</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td>Proline</td>
<td>[HL]/[H][L]</td>
<td>57.4 ± 1.6</td>
<td>-43.1 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>[H$_2$L]/[HL][H]</td>
<td>29.3 ± 0.8</td>
<td>-2.5 ± 0.4</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>[HL]/[H][L]</td>
<td>35.2 ± 0.8</td>
<td>-41.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>[H$_2$L]/[HL][H]</td>
<td>42.7 ± 0.4</td>
<td>0.4 ± 0.4</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>[HL]/[H][L]</td>
<td>52.7 ± 1.7</td>
<td>-38.5 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>[H$_2$L]/[HL][H]</td>
<td>22.2 ± 0.8</td>
<td>-3.8 ± 0.4</td>
</tr>
</tbody>
</table>

Table 2.3 Binary amino acid/copper concentration formation constants ($I_c = 0.1$ M KNO$_3$)

<table>
<thead>
<tr>
<th>System</th>
<th>Log$_{10}$ $\beta$</th>
<th>288.15</th>
<th>298.15</th>
<th>310.15</th>
<th>318.15</th>
<th>333.15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyproline</td>
<td>$\beta_{1001}$</td>
<td>8.63 ± 0.01</td>
<td>8.48 ± 0.03</td>
<td>8.22 ± 0.01</td>
<td>8.19 ± 0.01</td>
<td>8.06 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>$\beta_{1002}$</td>
<td>15.92 ± 0.02</td>
<td>15.6 ± 0.1</td>
<td>15.10 ± 0.01</td>
<td>15.04 ± 0.01</td>
<td>14.75 ± 0.02</td>
</tr>
<tr>
<td>Proline</td>
<td>$\beta_{1010}$</td>
<td>8.87 ± 0.01</td>
<td>8.73 ± 0.01</td>
<td>8.61 ± 0.02</td>
<td>8.50 ± 0.01</td>
<td>8.33 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>$\beta_{1020}$</td>
<td>16.37 ± 0.01</td>
<td>16.08 ± 0.03</td>
<td>15.8 ± 0.1</td>
<td>15.62 ± 0.03</td>
<td>15.3 ± 0.3</td>
</tr>
<tr>
<td>Valine</td>
<td>$\beta_{1010}$</td>
<td>8.1 ± 0.1</td>
<td>8.05 ± 0.01</td>
<td>7.94 ± 0.01</td>
<td>7.89 ± 0.03</td>
<td>7.35 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>$\beta_{1020}$</td>
<td>14.90 ± 0.02</td>
<td>14.79 ± 0.02</td>
<td>14.56 ± 0.01</td>
<td>14.47 ± 0.01</td>
<td>13.43 ± 0.05</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>$\beta_{1010}$</td>
<td>7.86 ± 0.01</td>
<td>7.71 ± 0.01</td>
<td>7.6 ± 0.1</td>
<td>7.56 ± 0.03</td>
<td>7.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>$\beta_{1020}$</td>
<td>14.76 ± 0.01</td>
<td>14.42 ± 0.02</td>
<td>14.1 ± 0.1</td>
<td>14.04 ± 0.02</td>
<td>13.80 ± 0.09</td>
</tr>
<tr>
<td>Leucine</td>
<td>$\beta_{1010}$</td>
<td>8.12 ± 0.02</td>
<td>8.1 ± 0.1</td>
<td>8.0 ± 0.1</td>
<td>7.92 ± 0.02</td>
<td>7.85 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>$\beta_{1020}$</td>
<td>14.9 ± 0.1</td>
<td>14.71 ± 0.01</td>
<td>14.55 ± 0.02</td>
<td>14.48 ± 0.03</td>
<td>14.34 ± 0.03</td>
</tr>
</tbody>
</table>
Table 2.4 Enthalpy and entropy changes for *mono- and bis-*binary complex formation over the temperature range $T = 298.15$ to $333.15$ K ($I_c = 0.1$ M KNO$_3$).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Equilibrium</th>
<th>$\Delta S_c$ (J.mol$^{-1}$.K$^{-1}$)</th>
<th>$\Delta H_c$ (kJ.mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine</td>
<td>[CuL]/[Cu][L]</td>
<td>116.8 ± 0.8</td>
<td>-11.3 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>[CuL$_2$]/[CuL][L]</td>
<td>92.5 ± 1.3</td>
<td>-10.5 ± 0.4</td>
</tr>
<tr>
<td>Valine</td>
<td>[CuL]/[Cu][L]</td>
<td>59.0 ± 0.4</td>
<td>-28.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>[CuL$_2$]/[CuL][L]</td>
<td>41.9 ± 0.8</td>
<td>-25.9 ± 0.4</td>
</tr>
<tr>
<td>Proline</td>
<td>[CuL]/[Cu][L]</td>
<td>94.6 ± 0.4</td>
<td>-21.8 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>[CuL$_2$]/[CuL][L]</td>
<td>65.3 ± 0.8</td>
<td>-22.6 ± 0.4</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>[CuL]/[Cu][L]</td>
<td>22.9 ± 0.3</td>
<td>-21.7 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>[CuL$_2$]/[CuL][L]</td>
<td>95.9 ± 1.7</td>
<td>-22.6 ± 0.8</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>[CuL]/[Cu][L]</td>
<td>69.9 ± 0.4</td>
<td>-27.2 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>[CuL$_2$]/[CuL][L]</td>
<td>39.8 ± 0.8</td>
<td>-28.9 ± 0.4</td>
</tr>
</tbody>
</table>
Table 2.5 Thermodynamic properties for the conversion reaction 
\[ \text{Cu}^{2+} + \text{CuL} \rightleftharpoons \text{CuL}^- + \text{Cu}^2+ \] at \( T^\circ = 298.15 \text{ K} \) (\( I_c = 0.1 \text{ M KNO}_3 \)).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Log_{10}K_B</th>
<th>( \Delta_r G_B^o ) (kJ.mol(^{-1}))</th>
<th>( \Delta_r H_B^o ) (kJ.mol(^{-1}))</th>
<th>( \Delta_r S_B^o ) (J.mol(^{-1}).K(^{-1}))</th>
<th>( \Delta G_r^o ) (kJ.mol(^{-1}))</th>
<th>( \Delta S_r^o ) (J.mol(^{-1}).K(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Valine</td>
<td>1.30</td>
<td>-7.39</td>
<td>-2.4</td>
<td>16.7</td>
<td>-2.23</td>
<td>-0.64</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.49</td>
<td>-8.50</td>
<td>-0.6</td>
<td>27.0</td>
<td>-3.34</td>
<td>9.26</td>
</tr>
<tr>
<td>Proline</td>
<td>1.38</td>
<td>-7.87</td>
<td>0.9</td>
<td>29.3</td>
<td>-2.71</td>
<td>11.90</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.99</td>
<td>-5.70</td>
<td>0.9</td>
<td>22.2</td>
<td>-0.53</td>
<td>4.88</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>1.37</td>
<td>-7.81</td>
<td>1.6</td>
<td>31.4</td>
<td>-2.65</td>
<td>14.10</td>
</tr>
</tbody>
</table>

Table 2.6 Ternary Cu(D' or L')(L-HyPro) concentration formation constants (\( I_c = 0.1 \text{ M KNO}_3 \)).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Log_{10} ( \beta )</th>
<th>T/K</th>
<th>288.15</th>
<th>298.15</th>
<th>310.15</th>
<th>318.15</th>
<th>333.15</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Proline</td>
<td>( \beta_{1011} )</td>
<td>16.52 ± 0.01</td>
<td>16.32 ± 0.03</td>
<td>15.84 ± 0.02</td>
<td>15.57 ± 0.02</td>
<td>15.02 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>D-Proline</td>
<td>( \beta_{1011} )</td>
<td>16.57 ± 0.01</td>
<td>16.37 ± 0.01</td>
<td>15.92 ± 0.03</td>
<td>15.69 ± 0.02</td>
<td>15.17 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>L-Valine</td>
<td>( \beta_{1011} )</td>
<td>15.87 ± 0.01</td>
<td>15.73 ± 0.02</td>
<td>15.25 ± 0.02</td>
<td>14.88 ± 0.01</td>
<td>14.0 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>D-Valine</td>
<td>( \beta_{1011} )</td>
<td>15.82 ± 0.02</td>
<td>15.64 ± 0.02</td>
<td>15.18 ± 0.01</td>
<td>14.75 ± 0.02</td>
<td>13.9 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>( \beta_{1011} )</td>
<td>15.88 ± 0.01</td>
<td>15.57 ± 0.02</td>
<td>15.14 ± 0.01</td>
<td>14.91 ± 0.01</td>
<td>14.47 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>D-Phenylalanine</td>
<td>( \beta_{1011} )</td>
<td>15.8 ± 0.1</td>
<td>15.46 ± 0.02</td>
<td>15.07 ± 0.02</td>
<td>14.84 ± 0.02</td>
<td>14.43 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>L-Leucine</td>
<td>( \beta_{1011} )</td>
<td>15.83 ± 0.02</td>
<td>15.69 ± 0.02</td>
<td>15.22 ± 0.03</td>
<td>14.97 ± 0.03</td>
<td>14.4 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>D-Leucine</td>
<td>( \beta_{1011} )</td>
<td>15.87 ± 0.02</td>
<td>15.68 ± 0.03</td>
<td>15.2 ± 0.1</td>
<td>14.92 ± 0.03</td>
<td>14.3 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

73
Table 2.7  Enthalpy and entropy changes for stepwise formation of ternary Cu(D' or L')(L-HyPro) complexes at 298.15 K ($I_c = 0.1$ M KNO$_3$).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>$\Delta_r S^o$ (J.mol$^{-1}$.K$^{-1}$)</th>
<th>$\Delta_r H^o$ (kJ.mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L - Leucine</td>
<td>88.8 ± 0.8</td>
<td>-14.7 ± 0.4</td>
</tr>
<tr>
<td>D - Leucine</td>
<td>74.5 ± 0.8</td>
<td>-18.9 ± 0.4</td>
</tr>
<tr>
<td>L - Valine</td>
<td>89.6 ± 0.8</td>
<td>-14.7 ± 0.4</td>
</tr>
<tr>
<td>D - Valine</td>
<td>81.6 ± 1.3</td>
<td>-16.3 ± 0.4</td>
</tr>
<tr>
<td>L - Proline</td>
<td>82.9 ± 0.8</td>
<td>-20.0 ± 0.4</td>
</tr>
<tr>
<td>D - Proline</td>
<td>89.6 ± 0.4</td>
<td>-18.4 ± 0.4</td>
</tr>
<tr>
<td>L - Phenylalanine</td>
<td>49.0 ± 0.4</td>
<td>-26.0 ± 0.4</td>
</tr>
<tr>
<td>D - Phenylalanine</td>
<td>59.5 ± 0.8</td>
<td>-22.2 ± 0.4</td>
</tr>
</tbody>
</table>

Table 2.8  Thermodynamic properties for the conversion reaction $\frac{1}{2}$CuA$_2$ + $\frac{1}{2}$CuB$_2$$\rightleftharpoons$CuAB at $T^\circ = 298.15$ K ($I_c = 0.1$ M KNO$_3$).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Log$_{10}$ $K_m$</th>
<th>$\frac{\Delta_r G^o_m}{(kJ.mol^{-1})}$</th>
<th>Log$_{10}$ $K_r$</th>
<th>$\Delta_r H^o_m$ (kJ.mol$^{-1}$)</th>
<th>$\Delta_r S^o_m$ (J.mol$^{-1}$.K$^{-1}$)</th>
<th>$\Delta S^o_r$ (J.mol$^{-1}$.K$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Valine</td>
<td>0.54</td>
<td>-3.06</td>
<td>0.23</td>
<td>13.4</td>
<td>54.9</td>
<td>49.0</td>
</tr>
<tr>
<td>D-Valine</td>
<td>0.46</td>
<td>-2.64</td>
<td>0.15</td>
<td>11.7</td>
<td>47.3</td>
<td>41.5</td>
</tr>
<tr>
<td>L-Proline</td>
<td>0.48</td>
<td>-2.76</td>
<td>0.18</td>
<td>2.9</td>
<td>18.4</td>
<td>13.0</td>
</tr>
<tr>
<td>D-Proline</td>
<td>0.54</td>
<td>-3.06</td>
<td>0.24</td>
<td>4.6</td>
<td>25.1</td>
<td>19.3</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>0.56</td>
<td>-3.22</td>
<td>0.26</td>
<td>2.9</td>
<td>20.1</td>
<td>14.7</td>
</tr>
<tr>
<td>D-Phenylalanine</td>
<td>0.45</td>
<td>-2.60</td>
<td>0.15</td>
<td>0.9</td>
<td>10.9</td>
<td>5.0</td>
</tr>
</tbody>
</table>
2.6 Figures

Figure 2.1  van't Hoff plots for stepwise protonation of proline: (a) $K_1$, (b) $K_2$
Figure 2.2 van’t Hoff plots for stepwise formation of mono- and bis-binary Cu$^{2+}$ complexes containing proline: (a) $K_1$, (b) $K_2$
Figure 2.3  van't Hoff plots for stepwise formation of ternary complex: \( \text{Cu(L-HyPro)}^+ + D^- \)

or \( L^- \xrightarrow{K_1} \text{Cu(L-HyPro)}(D' \text{or } L') \) for which the constant is \( K_t \). (a) leucine, (b) valine, (c) proline, (d) phenylalanine (filled squares: \text{L-amino acid}, filled circles: \text{D-amino acid})
2.7 References


Interpreting the Effects of Temperature and Solvent Composition on Separation of Amino-Acid Racemates by Chiral Ligand-Exchange Chromatography

3.1 Introduction

Invented by Davankov and Rogozhin (1971), chiral ligand-exchange chromatography (CLEC) is a widely used method for separating enantiomers of α-amino acids, dansyl amino acids, hydroxy acids, peptides, or amino alcohols, including potent therapeutic hormones and alkaloids such as epinephrine, ephedrine and norephedrine [1-3]. Separation is achieved through formation of transient diastereomeric metal complexes comprised of an immobilized bidentate chiral selector, a divalent cation, and either of the enantiomers to be resolved in the mobile phase. Each such mixed-ligand ternary metal complex formed at the stationary phase is stabilized by the binding of solvent molecules to the two axial coordination sites of the metal ion. However, steric and electronic effects imposed by the chirality of the selector and the bound enantiomer can prevent or weaken solvent coordination, resulting in small differences in the stabilities of the two ternary complexes. Additional intermolecular forces involved with chiral recognition, including polar interactions, hydrophobic effects, and hydrogen bonding, may also contribute to differences in the free energies of interaction and thereby provide an effective mechanism for chromatographic separation.

For a high-resolution separation to be realized, each ternary complex must form and
dissociate with reasonably fast kinetics [4-6]. Meeting this condition requires careful selection
and positioning of the electron-donor groups of the chiral selector and the type of metal ion
used. In general, CLEC is performed using a chiral selector that chelates a Zn(II), Ni(II), or
Cu(II) ion through a pair of coordinating electronegative (N, O, or S) atoms, each of which is
presented in the form of an amino, carboxy, hydroxy, amido, or thio functional group. Cu(II) is
the most often used metal ion, and it is generally added to the mobile phase to prevent loss of
the metal ion from the stationary phase during chromatographic processing. The racemate is
introduced as a pulse and eluted isocratically. Copper is then removed from the purified
enantiomer using an iminodiacetic-acid based chromatography column.

The use of CLEC in the purification of chiral therapeutics and in the general analysis of
chiral analytes has resulted in numerous publications on the technology. These studies have
primarily focused on the mechanism of separation [7,8], the discovery of new selector and
column chemistries [9,10], and various analytical and industrial applications of the technology
[11-13]. A large number of empirical studies have helped to define optimal column operating
conditions by mapping the dependence of the enantioselectivity and resolving power on
mobile-phase pH, Cu(II) concentration, and velocity. Temperature and the addition of
cosolvents also affect resolving power. For example, raising the column operating temperature
provides sharper elution peaks, but shorter retention times [14-16]. The resolving power of the
column can therefore be improved or diminished depending on the extent of peak sharpening.

While these previous on-column studies provide empirical guidelines for selecting
appropriate column operating conditions, they do not directly connect changes in column
performance to underlying changes in complex stabilities and speciation profiles within the column; nor do they specify and quantify rate-limiting mass-transfer processes within the column and their dependence on column operating conditions. As a result, describing and modeling CLEC-based separations at the molecular level remain difficult. The purpose of this study is to determine the influence of temperature and methanol concentration on chemical reaction equilibria and solute mass-transfer resistances within a Nucleosil-Chiral 1 column, a widely used CLEC column that uses L-hydroxyproline as the immobilized chiral selector (Figure 3.1), during separation of a racemic mixture of an α-amino acid. These fundamental data allow for a more complete understanding of the thermodynamic basis of the separation and the molecular origin of changes in CLEC column performance resulting from changes in operating temperature and solvent composition. Of particular concern is the dependence of the separation efficiency on the large number of complexes that form both within the mobile phase and at the stationary phase surface.

To fix ideas, we begin by distinguishing between the primary equilibrium driving separation in traditional chromatography columns, namely adsorption and desorption of analyte $A$

$$A^{\text{mobile}} \leftrightarrow A^{\text{stationary}},$$

(3.1)

from that driving CLEC-based separations, where the interaction between the analyte (in this case an enantiomer) and the immobilized chiral selector $CS$ does not occur through direct contact. Instead, the interaction is mediated by a metal ion $M$ that coordinates with the
enantiomer $A$ and the immobilized chiral selector to form a mixed-ligand ternary complex $MAS$. The primary equilibrium is therefore more accurately represented by

$$M^{2+} + A^- + CS^- \rightleftharpoons \beta_{1011} MACS$$  \hspace{1cm} (3.2)$$

where $\beta_{1011}$ is the equilibrium formation constant for the $MACS$ complex, and the subscripts define from left to right the number of metal ions (1), protons (0), enantiomer molecules (1), and chiral selector molecules (1) in the complex.

Equation (3.2) has been used to interpret CLEC chromatograms and their dependence on ligand density and metal-ion concentration [2,17,18]. However, while certainly much more appropriate than application of Equation (3.1), Equation (3.2) does not recognize important secondary equilibria that can influence the separation. When $A$ is an amino-acid enantiomer and $M$ is the Cu(II) ion, secondary chemical equilibria within CLEC columns include

$$H^+ + A^- \leftarrow \beta_{0110} HA, \hspace{1cm} H^+ + CS^- \leftarrow \beta_{0101} HCS$$  \hspace{1cm} (3.3)$$

$$2H^+ + A^- \leftarrow \beta_{0210} H_2A^+ \hspace{1cm} 2H^+ + CS^- \leftarrow \beta_{0201} H_2CS^+$$

$$M^{2+} + A^- \leftarrow \beta_{1010} MA^+ \hspace{1cm} M^{2+} + 2A^- \leftarrow \beta_{1020} MA_2$$

$$M^{2+} + CS^- \leftarrow \beta_{1001} MCS^+ \hspace{1cm} and \hspace{1cm} M^{2+} + 2CS^- \leftarrow \beta_{1002} MCS_2$$

for each enantiomer $A$ present in the sample and for the chiral selector $CS$. Equilibria (3.3) determine the amount of uncomplexed analyte $A$ and metal-ion $M$ available for binding to the
stationary phase through equilibrium Equation (3.2). As a result, they introduce additional stepwise equilibrium reactions through which a mixed-ligand ternary complex may form at the stationary phase. These additional reactions driving separation include

\[
MA^+ + CS^- \underset{K_1}{\overset{}{\longleftrightarrow}} MACS
\]

where \( K_1 = [MACS]/[MA][CS] \), (3.4)

\[
A^- + MCS^+ \underset{K_2}{\overset{}{\rightarrow}} MACS
\]

where \( K_2 = [MACS]/[A][MCS] \),

\[
MA^+ + MCS^+ \underset{K_3}{\overset{}{\rightarrow}} MACS + M^{2+}
\]

where \( K_3 = [MACS][M]/[MA][MCS] \),

\[
MA_2 + CS^- \underset{K_4}{\overset{}{\rightarrow}} MACS + A^-
\]

where \( K_4 = [MACS][A]/[MA_2][CS] \),

\[
MA_2 + MCS^+ \underset{K_5}{\overset{}{\rightarrow}} MACS + MA^+
\]

where \( K_5 = [MACS][MA]/[MA_2][MCS] \)

\[
A^- + MCS_2 \underset{K_6}{\overset{}{\rightarrow}} MACS + CS^-
\]

where \( K_6 = [MACS][CS]/[A][MCS_2] \),

\[
MA^+ + MCS_2 \underset{K_7}{\overset{}{\rightarrow}} MACS + MCS^+
\]

where \( K_7 = [MACS][MCS]/[MA][MCS_2] \),

\[
MA_2 + MCS_2 \underset{K_8}{\overset{}{\rightarrow}} 2MACS
\]

where \( K_8 = [MACS]^2/[MA_2][MCS_2] \),

as well as an additional set of equilibria for formation of the mixed-ligand ternary complex from protonated and hydroxylated reactants. As a result, the resolution of a racemic mixture is expected to show a complex dependence on pH and solvent composition, Cu(II) concentration, immobilized chiral selector density, and temperature that is not captured in Equation (3.2) alone.

### 3.2 Experimental

Proline and L-hydroxyproline enantiomers, nitric acid (0.0983-M HNO₃ standard), potassium nitrate (KNO₃), copper nitrate (Cu(NO₃)₂), and copper sulfate pentahydrate
(CuSO₄•5H₂O), were purchased from Sigma-Aldrich Chemicals Canada Ltd. (Oakville, ON). These reagents have reported purities of greater than 99% and were used without further purification. Methanol (HPLC grade) was purchased from Fisher Chemicals (Fair Lawn, NJ). Dextran fraction (T110) with a Mₙ of 110,000 (Da) was purchased from GE Healthcare (Que., Canada). For the preparation of all aqueous solutions, water was first double distilled and then treated with a NANOpure® II ultrafiltration system (Barnstead; Dubuque, IW). KOH standard (0.1 M) was prepared by diluting KOH Titrisol ampoules (Merck) according to the manufacturer’s instructions. All reagents and solutions were prepared immediately prior to use in an experiment.

3.2.1 Chiral Ligand Exchange Chromatography Experiments

Aqueous solutions for use in CLEC experiments were first filtered through an Acrodisc 0.2 μm PVDF syringe filter (Gelman) and then degassed at ambient temperature for 20 minutes under magnetic stirring and a humidified nitrogen atmosphere. Anhydrous methanol was added to the mobile phase after degassing. Elution chromatograms from the Nucleosil Chiral-1 column (Macherey-Nagel Inc.; Easton, PA) were measured on a Waters Inc. HPLC system consisting of a model 717 Autosampler, a model 486 UV detector, a model 410 differential refractometer and a model 600S controller. The CLEC column operating temperature was controlled with a Waters model 610 column heater, which also provided for preheating of all feed solutions. The Nucleosil Chiral-1 column (length L = 250 mm, inner column radius Rₑ = 2 mm) contains a stationary phase of porous silica particles with an average particle radius (Rₚ) of 2.5 μm and an average pore diameter of 120 Å. L-hydroxyproline serves as the chiral selector and is covalently immobilized onto the silica matrix through its α-amino group (Figure 3.1). Additional relevant properties of the Nucleosil Chiral-1 column are provided in Table 3.1.
Prior to sample loading, the Nucleosil Chiral-1 CLEC column was equilibrated to the desired column temperature, pH and eluent-phase composition by passing through the column at least 10 column volumes of eluent at a flowrate of 1 mL min\(^{-1}\). Sample solutions (10 μL) containing 10 mM L-proline and/or 10 mM D-proline in 0.5 mM CuSO\(_4\), adjusted to the desired pH using anhydrous sulfuric acid (from Fischer Scientific, Nepean, ON), were injected as a pulse onto the CLEC column at a mobile phase flowrate of 1 mL min\(^{-1}\). Elution chromatograms were monitored by in-line UV absorbance at 280 nm. The Cu(II) loaded CLEC column was operated at a constant pH between 2 and \(ca. 5.6\), above which hydroxylated ions (CuOH\(^+\)) and species (Cu(OH)\(_2\)) begin to form in appreciable amounts.

### 3.2.2 Potentiometric Titration Experiments

Protonation constants and equilibrium formation constants for all species formed in the system at each solvent condition were determined by potentiometric titration according to the protocol described in Sanaie and Haynes [19]. Measurements include the protonation constants for the \(\alpha\)-amino and \(\alpha\)-carboxy groups of proline and L-hydroxyproline, and formation constants for binary amino-acid-Cu\(^{2+}\) complexes and for ternary amino-acid-Cu\(^{2+}\)-(L-hydroxyproline) complexes at each solvent composition and system temperature. Titrations involving methanol-containing solutions were performed under methanol-stat conditions by preparing titrant (0.1-M KOH) and nitric-acid standard solution for electrode calibration to include methanol at the same concentration as in the sample titrand solution.

Protonation constants and formation constants were regressed from potentiometric data using the program CHEMEQ previously developed by our group [20]. CHEMEQ minimizes...
the weighted sum $U$ of the squared residuals between observed ($E_{i}^{obs}$) and calculated ($E_{i}^{calc}$) electrode potentials

$$ U = \sum_{i=1}^{n} \left( \frac{E_{i}^{obs} - E_{i}^{calc}}{\sigma_{i}} \right)^{2} = \sum_{i=1}^{n} W_{i} \left( E_{i}^{obs} - E_{i}^{calc} \right)^{2} $$  \hspace{1cm} (3.5)

The weighting factor ($W_{i}$) is defined as the reciprocal of the square of the total estimated error ($\sigma_{i}$) due to errors in the electrode potential ($\sigma_{E} = 0.1$ mV), reagent concentrations ($\sigma_{C} = 0.01$ mM), and the titrant volume readings ($\sigma_{V} = 0.002$ cm$^{3}$) [21]

$$ \sigma_{i}^{2} = \sigma_{E}^{2} + \left( \frac{\partial E_{i}^{obs}}{\partial C} \right)^{2} \sigma_{C}^{2} + \left( \frac{\partial E_{i}^{obs}}{\partial V} \right)^{2} \sigma_{V}^{2} $$  \hspace{1cm} (3.6)

A detailed description of the program CHEMEQ, which improves upon the implicit differentiation routine of Gans et al. [21,22], is provided elsewhere [20]. Each reported parameter (protonation constant, formation constant) was regressed from the combined titration data for 10-15 experimental runs, with the standard deviation computed and reported in each case.

3.2.3 Determination of Chemical Equilibria

Chemical equilibria in chiral ligand-exchange chromatography systems are governed by a set of equilibrium formation reactions defined by the generalized form of Equation (3.2)
\[ aM^{2+} + bH^+ + cL^- + dL'^- \xrightarrow{\beta_{abcd}} M^c_a H^b_b L^e_e L'^d_d \quad (\text{const } P, T) \] (3.7)

where \( M^{2+} \) is the metal ion (i.e., \( Cu^{2+} \)), \( H^+ \) is the proton, \( L^- \) is the deprotonated (free) enantiomer, \( L'^- \) is the deprotonated chiral selector (i.e., \( L^-\)-hydroxyproline), and \( a, b, \ldots \) are the stoichiometric coefficients for the complexation reaction. The formation constant \( \beta_{abcd} \) for the complex is defined in terms of equilibrium concentrations of the reactants. We therefore designate \( \beta_{abcd} \) data as concentration formation constants to clearly differentiate them from standard formation constants based on the activities of the reactants. A negative value for the stoichiometric coefficient \( b \) indicates the presence and number of hydroxyl ions in the complex.

Chemical equilibria Equations (3.7) are combined with total mass balance equations for \( M^{2+}, H^+, L^- \), and \( L'^- \) to obtain a set of nonlinear algebraic equations that may be solved to determine the equilibrium composition at any solution temperature and pH. For example, the total mass balance for the metal ion is given by

\[
T_M = \left[ M^{2+} \right] + \sum_k a_k \left[ M^+ \right] c_k \left[ H^+ \right] ^b_k \left[ L^- \right] ^{c_k} \left[ L'^- \right] ^{d_k} (3.8)
\]

where \( T_M \) represents the total concentration of the metal ion (mol/L) in the system and \( \beta_{a_k b_k c_k d_k} \) is the formation constant for complex \( k \).

3.3 Results and Discussion

3.3.1 Mass Transfer Limitations

Table 3.2 reports, as functions of the column operating temperature, the axial dispersion coefficient \( D_L \) (m\(^2\) s\(^{-1}\)), the film mass transfer coefficient \( k_f \) (m s\(^{-1}\)) and the pore diffusion
coefficient $D_p \ (m^2 \ s^{-1})$ for the transport of $L$-proline within the Nucleosil Chiral-1 column. The column void fraction $\varepsilon, D_L$, and the overall mass transfer coefficient $K_m \ (s^{-1})$ for the solute were determined by application of moments analysis [23] and plate theory [24,25] to elution peaks such as those shown in Figures 3.2 and 3.3 for valine and proline, respectively. For a Gaussian peak, the first central moment $\mu_1$ is given by

$$\mu_1 = t_R - t_0 = \frac{L}{u} \left( \varepsilon + (1 - \varepsilon) \varepsilon_p \right)$$

(3.9)

where $t_R \ (s)$ is the solute retention time and $t_0 \ (s)$ is the system dead time, $L$ is the column length (m), $u$ is the superficial velocity (m s$^{-1}$), and $\varepsilon_p$ is the porosity of the stationary phase. Equation (3.9) was applied to elution data as a function of $u$ for Dextran T110, a high molecular weight polymer that does not partition into the 12 nm pores of the stationary phase of the Nucleosil Chiral-1 column, to estimate $\varepsilon$. The experiment was then repeated using glucose, a penetrating but non-interacting solute of similar size to that of amino acids, to estimate $\varepsilon_p$.

For a column operating under solute-binding conditions, moments analysis and plate theory yield the following expression for the second central moment $\mu_2$, given by the square of the standard peak variance $\sigma^2$, and the height equivalent to a theoretical plate (HETP (m))

$$u_o \ HETP = \frac{\mu_2 L}{\mu_1^2} u_o = 2D_L + 2u_o \left[ \frac{l}{K_m} \right] \left( \frac{\varepsilon}{1 - \varepsilon} \right) \left( \frac{k_l}{1 + k_l} \right)^2$$

(3.10)

where
\[ \frac{1}{K_m} = \frac{R_p^2}{15D_p} + \frac{R_p}{3k_f} + \left( \frac{k_p}{1 + k_p} \right)^2 \frac{1}{k_{ads}}, \]  
(3.11)

and

\[ k_f = \frac{(1-\varepsilon)}{\varepsilon} (\varepsilon_p + (1-\varepsilon)K_a) \]  
(3.12)

In Equations (3.10-3.12), \( u_0 \) is the interstitial velocity (m s\(^{-1}\)), \( k_{ads} \) is the intrinsic rate constant (s\(^{-1}\)) for solute adsorption to the stationary phase, \( K_a \) is the stepwise equilibrium binding constant for the amino-acid enantiomer (\( A' \) - activated chiral selector (\( MS^+ \)) complex, and

\[ k_p = \frac{1-\varepsilon}{\varepsilon_p} K_a. \]  
(3.13)

Equation (3.10) was first applied to elution data as a function of \( u_0 \) for an amino-acid sized solute (glucose) that does not interact with the stationary phase of the Nucleosil Chiral-1 column. Both \( k_{ads} \) and \( K_a \) are vanishingly small for glucose, allowing direct regression for \( K_m \) and \( D_L \) from a plot of \( u_0 \cdot HETP \) versus \( u_0^2 \). At each operating temperature, an estimate of \( k_f \) was obtained from the dimensionless correlation of Wilson and Geankopolis [26] for flow through a packed bed of spherical particles at low Reynolds numbers (0.0015 \(< \text{Re} < 55\)

\[ Sh = \frac{2R_p k_f}{D_m} = \frac{1.09}{\varepsilon} \text{Re}^{0.33} \text{Sc}^{0.33} \]  
(3.14)

where \( Re \) and \( Sc \) are the Reynolds (\( Re = 2R_pu/\nu \)) and Schmidt (\( Sc = \nu/D_m \)) numbers, respectively, \( \nu \) is the kinematic viscosity of the mobile phase (e.g., \( \nu = 9.09 \times 10^{-7} \text{ m}^2 \text{ s}^{-1} \) at 298°K).
K) taken from [27], and $D_m$ is the free molecular diffusion coefficient of L-proline in water (e.g., $D_m = 8.79 \times 10^{-10}$ m$^2$ s$^{-1}$ at 298 K) taken from [28]. Regression of Equation (3.10) to L-proline elution data under normal column operating conditions (pH 5) then allowed estimation of the magnitude of the last term on the right-hand side of Equation (3.11), from which an estimate of $k_{ads}$ could be obtained.

Results from this analysis were used to compute as a function of operating temperature a set of dimensionless groups from which the rate-limiting resistance to solute mass transfer within the column could be determined. Values for the Peclet ($Pe$) number, the Biot ($Bi$) number, and the Damkohler number ($Da$) are reported in Table 3.3. The last term on the right-hand side of Eq. (3.11) was found to contribute less than 0.01% (the statistical limit) to the overall resistance to mass transfer for L-proline, indicating that $k_{ads} > 7.0 \times 10^4$ (s$^{-1}$). Thus, the Damkohler number, which expresses the intrinsic rate of solute adsorption to the rate of solute diffusion through the pores of the stationary phase, is always greater than $10^4$. This, combined with the large values of $Bi$, shows that solute diffusion through the pores of the stationary phase limits the rate of solute adsorption to the porous resin at all column temperatures. Thus, at all operating temperatures studied, solute and sorbate are always found to be in local equilibrium throughout the column. This result challenges an assertion by Hyun et al. [17] that changes in elution peak shape and time with increasing temperature may be due to concomitant changes in the kinetics of ternary complex formation at the stationary phase. While a decrease in temperature undoubtedly slows the rate of ternary complex formation, our results indicate that, at all temperatures, the overall rate of L-proline binding to the stationary phase is controlled by the rate of solute diffusion through the pores of the stationary-phase resin. Very similar results and conclusions were obtained for valine (data not shown). As the number of theoretical plates (NTUs) offered by the column is large and relatively constant with temperature (Table 3.3),
changes in the performance of the Nucleosil Chiral-1 column with temperature are found to be
due to changes in chemical equilibria rather than changes in adsorption kinetics as previously
reported [17].

3.3.2 Influence of Temperature on Chemical Equilibria

Elution profiles for proline enantiomers (Figure 3.3) on a Nucleosil Chiral-1 column
show that the D enantiomer is retained longer, regardless of column operating temperature.
This implies that the hetero-chiral ternary metal complex ((D-proline) – Cu$^{2+}$ – (L-
hydroxyproline)) is more stable than the corresponding homo-chiral ternary metal complex ((L-
proline) – Cu$^{2+}$ – (L-hydroxyproline)). Ligand protonation constants and equilibrium formation
constants for binary and ternary metal-ligand complexes are reported for a range of solution
temperatures in Tables 3.4 and 3.5. In agreement with elution data, the results in Table 3.5
show that the heterochiral ternary complex is more stable over the entire temperature range
studied.

Figure 3.3 also shows that retention of each proline enantiomer decreases with
increasing temperature, presumably due to changes in chemical equilibria within the system
that reduce partitioning of the enantiomer into the stationary phase. A theoretical relationship
between solute retention time $t_R$ and chemical equilibria within a chromatography column was
first formulated by Devault [29], who showed for the reversible partitioning of solute $A$ into a
stationary phase that

$$t_R = t_0 (1 + \phi K_P) \quad (3.15)$$
where $\phi$ is the phase ratio ((1-\(\bar{c}\))/\(\bar{c}\)) and $K_p$ is the solute partition coefficient, given by the concentration of the solute in the stationary phase over that in the mobile phase at equilibrium. Martin [30] later extended Devault's theoretical analysis to include cases where $A$ can complex with itself or with other solutes in the mobile phase, such as through the formation of the metallo-complexes $AM^+$ and $A_2M$. Using the approach taken by Martin, we find that the retention of an amino-acid enantiomer within a CLEC column is given by

$$t_R = t_0 \left(1 + \phi K_p^* \right)$$  \hspace{1cm} (3.16)

where $K_p^*$ is the effective partition coefficient of the enantiomer, given by

$$K_p^* = K_A \left(1 + \beta_{1010} [M]_s + \left(\frac{\beta_{1020}}{\beta_{1010}}\right)[AM]_s + \left(\frac{\beta_{1011}}{\beta_{1001}}\right)[ML]_s \right) \left(1 + \beta_{1010} [M]_m + \left(\frac{\beta_{1020}}{\beta_{1010}}\right)[AM]_m \right)^{-1}$$  \hspace{1cm} (3.17)

where the large term in brackets gives the ratio of the total enantiomer concentration in the stationary phase over that in the mobile phase, and $K_A$ is the partition coefficient of the uncomplexed enantiomer under conditions where it does not bind to the stationary phase resin. Figure 3.4 compares the temperature dependence of the retention time for D-proline as predicted by Equations (3.16) and (3.17) with that measured in our experiments. In both cases, \(\ln((t_R-t_0)/t_0)\) increases linearly with inverse temperature with a slope of ca. 2 \(\times 10^3\) (K\(^{-1}\)). Retention times predicted from Equations (3.16) and (3.17) are in good agreement with those measured in the column.
By analogy with more conventional modes of chromatography, it is tempting to assume that the observed decrease in L-proline retention times (and \( K_p^* \)) with increasing temperature is exclusively due to the associated decrease in \( \beta_{1011} \), the equilibrium formation constant for the bidentate ternary complex. However, the value of \( \beta_{1011} \) defines the equilibrium concentration of the ternary complex relative to that of the uncomplexed deprotonated (free) enantiomer in solution. At all column conditions studied, the concentration of the free enantiomer is vanishingly small. Instead, the enantiomer is almost exclusively present in solution in mono- and bis-binary complexes with \( \text{Cu}^{2+} \), making \( K_p^* \) more strongly dependent on the stabilities of these binary complexes, as indicated in Equation (3.17). Enantiomer partitioning also depends on the concentration of activated ligand (i.e., the \( \text{Cu}^{2+} – (\text{L-hydroxyproline}) \) complex), so that \( K_p^*(T) \) is a function of \( \beta_{1010}, \beta_{1020}, \beta_{1001}, \text{and } \beta_{1002} \), as well as \( \beta_{1011} \). As a result, Equation (3.17) predicts that \( K_p^* \) can either decrease or increase with temperature depending on the degree of change in the binary formation constants with temperature relative to that of \( \beta_{1011} \). In the case of L-proline partitioning in the Nucleosil Chiral-1 column operating at pH 5, the decreases in \( \beta_{1010}, \beta_{1020}, \beta_{1001}, \text{and } \beta_{1002} \) with temperature (Table 3.4) are less severe than that observed for \( \beta_{1011} \) (Table 3.5). As a result, the concentration of adsorbed enantiomer decreases with temperature, resulting in the observed decrease in \( K_p^* \), while the concentrations of the two \( \text{bis} \)-binary complexes increase with temperature despite their reduced stabilities relative to the free enantiomer (Figure 3.5).

### 3.3.3 Influence of Solvent Composition on Chemical Equilibria

The need to account for all speciation to accurately describe CLEC elution data is unequivocally illustrated through the effects of solvent composition on solute retention times.
The addition of methanol (MeOH) to the mobile phase reduces the $t_R$ of both valine (Figure 3.6) and proline (Figure 3.7) enantiomers such that $\ln t_R$ shows a linear dependence on % (v/v) methanol. However, unlike when the column operating temperature is raised, increasing the % MeOH in the mobile phase results in a linear increase in the formation constant for the ternary complex (Table 3.6), which on its own would suggest that $t_R$ will increase with % MeOH as well. The fact that it does not recognizes that the primary equilibria driving separation in the CLEC column are those between the ternary complex formed at the stationary phase and the various mono- and bis-binary complexes present in the mobile phase.

Amino-acid protonation constants and formation constants for binary Cu$^{2+} -$ (amino acid) complexes are reported in Table 3.6 as a function of % MeOH. As noted previously by Rorabacher et al. [31], the addition of MeOH leads to a reduction in the dielectric permittivity of the mobile phase. Water has a very high dipole moment due to the electronegativity of oxygen and the relatively short hydrogen-oxygen bond. The simple replacement of a hydrogen atom with a methyl group reduces the dipole moment of MeOH and thereby reduces its dielectric permittivity by more than a factor of two. As the pair potential $U(r) = q_i q_j/(4\pi \varepsilon \varepsilon_0 r)$ between two ions is inversely proportional to the dielectric permittivity $\varepsilon$ of the solvent, lowering $\varepsilon$ by a factor of two favors pairing of ions of opposite charge and disfavors the creation of charged species within the medium. As a result, the addition of MeOH decreases $\beta_{0110}$ and $\beta_{0101}$, the protonation constants for the $\alpha$-amino groups of each amino acid, and increases $\beta_{0210}$ and $\beta_{0201}$, the protonation constants for the respective carboxylic group; that is, MeOH addition disfavors the zwitterionic state in favor of the neutral form of each amino acid as previously reported by Shehata et al. [32] and Kõseoglu et al. [33]. The presence of MeOH also favors formation of metal-ion complexes, including mono-binary, bis-binary and ternary...
complexes, due to the lower dielectric permittivity of the solvent and the lower hydrogen bonding potential of MeOH, which disfavors solvation of Cu(II) through coordination of the oxygen of MeOH to one of the four planar or two distal coordination sites. As a result, the displacement of MeOH from the coordination sites of Cu(II) by a bidentate amino-acid ligand or chiral selector is favored relative to the same reaction in aqueous solution. The formation constants for all binary and ternary complexes were found to increase linearly with % MeOH (Table 3.6). These trends agree with previous studies by Khan et al. [34] on Cu(II) halides, by Sondwale and Narwade [35] on the interaction of Cu(II) with glycine tripeptide (GGG), and by Davankov et al. [36] on select binary Cu$^{2+}$ – (amino acid) complexes.

Equation (3.16) predicts that $t_R$ will decrease when % MeOH is increased if the rate of increase in the formation constants for one or more of the mono- and bis-binary complexes is larger than that for the ternary complex. As shown in Figure 3.8, the increase in $\beta_{1020}$ is more than four times that of $\beta_{1011}$, while $\beta_{1010}$ increases over twice as much. As a result, an increase in % MeOH favors formation of the (proline)$_2$ – Cu$^{2+}$ complex at the expense of the ternary (proline) – Cu$^{2+}$ – (L-Hypro) complex, leading to a decrease in both $K_p^*$ and $t_R$ (Figure 3.7).

### 3.4 Conclusions

Mass transfer resistances and chemical reaction equilibria within a Nucleosil Chiral-1 column were determined over a range of standard column operating conditions to elucidate the dominant resistance to solute mass transfer and the underlying mechanism for the strong dependence of CLEC separation efficiency on temperature and mobile-phase solvent composition. Regardless of the column operating temperature, the overall rate of solute
binding to the stationary phase is limited by the rate of solute diffusion through the pores of the stationary phase resin. The pore liquid and stationary phases are therefore in local equilibria throughout the column and changes in enantiomer retention times are found to correlate closely with changes in chemical equilibria within the column. The dependence of the solute partition coefficient on chemical equilibria within the column is defined and used to show that subtle changes in the formation constants for binary and ternary bidentate complexes with Cu(II) can result in significant changes in solute elution behaviour. As a result, in contrast to what is implied in currently available models of CLEC [37,38], enantiomer separations within a CLEC column are not simply governed by ternary complex formation at the stationary phase. Our results reveal that the modeling of enantiomer elution chromatograms from CLEC columns must account for all chemical equilibria within the column, including protonation and binary-complex equilibria, to accurately predict the dependence of separation performance on column operating conditions.
3.5 Tables

Table 3.1 Properties of the Nucelosil Chiral 1 column

<table>
<thead>
<tr>
<th>Column Property</th>
<th>$R_c$ (m)</th>
<th>$R_p$ (m)</th>
<th>$L$ (m)</th>
<th>$\varepsilon$</th>
<th>$\varepsilon_p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column Radius</td>
<td></td>
<td>$2.0 \times 10^{-3}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Particle Radius</td>
<td></td>
<td>$2.5 \times 10^{-6}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Column Length</td>
<td></td>
<td></td>
<td>0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Column Void</td>
<td></td>
<td></td>
<td></td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>Stationary Phase Porosity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.65</td>
</tr>
</tbody>
</table>

Table 3.2 Temperature dependence of mass transfer coefficients for L-proline injected onto a Nucelosil Chiral 1 column as a 20-μL pulse at concentration of 1 mM and a flow rate of 1 ml min$^{-1}$. The mobile phase contained 0.5 mM CuSO$_4$ at pH 5.4.

<table>
<thead>
<tr>
<th>Temperature (K)</th>
<th>$D_L$ (m$^2$/sec)</th>
<th>$K_m$ (sec$^{-1}$)</th>
<th>$D_p$ (m$^2$/sec)</th>
<th>$k_f$ (m/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>298.15</td>
<td>$3.20 \pm 0.21 \times 10^{-8}$</td>
<td>$18.99 \pm 0.45$</td>
<td>$1.24 \pm 0.03 \times 10^{-11}$</td>
<td>$7.05 \times 10^{-4}$</td>
</tr>
<tr>
<td>310.15</td>
<td>$3.24 \pm 0.11 \times 10^{-8}$</td>
<td>$19.61 \pm 0.37$</td>
<td>$1.28 \pm 0.02 \times 10^{-11}$</td>
<td>$7.24 \times 10^{-4}$</td>
</tr>
<tr>
<td>318.15</td>
<td>$2.89 \pm 0.13 \times 10^{-8}$</td>
<td>$20.42 \pm 0.39$</td>
<td>$1.34 \pm 0.03 \times 10^{-11}$</td>
<td>$7.37 \times 10^{-4}$</td>
</tr>
<tr>
<td>333.15</td>
<td>$2.10 \pm 0.12 \times 10^{-8}$</td>
<td>$20.74 \pm 0.71$</td>
<td>$1.36 \pm 0.05 \times 10^{-11}$</td>
<td>$7.60 \times 10^{-4}$</td>
</tr>
</tbody>
</table>
Table 3.3 Temperature dependence of dimensionless parameters characterizing the transport of L-proline injected onto a Nucelosil Chiral 1 column as a 20-μL pulse at concentration of 1 mM and a flow rate of 1 ml min⁻¹. The mobile phase contained 0.5 mM CuSO₄ at pH 5.4.

<table>
<thead>
<tr>
<th>Temperature (K)</th>
<th>$Pe = Lu/D_L$</th>
<th>$Bi = k_f R_p / D_p$</th>
<th>$Da = k_{ads} R_p^2 / D_p$</th>
<th>NTUs</th>
</tr>
</thead>
<tbody>
<tr>
<td>298.15</td>
<td>$1.04 \pm 0.07 \times 10^4$</td>
<td>$142 \pm 4$</td>
<td>$\geq 3.83 \times 10^4$</td>
<td>$3.34 \pm 0.1 \times 10^3$</td>
</tr>
<tr>
<td>310.15</td>
<td>$1.02 \pm 0.04 \times 10^4$</td>
<td>$141 \pm 2$</td>
<td>$\geq 1.68 \times 10^5$</td>
<td>$4.20 \pm 0.1 \times 10^3$</td>
</tr>
<tr>
<td>318.15</td>
<td>$1.15 \pm 0.05 \times 10^4$</td>
<td>$137 \pm 3$</td>
<td>$\geq 2.04 \times 10^5$</td>
<td>$4.40 \pm 0.2 \times 10^3$</td>
</tr>
<tr>
<td>333.15</td>
<td>$1.58 \pm 0.09 \times 10^4$</td>
<td>$140 \pm 3$</td>
<td>$\geq 2.82 \times 10^5$</td>
<td>$4.62 \pm 0.3 \times 10^3$</td>
</tr>
</tbody>
</table>

Table 3.4 Protonation and binary formation constants for proline and L-hydroxyproline in aqueous solution ($I_c = 0.1$ M KNO₃) over the temperature range 298.15 to 333.15K

<table>
<thead>
<tr>
<th>System</th>
<th>$\log \beta$</th>
<th>Temperature (K)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>298.15</td>
</tr>
<tr>
<td>H, Proline</td>
<td>$\rho_{010}$*</td>
<td>10.50 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>$\rho_{020}$</td>
<td>12.46 ± 0.02</td>
</tr>
<tr>
<td>H, Hydroxyproline</td>
<td>$\rho_{010}$</td>
<td>9.46 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>$\rho_{020}$</td>
<td>11.31 ± 0.02</td>
</tr>
<tr>
<td>Cu, H, Proline</td>
<td>$\rho_{010}$</td>
<td>8.73 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>$\rho_{020}$</td>
<td>16.08 ± 0.03</td>
</tr>
<tr>
<td>Cu, H, Hydroxyproline</td>
<td>$\rho_{000}$</td>
<td>8.48 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>$\rho_{002}$</td>
<td>15.6 ± 0.1</td>
</tr>
</tbody>
</table>

* The subscript ‘abcd’ on the $\beta_{abcd}$ indicates the number of molecules of Cu²⁺ (a), protons (b), amino-acid enantiomer (c) and L-hydroxyproline (d)
Table 3.5  Ternary Cu(D- or L-proline)(L-HyPro) formation constants ($I_c = 0.1$ M KNO$_3$) for the temperature range 298.15 to 333.15 K.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Log$_{10}$$\beta$</th>
<th>Temperature (K)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>288.15</td>
<td>298.15</td>
<td>310.15</td>
<td>318.15</td>
</tr>
<tr>
<td>L-Proline</td>
<td>$\beta_{1011}$</td>
<td>16.52 ± 0.01</td>
<td>16.32 ± 0.03</td>
<td>15.84 ± 0.02</td>
<td>15.57 ± 0.02</td>
</tr>
<tr>
<td>D-Proline</td>
<td>$\beta_{1011}$</td>
<td>16.57 ± 0.01</td>
<td>16.37 ± 0.01</td>
<td>15.92 ± 0.03</td>
<td>15.69 ± 0.02</td>
</tr>
</tbody>
</table>

Table 3.6  Protonation and equilibrium formation constants as a function of methanol concentration (298.15 K, $I_c = 0.1$ M KNO$_3$)

<table>
<thead>
<tr>
<th>Complex</th>
<th>Log$_{10}$$\beta$</th>
<th>0% MeOH</th>
<th>20% MeOH</th>
<th>40% MeOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>H, Proline</td>
<td>$\beta_{0110}$</td>
<td>10.50 ± 0.01</td>
<td>10.30 ± 0.05</td>
<td>10.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>$\beta_{0210}$</td>
<td>12.46 ± 0.02</td>
<td>12.52 ± 0.05</td>
<td>12.6 ± 0.1</td>
</tr>
<tr>
<td>H, Hydroxyproline</td>
<td>$\beta_{0101}$</td>
<td>9.46 ± 0.01</td>
<td>9.4 ± 0.1</td>
<td>9.34 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>$\beta_{0201}$</td>
<td>11.31 ± 0.02</td>
<td>11.56 ± 0.13</td>
<td>11.8 ± 0.1</td>
</tr>
<tr>
<td>Cu, H, Proline,</td>
<td>$\beta_{1010}$</td>
<td>8.73 ± 0.01</td>
<td>8.91 ± 0.02</td>
<td>9.11 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>$\beta_{1020}$</td>
<td>16.08 ± 0.03</td>
<td>16.42 ± 0.02</td>
<td>16.74 ± 0.02</td>
</tr>
<tr>
<td>Cu, H, Hydroxyproline</td>
<td>$\beta_{1001}$</td>
<td>8.48 ± 0.03</td>
<td>8.54 ± 0.01</td>
<td>8.61 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>$\beta_{1002}$</td>
<td>15.6 ± 0.1</td>
<td>15.64 ± 0.03</td>
<td>15.72 ± 0.02</td>
</tr>
<tr>
<td>Cu, H, Proline, L-Hydroxyproline</td>
<td>$\beta_{1011}$ (L)</td>
<td>16.32 ± 0.03</td>
<td>16.39 ± 0.01</td>
<td>16.47 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>$\beta_{1011}$ (D)</td>
<td>16.37 ± 0.01</td>
<td>16.45 ± 0.01</td>
<td>16.5 ± 0.1</td>
</tr>
</tbody>
</table>
3.6 Figures

Figure 3.1  Structure of the immobilized L-hydroxyproline ligand bound to the stationary phase of the Nucleosil Chiral 1 Column
Temperature dependence of the elution chromatogram for a 10-μL pulse injection of 1 mM \(D,L\)-valine onto the Nucleosil Chiral-1 column at 1 mL min\(^{-1}\). The mobile phase contained 0.5 mM CuSO\(_4\) at pH=5.4.
Figure 3.3 Temperature dependence of the elution chromatogram for a 10-μL pulse injection of 1 mM D,L-proline onto the Nucleosil Chiral-1 column at 1 mL min⁻¹. The mobile phase contained 0.5 mM CuSO₄ at pH=5.4.
Figure 3.4  Comparison between experimental (filled circles) and predicted (filled inverse triangles) retention times for 0.5 mM D-proline injected as a 10-μL pulse onto the Nucleosil Chiral-1 column. The mobile-phase (0.5 mM CuSO₄, pH 5.4) flow rate was 1 ml min⁻¹.
Figure 3.5  Temperature dependence of Cu(II)-containing complexes at equilibrium in an aqueous solution (pH 5.4) containing 10 mM L-hydroxyproline, 10 mM L-proline and 10 mM Cu(II). Dominant complexes present at equilibrium include 1010 (open squares), 1020 (open diamonds), 1001 (filled squares), 1002 (filled diamonds), and the ternary complex 1011 (filled stars).
Figure 3.6 The dependence on mobile-phase methanol content of the elution chromatogram for a 10-μL pulse injection of 1 mM D,L-valine onto the Nucleosil Chiral-1 column at a flow rate of 0.6 mL min⁻¹. The mobile phase contained 0.5 mM CuSO₄ at pH 5.4 and varying amounts of methanol: 20% (v/v) MeOH – continuous line, 0% (v/v) MeOH – dashed line. The column temperature was 298.15 K.
Figure 3.7  The dependence on mobile-phase methanol content of the elution chromatogram for a 10-μL pulse injection of 1 mM \(D,L\)-proline onto the Nucleosil Chiral-1 column at a flow rate of 1 mL min\(^{-1}\). The mobile phase contained 0.5 mM \(\text{CuSO}_4\) at pH 5.4 and varying amounts of methanol: 40% (v/v) \(\text{MeOH}\) – continuous line, 20% (v/v) \(\text{MeOH}\) – dashed-dotted line, 0% (v/v) \(\text{MeOH}\) – dashed line. The column temperature was 298.15 K.
Figure 3.8 The dependence of formation constants for binary and ternary complexes on solvent composition. Formation constants include $\beta_{1010}$ (squares), $\beta_{1020}$ (circles), $\beta_{1001}$ (triangles), $\beta_{1002}$ (stars), and $\beta_{1011}$ (hexagons). Superscript 'aq' refers to the cosolvent-free system.
3.7 References


4 A Multiple Chemical Equilibria Approach to Modeling and Interpreting the Separation of Amino Acid Enantiomers by Chiral Ligand-Exchange Chromatography *

4.1 Introduction

Lessons learned from the unexpected but devastating teratogenic effects of the sedative thalidomide have led to an increased awareness in pharmacology and medicine of the importance of chirality in the human biosystem [1,2]. Nearly half of all synthetic drugs contain a single chiral center, and many are now marketed in an enantiomerically pure form [3,4]. Regulatory agencies such as the FDA demand stringent pharmacological investigations of the safety and effectiveness of each enantiomer of synthetic therapeutics [5]. This has intensified the need for effective, inexpensive methods for resolving and analyzing optical isomers. Similarly, there is a growing need for new technologies to purify the chiral precursors required for direct asymmetric synthesis of complex chiral therapeutics.

The nearly identical physical properties of optical isomers make their separation one of the more challenging problems in the purification sciences. Current industrial methods for resolving enantiomers include preferential [6] and diastereomeric [7] crystallization, directed chemical or enzymatic resolution [8], and chiral chromatography [9,10]. Preferential

* A version of this chapter has been accepted for publication in the Journal of Chromatography A. [Reference: Sanaie, N. and Haynes, C.A., A Multiple Chemical Equilibria Approach to Modeling and Interpreting the Separation of Amino Acid Enantiomers by Chiral Ligand Exchange Chromatography. J. Chromatogr. A.]
crystallization, while highly cost effective, is limited to racemates where the heats of formation of the two enantiomeric crystals are sufficiently different to overcome the large entropic penalty of demixing [6]. Diastereomeric crystallization and various modes of chiral chromatography, including both Pirkle-type [11,12] and chiral ligand-exchange chromatography (CLEC) [13,14], therefore remain the most general methods for high-resolution separation of optical enantiomers despite the challenges associated with scale-up [15]. CLEC is widely used for separating enantiomers of barbiturates, pyridone carboxylic acids (e.g., the chemotherapeutic agent ofloxacin), α-amino acids, dansyl amino acids, hydroxy acids, peptides, amino alcohols, alkaloids, β-blockers and other adrenergic drugs [16-18]. It is based on the formation of reversible ternary complexes between a ligand, one of the enantiomers to be separated, and a central transition metal ion that offers unfilled inner-shell d-orbitals for ligand binding. The stability of the ternary complex is stereochemically dependent, due in part to electronic and steric effects imposed by the chirality of the selector that can strengthen or weaken both solvent and enantiomer coordination [19]. Optical resolution by CLEC is therefore possible through reversible binding of each (typically bidentate) enantiomer with a metal-ion-bearing chiral selector presented on the stationary phase.

CLEC stationary phases are prepared by grafting or physically adsorbing an optically pure moiety onto an achiral chromatographic matrix. Davankov [20], and many others [21,22] synthesized and tested a wide range of stationary phases to find that the most promising immobilized chiral ligands for separating racemates of dansyl amino acids, α-alkyl amino acids and α-amino acids are themselves derivatives of cyclic or aromatic amino acids. Several such grafted ligand exchange phases are commercially available, including immobilized derivatives of L-hydroxyproline (the Nucleosil Chiral-1 column, Macherey-Nagel) or L-proline (the Chiral
ProCu column, Serva; the Cosmosil 5CSP-HPR column, Nacalai-Teseque). Examples of physi-
sorbed CLEC stationary phases include the Davankov A column of Regis Technologies Inc.
and the use of N\textsuperscript{1}-n-decyl-L-histidine immobilized onto a C10 reversed phase matrix for use in
separating most \( \alpha \)-amino acid racemates [21].

Due to the generally low loading capacities of CLEC columns [23], proper selection of the
ligand and column operating conditions (\textit{i.e.}, temperature, flow rate, pH, solvent composition,
etc.) can have a dramatic impact on resolution [24]. Experiments have helped to define optimal
column operating conditions by mapping the dependence of the enantioselectivity and
resolution on mobile phase pH, Cu(II) concentration, temperature, and velocity (for a
comprehensive review, see [13,14] and references therein). Kurganov \textit{et al.} [25], for example,
studied peak distortion during CLEC separation of a range of enantiomers on a C18 column
loaded with Cu(II) • [N-(n-octadecyl)-S-proline]\textsubscript{2}. They observed that resolution is strongly
affected by the copper (II) concentration. Resolving power is also influenced by temperature.
Raising the column operating temperature generally provides sharper elution peaks and shorter
retention times [26,27].

These empirical studies, along with theoretical work aimed at defining the equilibrium
structures of ternary mixed-ligand complexes [19,28], provide useful guidelines for selecting an
appropriate stationary phase ligand and suitable column operating conditions. However, they
do not directly connect elution behavior to underlying changes in chemical equilibria within the
column, particularly within the mobile phase; nor do they specify and quantify rate-limiting
mass-transfer processes and their dependence on column operating conditions. Jacobson \textit{et al.}
[29] were the first to show that nonlinear chromatography theory, when combined with a
competitive adsorption isotherm, can be used to model elution band profiles from chiral chromatography columns [30]. They successfully predicted elution profiles for N-benzoylalanine enantiomers obtained on a chiral stationary phase bearing immobilized bovine serum albumin (BSA). Seidel-Morgenstern and Guiochon [31,32] extended this concept to a different mode of chiral chromatography by combining the equilibrium-dispersion equation of chromatography with the ideal adsorbed solution theory of Radke and Prausnitz [33] to model changes in the elution chromatogram when the two enantiomers of Tröger's base are loaded either at low concentration, where they are predicted to adsorb independently, or at high concentrations, where they adsorb competitively, on a chiral cellulose triacetate (CTA) column.

Separation of enantiomers on BSA and CTA columns does not occur through ligand exchange. As a result, the above models are not directly applicable to CLEC separations, in part because they do not explicitly account for the complex chemical equilibria that occur in a ligand-exchange type chromatographic separation. Nevertheless, both models [29,31] address the potential to interpret and simulate chiral chromatography data by properly accounting for adsorption equilibria and solute mass transfer resistances.

We describe a model of enantiomer transport and chemical equilibria within a CLEC column (the Nucleosil Chiral-1 column) that allows prediction of elution band profiles as a function of key operating parameters such as Cu(II) concentration and pH. Recently we have shown that changes in separation performance in CLEC columns correlate closely with changes in chemical equilibria [27]. Each enantiomer participates in a large number of complexes both at the stationary phase surface and within the mobile phase. As a result, the thermodynamic driving force for separation is unusually complex, allowing subtle changes in column operating
conditions to mediate significant changes in elution band profiles and resolution. Standard thermodynamic databases [34,35] now report protonation constants for single stereocenter enantiomers, as well formation constants for complexes formed with transition metal ions. This permits the development of a model of CLEC that is comprehensive in its treatment of chemical equilibria and therefore capable of providing insights into the overall separation mechanism by connecting elution band profiles to temporal and spatial changes in the distribution of equilibrium complexes as the components migrate through the column. It is developed and used here to interpret and simulate elution profiles for hydrophobic amino acid racemates on the Nucelosil Chiral-1 column as a function of mobile phase composition, pH, and temperature.

4.2 Materials and Methods

Amino acid enantiomers and copper sulfate (CuSO₄) pentahydrate were purchased from Sigma-Aldrich Chemical Canada Ltd. (Oakville, ON). All reagents were of the highest available purity (>99%) and were used without further purification. For the preparation of all aqueous solutions, water was first double distilled and then treated with a Barnstead NANOpure® II ultrafiltration system (Dubuque, WI). All solutions for HPLC studies were filtered through a 0.22 μm (GV) Durapore filter (Millipore) and degassed prior to use. Chromatograms were measured on a Waters HPLC system consisting of a model 717 autosampler, a model 486 UV detector, a model 410 differential refractor and a model 600S controller. The column operating temperature was controlled with a Waters model 610 column heater, which also provided for preheating of all feed solutions.

CLEC studies were performed on one of two Nucleosil Chiral-1 columns (Macherey-Nagel Inc.; Easton, PA) containing porous silica beads with an average particle size of 5 μm and pore
diameter of 120 Å bearing L-hydroxoprolne immobilized through its α-amine at a density of 310 mM or 510 mM (Appendix I). The stainless-steel column was 250 mm in length, with an inner diameter $d_i$ of 4.0 mm. The filtered (Acrodisc 0.2 μm PVDF syringe filter; Gelman Scientific) and degassed mobile phase contained CuSO$_4$ at a specified concentration in nanopure water. The pH of the mobile phase was adjusted by appropriate addition of 99% sulfuric acid (Fisher Scientific; Nepean, ON). Prior to sample loading, the Nucleosil Chiral-1 column was equilibrated to the desired column temperature, pH and eluent-phase composition by passing through the column at least 10 column volumes of eluent at the flow rate corresponding to the experiment. A 10 μL sample solution containing 10 mM of one or both enantiomers in the degassed mobile phase was then injected as a pulse onto the CLEC column at the specified mobile phase flow rate. The elution chromatogram was monitored by in-line UV absorbance at 280 nm. A standard chromatogram for $L,D$-valine (pH 5.4, 25°C) was measured each week to determine if the density of active immobilized chiral selector or the packing and pore properties of the stationary phase were changing over time. No change in retention time ($\pm$ 0.2 min) was observed over the six-month period in which all reported experiments were conducted.

4.3 Theory

4.3.1 Column Model Development

As will be shown through analysis of dimensionless groups determined from first and second moment analyses, mass transfer of amino acid enantiomers within the Nucleosil Chiral-1 column is limited at all operating conditions by the rate of solute diffusion within the pores of the stationary phase [27]. We have therefore assumed local equilibrium within the cylindrical
CLEC column of length $L$ packed with spherical sorbent particles of radius $R_p$ and porosity $\varepsilon_p$. Figure (4.1). The one-dimensional (1-D) continuity equation applied to each enantiomer $i$ present in the mobile phase is given by

$$\frac{\partial c_i}{\partial t} = D_L \frac{\partial^2 c_i}{\partial z^2} - \frac{u}{\varepsilon} \frac{\partial c_i}{\partial z} - \frac{(1 - \varepsilon)}{\varepsilon} \frac{\partial s_i}{\partial t}$$

(4.1)

where $D_L$ is the axial dispersion coefficient ($m^2 s^{-1}$), $u$ is the superficial liquid velocity ($m s^{-1}$), and $\varepsilon$ is the column void fraction. In Equation (4.1), $c_i(z,t)$ (mol m$^{-3}$ bulk fluid) represents the total concentration of solute component $i$ in the mobile phase. The model therefore differs from traditional 1-D nonlinear nonideal chromatography theory in that each enantiomer can be present in the mobile phase in a number of metal-ion containing complexes, as well as in its deprotonated, zwitterionic and fully protonated states. As the transport properties of these complexes cannot be determined independently, we treat each solute as a pseudo-solute characterized by a single set of transport parameters (e.g., a single characteristic axial dispersion coefficient, pore diffusion coefficient, etc.).

The average total concentration $s_i(z,t)$ (mole/m$^3$ adsorbent particle) of a given pseudo-solute component within the stationary phase particles at column position $z$ at time $t$ is given by

$$s_i(z,t) = \frac{4\pi}{V_p} \int_0^{R_p} \left[ \varepsilon_p c_i^*(r,z,t) + (1 - \varepsilon) c_i^*(r,z,t) \right] r^2 dr$$

(4.2)
where $V_p$ and $\varepsilon_p$ are the volume (m$^3$) and porosity of a stationary phase particle, respectively, and $c_i^*$ (mole/m$^3$ pore fluid) and $q_i^*$ (mole m$^{-3}$ sorbent particle) are the total equilibrium concentrations of the pseudo-solute in the intraparticle fluid and adsorbed to the sorbent surface, respectively, at column position $z$ and time $t$. Solution of the continuity equation for the concentration of a pseudo-solute at any time $t$ and column position $z$ therefore requires knowledge of the rate of uptake $\frac{\partial s_i}{\partial t}$ within the porous sorbent particles, given by

$$\frac{\partial s_i}{\partial t} = \frac{3}{R_p} N_0 = \frac{3}{R_p} D_p \left( \frac{\partial c_i^*}{\partial r} \right)_{r=R_p}$$  \hspace{1cm} (4.3)$$

where $D_p$ is the intraparticle diffusivity of the pseudo-solute (m$^2$ s$^{-1}$). This relation can be simplified by noting that the flux $N_o$ of pseudo-solute $i$ into the particle (mol m$^{-2}$ s$^{-1}$) is equal to the rate of transfer through the stagnant fluid film surrounding each particle, so that

$$D_p \left( \frac{\partial c_i^*}{\partial r} \right)_{r=R_p} = k_f \left( c_i - c_i^* \right)_{r=R_p}$$  \hspace{1cm} (4.4)$$

where $k_f$ is the film mass transfer coefficient (m s$^{-1}$).

The spatially resolved concentration of pseudo-solute within the pores of the sorbent particles is determined through an intraparticle solute mass balance
Solution of Equation (4.5) requires an equilibrium model relating $q_i^*(r,z,t)$ to the local concentration $c_i^*(r,z,t)$ of the component in the pore fluid, which is typically modeled using either a noncompetitive or a competitive form of the Langmuir isotherm [36]. These approaches have proven appropriate in modeling chromatographic systems where solute component $i$ in solution does not complex with itself or with any other solutes and therefore can bind to the sorbent without undergoing any dissociation reactions. However, the solution and sorbent surface environments in CLEC fail to satisfy this condition. Consider, for example, a relatively simple CLEC system in which a racemic mixture of an $\alpha$-amino acid with a uncharged side chain is separated on a Nucleosil Chiral-1 column containing a constant concentration of Cu(II) ion in the mobile phase. Components present in the mobile phase then include the Cu(II) ion, the proton, and the fully deprotonated $L$- and $D$-amino acid enantiomers. The chemical equilibria when these three components are present in aqueous solution depend on pH, temperature and the relative concentrations of the three components [37-39]. Generally, CLEC separations are performed under acidic conditions near the $pK_a$ of the $\alpha$-carboxylic group of the amino acid enantiomers to be separated. Each enantiomer is therefore present in solution in both its zwitterionic and fully protonated states. In addition, each enantiomer can participate in the formation of a set of mono- and bis-binary complexes with the Cu(II) ion. As a result, each enantiomer is present in the mobile phase in ca. a half dozen complexes. For the $L$ enantiomer ($L$), these equilibria and complexes include
\[
H^+ + L^- \rightleftharpoons \beta_{01100} HL,
\]
\[
2H^+ + L^- \rightleftharpoons \beta_{02100} H_2L^+,
\]
\[
M^{2+} + L^- \rightleftharpoons \beta_{10100} ML^+,
\]
\[
M^{2+} + 2L^- \rightleftharpoons \beta_{10200} ML_2,
\]
and
\[
M^{2+} + L^- + D^- \rightleftharpoons \beta_{10110} MLD
\]

where, for example, \( \beta_{10110} \) is the equilibrium formation constant for the hetero \( bis\)-binary complex \( MLD \), and the subscripts define from left to right the number of metal ions (1), protons (0), \( L \)-enantiomer molecules (1), \( D \)-enantiomer molecules (1), and immobilized chiral selector molecules (0) in the complex. The formation constant \( \beta_{abcde} \) for each equilibrium complex is therefore defined as

\[
\beta_{abcde} = \frac{[A^a B^b C^c D^d E^e]}{[A]^b [B]^c [C]^d [D]^e [E]^f}
\]

where \([A^a B^b C^c D^d E^e]\) and \([A]\) are the molar concentrations of the complex and the uncomplexed metal ion, respectively. We therefore designate all \( \beta_{abcde} \) as concentration formation constants to clearly differentiate them from standard formation constants based on the activities of the reactants. A negative value for the stoichiometric coefficient \( b \) indicates the presence and number of hydroxyl ions in the complex.
The concentration of each complex formed is constrained by the total mass balance equation for each component. For example, the total mass balances for uncomplexed components A and B are given by:

\[ T_A = [A] + \sum_k a_k \left[ A \right]^{a_k} [B]^{b_k} [C]^{c_k} [D]^{d_k} [E]^{e_k} \]

\[ T_B = [B] + \sum_k b_k \left[ A \right]^{a_k} [B]^{b_k} [C]^{c_k} [D]^{d_k} [E]^{e_k} \] (4.8)

where \( T_A \) represents the total concentration of component A (mol L\(^{-1}\)) and there are \( a_k \) molecules of A in each complex \( k \). This result can be rewritten in terms of the free concentration of each component by using the equilibrium formation constant \( \beta_{abcde} \) for every complex present, \( i.e., \)

\[ T_A = [A] + \sum_k a_k \beta_k [B]^{b_k} [C]^{c_k} [D]^{d_k} [E]^{e_k} \]

\[ T_B = [B] + \sum_k b_k \beta_k [A]^{a_k} [B]^{b_k} [C]^{c_k} [D]^{d_k} [E]^{e_k} \] (4.9)

where the index \( k \) sums over all equilibrium complexes in which the component is present.

In this model, Equation (4.5) is solved for each enantiomer \( i \), but not for Cu\(^{2+}\) or the proton H\(^+\). The total concentrations of Cu\(^{2+}\) and H\(^+\) are assumed to be constant and uniform within the column, eliminating concentration gradients for these two components. Solution of the
continuity equation (i.e., Equation (4.1)) and Equation (4.5) describing pseudo-solute uptake within the stationary phase particles requires updated values of $s_i$, $c_i^*$, and $q_i^*$ as a function of time and position. Together, Equations (4.2), (4.7) and (4.9) provide this information by defining the concentrations of all complexes formed within an equilibrium system of total composition $T_A$, $T_B$, etc., allowing one to determine chemical equilibria within any volume element of the CLEC column (Appendix II). Both $c_i^*$ and $q_i^*$ represent total concentrations of the pseudo-solute, which for the Cu(II) ion, the L-enantiomer and the proton are given by

$$ c_{Cu^{2+}}^* = \left[ Cu^{2+} \right] + \beta_{10100} \left[ Cu^{2+} \right][L^-] + \beta_{10210} \left[ Cu^{2+} \right][L^-]^2 + \beta_{10010} \left[ Cu^{2+} \right][D^-] + \beta_{10020} \left[ Cu^{2+} \right][D^-]^2 $$

$$ + \beta_{10110} \left[ Cu^{2+} \right][D^-][L^-] $$

(4.10)

$$ c_L^* = \left[ L^- \right] + \beta_{01100} \left[ H^+ \right][L^-] + \beta_{02100} \left[ H^+ \right][L^-]^2 + \beta_{10100} \left[ Cu^{2+} \right][L^-] + 2\beta_{10200} \left[ Cu^{2+} \right][L^-]^2 + \beta_{10110} \left[ Cu^{2+} \right][L^-][D^-] $$

$$ \beta_{10110} \left[ Cu^{2+} \right][L^-][D^-] $$

$$ c_{H^+}^* = \left[ H^+ \right] + \beta_{01100} \left[ H^+ \right][L^-] + \beta_{02100} \left[ H^+ \right][L^-]^2 + \beta_{00110} \left[ H^+ \right][D^-] + \beta_{02010} \left[ H^+ \right][D^-]^2 $$

and

$$ \text{and} $$

129
where \([CS^-]\) is the concentration of the uncomplexed immobilized chiral selector. Similar relations can be written for the remaining feed components.

This study focuses on interpreting and simulating the separation of racemates of valine or phenylalanine as a function of column operating conditions. The values of the required formation constants and reaction stoichiometric coefficients for all equilibrium complexes formed within the mobile phase and stationary phase pore liquid have been reported previously [39]. Solution of Equations (4.9) also requires protonation constants for the chiral selector and formation constants for all complexes formed at the stationary phase surface. As first reported by Zolotarev et al. [40], we assume that the first protonation constant \((\beta_{01001})\) of the L-hydroxyproline chiral selector is not significantly altered by selector immobilization due to the small size of the proton. Similarly, \(\beta_{10001}\) and \(\beta_{10002}\) are assumed to be unaltered by immobilization of L-hydroxyproline, permitting those formation constants previously determined by solution-phase potentiometric titration [39] to be used in our column model.
Finally, immobilization of L-hydroxyproline through its α-amino group eliminates the potential to form the 02001 complex (i.e., the H₂CS⁺ complex) within the CLEC column.

As first described by Davankov and Rogozhin [19], chemical conjugation of the chiral selector to the stationary phase can result in subtle changes in steric packing effects, solvent dielectric properties, and nonspecific interactions with the underlying stationary-phase matrix that alter the stability of the immobilized ternary mixed-ligand complex relative to that in solution. In certain cases, ligand immobilization can result in inversion of retention order [28]. We therefore define a factor \( k_l \)

\[
\log(\beta_{ijklm}^*) = (1 + k_l)\log(\beta_{ijklm})
\]  

(4.12)

that corrects the independently measured \( \beta_{ijklm} \) by accounting for changes in the stability of the mixed-ligand complex due to ligand immobilization. The on-column formation constant \( \beta_{ijklm}^* \) is then given by Equation (4.12).

### 4.3.2 Boundary Conditions and Solution Algorithm

The set of coupled transport (Equations (4.1) to (4.5)) and chemical-equilibria (Equations (4.7) and (4.9)) equations were solved numerically by a finite-difference iteration scheme written in FORTRAN 90. Initial and boundary conditions for the continuity equation are:
where \( c_i^o \), \( c_i^{feed} \), and \( c_i^m \) (mole/m\(^3\)) are the total concentration of component \( i \) in the column prior to sample injection, in the injected sample, and in the mobile phase buffer solution, respectively. The sample is injected into the column as a pulse of duration \( t_{feed} \), given by \( V_{feed}Q \), where \( V_{feed} \) and \( Q \) are the sample volume (m\(^3\)) and the volumetric flow rate (m\(^3\) s\(^{-1}\)), respectively. Other inlet boundary conditions, including \( D_L \frac{\partial c_i}{\partial z} \bigg|_{z=0} = u \left[ c_i \bigg|_{z=0} - c_i^{feed} \right] \), were also examined, and the resulting predicted chromatograms were found to be equivalent to those computed using Equation (4.13) due to the short duration (0.6 sec) of the feed pulse relative to the elution peak widths. The complementary set of initial and boundary conditions for Equation (4.5), describing pseudo-solute diffusion and binding within the stationary phase particle volume, are

\[
\begin{align*}
  c_i^* &= 0 & t = 0, \quad 0 \leq r \leq R_p \\
  \frac{\partial c_i^*}{\partial r} &= 0 & r = 0, \quad t > 0 
\end{align*}
\]  

Equation (4.4) provides the final boundary condition by specifying that \( c_i^*(r = R_p, z, t) \) and \( c_i(z, t) \) are coupled by the rate of mass transfer through the fluid film.
Time and space domains were discretized by a Crank-Nicolson scheme [41] to approximate differentials by a central difference in time and an average central difference in space (Appendix II). The column was meshed in the z dimension to match (or slightly exceed) the number of column NTUs and the number of radial volume elements within the stationary phase particle was set equal to 5. Finer meshing within the stationary phase significantly increased computational time without a noticeable improvement in model accuracy. This discretization of the diffusion-reaction equations yielded a set of tridiagonal linear algebraic equations that were solved by the Thomas algorithm [42] and the application of a first-order upwind-corrected power-law scheme [43] to ensure diagonally dominant matrices. Time increments for solution of Equations (4.1) and (4.5) were set at 0.01t_{feed} and 0.002t_{feed}, respectively.

4.4 Results and Discussion

4.4.1 Column Properties and Solute Transport Characteristics

Table 4.1 reports the geometric properties of the Nucleosil Chiral-1 column, as well as mass transfer coefficients and related parameters describing the transport of \((L,D)\)-valine within the mobile and stationary phases of the column. The fraction of external voids \((\varepsilon)\) was regressed from elution data for pulse injections of the non-binding, non-penetrating polymer, dextran T110, for which \(\varepsilon_p = 0\) (Figure 4.2). Elution peaks were Gaussian, allowing the first moment \((\mu_1)\) of the elution peak to be directly related to the retention time \(t_R(s)\) according to the classic theory of Haynes and Sarma [44]

\[
\mu_1 = t_R \cdot t_0 = \frac{L}{u} \left( \varepsilon + (1 - \varepsilon) \varepsilon_p \right) \tag{4.15}
\]
where \( t_0 \) is the system dead time (s).

For a column operating under solute binding conditions, the second central moment \( \mu_2 \), given by the square of the standard peak variance \( \sigma^2 \) for a Gaussian elution peak, and the height equivalent to a theoretical plate (HETP (m)) are given by [45,46]

\[
u_0 HETP = \frac{\mu_2 L}{\mu_1} = 2D_L + 2u_0^2 \left( \frac{1}{K_m} \right) \left( \frac{\varepsilon}{1-\varepsilon} \right) \left( \frac{k_f}{1+k_f} \right)^2
\]

(4.16)

where

\[
\frac{1}{K_m} = 15D_p + \frac{R_p}{3k_f} + \left( \frac{k_p}{1+k_p} \right)^2 \frac{1}{k_{ads}}
\]

(4.17)

and

\[
k_f = \frac{(1-\varepsilon)}{\varepsilon} (\varepsilon_p + (1-\varepsilon_p)K_a)
\]

(4.18)

In Equations (4.16) to (4.18), \( u_0 (= u/\varepsilon) \) is the solvent interstitial velocity (m s\(^{-1}\)), \( k_{ads} \) is the intrinsic rate constant (s\(^{-1}\)) for solute adsorption to the stationary phase, \( K_a (M^{-1}) \) is the stepwise equilibrium constant for binding of an amino acid enantiomer to the immobilized chiral selector complexed with a Cu\(^{2+}\) ion, and

\[
k_p = \frac{1-\varepsilon_p}{\varepsilon_p} K_a
\]

(4.19)

Elution data as a function of \( u_0 \) were first acquired for a solute that does not interact with the stationary phase of the Nucleosil Chiral-1 column (Figure 4.3). Given that its size is similar to that of an amino acid, glucose was used for this purpose. \( K_a \) is vanishingly small for glucose, allowing direct regression of \( K_m \) and \( D_L \) from a plot of \( u_0 \cdot HETP \) versus \( u_0^2 \). At each operating temperature, an estimate of \( k_f \) was obtained from the dimensionless correlation of Wilson and
Geankopolis [47] for flow through a packed bed of spherical particles at low Reynold numbers (0.0015 < Re < 55)

\[ Sh = \frac{2R_p k_f}{D_m} = \frac{1.09 \epsilon}{Re^{0.33} Sc^{0.33}} \]  

where Re and Sc are the Reynolds (\(Re = 2R_p u/\nu\)) and Schmidt (\(Sc = \nu/D_m\)) numbers, respectively, \(\nu\) is the kinematic viscosity of the mobile phase (e.g., \(\nu = 9.09 \times 10^{-7} \text{ m}^2/\text{sec} \) at 298 K) taken from [48], and \(D_m\) is the free molecular diffusion coefficient of the amino acid in water (e.g., for valine, \(D_m = 6.90 \times 10^{-10} \text{ m}^2/\text{sec} \) at 298 K) taken from [49]. Regression of Equation (4.16) to enantiomer elution data (Figure 4.3) under normal column operating conditions (pH 5) then allowed estimation of the magnitude of the last term on the right-hand side of Equation (4.17), from which an estimate of \(k_{ads}\) could be obtained.

Results from this analysis were used to compute dimensionless groups that allowed determination of the rate-limiting resistance to solute mass transfer within the column. Values for the Biot (Bi) number, the Peclet (Pe) number, and the Damköhler number (Da) are reported in Table 4.1. For both valine and phenylalanine, the last term on the right-hand side of Equation (4.17) contributes less than 0.01% to the overall resistance to mass transfer, indicating that \(k_{ads} > 7.0 \times 10^4 \text{ (s}^{-1})\). Thus, \(Da\) is always greater than \(10^4\). This, combined with the large value of \(Bi\), shows that solute diffusion through the pores of the stationary phase limits the rate of solute adsorption to the stationary phase, permitting the local equilibrium approximation to be applied throughout the column.
4.4.2 Model Evaluation

Table 4.2 reports equilibrium formation constants at 25°C for ternary mixed-ligand complexes formed between a free enantiomer, a Cu\(^{2+}\) ion, and a soluble analogue of the immobilized chiral selector (L-hydroxyproline). Each \(\beta_{ijklm}\) was determined by potentiometric titration according to the procedure described in Sanaie and Haynes [39]. Reported in Tables 4.2 and 4.3, \(k_i\) values were determined by model regression to a single elution band profile for a 10 \(\mu\)L pulse injection of pure enantiomer (2 mM) onto the Nucleosil Chiral-1 column operating at a flow rate of 1 mL min\(^{-1}\) and a mobile phase pH and CuSO\(_4\) concentration of 5.4 and 0.5 mM, respectively. For each enantiomer, \(k_i\) provides no more than a 4% correction to the independently measured value of \(\beta_{ijklm}\), indicating that the underlying stationary phase does not significantly alter the stability of the immobilized ternary mixed-ligand complex relative to that in solution. Nevertheless, as observed in Figure 4.4, the influence of the base matrix is sufficient to create an inversion of stereoselectivity, as has been observed previously by others [28].

With all required parameters determined, the performance of the model was evaluated through comparison to experimental chromatograms collected over a range of flow rate, pH, Cu\(^{2+}\) concentration, and feed concentration. Figure 4.4, for example, compares with experiment the predicted chromatogram for a 10 \(\mu\)L pulse injection of 2 mM racemate of D,L-valine onto the Nucleosil Chiral-1 column at operating conditions specified by a flow rate of 1 mL min\(^{-1}\), a temperature of 25 °C, and a mobile phase pH of 5.4. However, unlike the conditions used to regress \(k_i\), the mobile phase Cu\(^{2+}\) concentration is now 1 mM. The model accurately predicts both the retention time and peak profile of each enantiomer. Similar results are obtained for
L,D-phenylalanine (Figure 4.5), and these results are indicative of model performance over a wide range of operating conditions.

4.4.2.1. Copper Concentration Effects

Several experimental studies have shown that the resolution and retention times of enantiomers on CLEC columns depend nonlinearly on mobile phase Cu$^{2+}$ concentration [21,50-53]. For example, at pH 5.4, an increase in [Cu$^{2+}$] results in a nonlinear decrease in the retention times of L- and D-valine (Figure 4.6), with the decrease in the retention of the more retained enantiomer being considerably more pronounced. As a result, increasing [Cu$^{2+}$] from 0.5 to 3 mM reduces the separation factor. However, when mobile-phase chemical equilibria are not considered in our model, one predicts that enantiomer retention times should increase with increasing Cu$^{2+}$ concentration since the density of the Cu$^{2+}$-activated chiral selector [Cu•CS$^+$] (i.e., the stationary phase ligand density) is increased through the following equilibria

\[
Cu^{2+} + CS^- \rightleftharpoons \beta_{10001} \rightarrow CuCS^+
\]

\[
Cu^{2+} + Cu(CS)_2 \left(\beta_{10001}^{\beta_{10002}}\right) \rightarrow 2CuCS^+
\]

The observed decrease in elution times with increasing [Cu$^{2+}$] therefore makes clear the importance of defining all chemical equilibria in the system. Consider Figure 4.7 which, for the injection of L-valine onto the Nucleosil Chiral-1 column, compares the computed composition within a central volume element of the enantiomer (L-valine) elution band when the CLEC column is operated at two different mobile phase Cu$^{2+}$ concentrations: 0.5 mM CuSO$_4$ and 3 mM CuSO$_4$. At least 8 equilibrium complexes are formed at either mobile phase Cu$^{2+}$
concentration. However, only those complexes present at concentrations large enough to influence the elution behavior of the enantiomer are shown. At pH 5.4, the mobile phase pH used to collect the data shown in Figure 4.7, increasing the concentration of Cu$^{2+}$ in the mobile phase increases the concentration of activated chiral selector ($C_{10001}$) at the expense of the bis chiral selector complex ($C_{10002}$), as predicted by Equation (4.21). However, as shown in Figure 4.7b, mass action also favors formation of the mono Cu$^{2+}$·L-valine complex

$$Cu^{2+} + L \rightleftharpoons \beta_{10100} CuL^+$$

(4.22)

when the Cu$^{2+}$ concentration is increased. Formation of the ternary complex (10101) at the stationary phase then requires release of a Cu$^{2+}$ ion into the solution phase

$$CuL^+ + CuCS^+ \rightleftharpoons \beta_{10101}(\beta_{10100} \beta_{10001}) CuLCS + Cu^{2+},$$

(4.23)

which is predicted by Equation (4.23) and by our model to be thermodynamically disfavored at high Cu$^{2+}$ concentrations. As a result, although it increases the density of active stationary phase ligand, an increase in Cu$^{2+}$ in the mobile phase reduces formation of the ternary mixed-ligand complex, leading to the observed decrease in enantiomer retention time. By accounting for all chemical equilibria, the model properly predicts the retention times of both enantiomers of valine and their nonlinear decrease with increasing Cu$^{2+}$ concentration (Figure 4.6).

4.4.2.2. pH Effects

As reported by Belov et al. [54] and others [24,55], CLEC separations of racemic mixtures show a complex dependence on mobile phase pH. Figure 4.8 compares the
experimental and predicted pH dependence of the retention time \( t_R = t_e - t_0 \), where \( t_e \) is the enantiomer elution time and \( t_0 \) is the dead time of the system) for valine enantiomers on the Nucleosil Chiral-1 column. As is generally observed in CLEC separations, the on-column retention times for both enantiomers decrease nonlinearly with decreasing pH, in this case with the \( t_R \) value for the D-enantiomer decreasing much more sharply. At pH 3.2 and below, the retention times for both enantiomers coincide and no separation is observed.

The ability of the model to accurately capture these pH effects permits its use in interpreting the elution behavior in terms of chemical equilibria and their pH dependence. Decreasing the mobile phase pH increases the protonated 01001 state of the immobilized chiral selector (Figure 4.7), severely diminishing the concentration of the activated chiral selector (i.e., the 10001 species). The concentration of the ternary complex (i.e., the 10101 species) is likewise decreased, as formation of the ternary complex is destabilized under acidic conditions (e.g., pH 2-3) because both the free enantiomer and the uncomplexed chiral selector primarily exist in their fully protonated states, LH\(_2^+\) and CSH, respectively. As a result, the model reveals that formation of a ternary complex largely proceeds through the equilibrium reaction

\[
Cu^{2+} + LH^+ + CSH \leftrightarrow \beta_{10101}'\left(\beta_{02100}\left(\beta_{02001} - \beta_{01001}\right)\right)CuLCS + 3H^+, \quad (4.24)
\]

which is thermodynamically less favorable - \( K_a = \beta_{10101}'\left(\beta_{02100}\left(\beta_{02001} - \beta_{01001}\right)\right) = 1.35 \times 10^2 \) - than the corresponding dominant complex-formation reaction at pH 5.4,

\[
Cu^{2+} + LH + CS^- \leftrightarrow \beta_{10101}'\beta_{01100}CuLCS + H^+, \quad (4.25)
\]

139
for which $K_a = \frac{\beta_{0101}}{\beta_{01100}} = 1.56 \times 10^6$. The model therefore correctly predicts that the retention time for each enantiomer decreases sharply with decreasing pH until the interaction of either enantiomer with the stationary phase becomes sufficiently weak to permit both enantiomers to elute close to or with the column void (2.8 min).

4.4.2.3. Sample Loading Effects

Figure 4.9 reports the dependence of the elution band profiles for $D,L$-valine on the concentration of the racemate in the feed pulse. Peak distortion away from a Gaussian shape occurs with increasing feed concentration, as is commonly observed under column overload conditions due to the combined shelf-sharpening of the peak front and tailing of the peak end associated with operation within the nonlinear region of the isotherm. As both the peak width and the self-sharpening effect are necessarily larger for the more retained enantiomer, a more pronounced reduction in the retention time of that enantiomer is observed with increasing sample load. Similar observations have previously been reported by Kurganov et al. [25], who studied the impact of overloading on the elution peak profiles of various amino acids separated by ligand exchange chromatography.

Equations (4.9) specify equilibria within the CLEC column at any operating condition, including nonlinear and overload (i.e. ligand saturation) operation of the column. As a result, the band distortion and reduced elution times observed experimentally are predicted by the model (Figure 4.9), allowing it to be used to more carefully explore column-loading effects on separation performance. We find that elution peak distortion in CLEC columns is also predicted at conditions within the linear regime of column operation. For example, significant
tailing is both seen and predicted in the elution peaks corresponding to a feed pulse containing $D,L$-valine at a concentration (10 mM) far below the local density of the immobilized chiral selector (510 mM) (Figure 4.9). This is consistent with experimental results of Fornstedt et al. [56,57], who observed peak tailing in chiral phases at low concentrations of feed analyte. Almost all models of linear elution chromatography under local equilibrium conditions predict Gaussian peak profiles unless the column efficiency is very poor - below ca. 50 NTUs. Fornstedt et al. therefore assumed that peak tailing at low enantiomer feed concentrations was a consequence of heterogeneity of the stationary phase, concluding that the stationary phase offers at least two different types of binding sites: one that involves specific chiral interactions and the other nonspecific interactions. However, our model suggests that perturbations in peak symmetry within the linear binding regime may also arise from the strong sensitivity of chemical equilibria within a volume element of the column to the total enantiomer concentration within that element. The total enantiomer concentration is a maximum at the elution band apex, which leads to an increase in $bis$-binary complex formation, a decrease in the enantiomer distribution coefficient ($s/c_i$), and an associated increase in the effective interstitial migration velocity of the enantiomer out of that volume element.

4.4.2.4. Temperature Effects

Raising the column operating temperature provides sharper elution peaks, but shorter retention times [58,59]. Recently, we reported on the temperature dependence of amino acid transport within the Nucleosil Chiral-1 column and found it to be limited by pore diffusion at all column operating temperatures, indicating that the condition of local equilibria is maintained over a wide temperature range [27]. In general, an increase in temperature decreases the viscosity of the mobile phase, increasing the diffusion coefficient of the analyte in the mobile
phase and within the pore liquid of the stationary phase, and thereby improving the separation performance through what is collectively termed the kinetic effect. However, the kinetic effect is weak for separation of amino acid racemates within the Nucleosil Chiral-1 column; raising the column temperature from 10 to 60 °C enhances pore diffusion and film mass transfer of the analyte by only a small amount. Instead, changes in separation performance with temperature correlate more closely with changes in chemical equilibria. This is reflected in Figure 4.10, which shows model predictions of the temperature dependence of retention times for L-valine on the Nucleosil Chiral-1 column. Good agreement with experiment is observed, with the model indicating that the reduction in $t_R$ with increasing temperature is largely due to a more severe weakening of the stability of the ternary complex as compared to the stabilities of the various binary complexes formed in the mobile phase.

4.5 Optimization of CLEC Operating Conditions

As illustrated in Figures 4.4 to 4.10, CLEC column performance is sensitive to a number of operating variables, including temperature, pH, Cu(II) concentration, and sample load. An appropriately constructed composite design of experiments could be used to map separation efficiency onto this multivariable landscape, thereby permitting identification of optimal column operating conditions. Our model can also be used for this purpose. This latter approach is of course tempered by the computing time required to solve the model, which can be significant because of the large set of chemical equilibria equations that must be solved. Our model is currently programmed in FORTRAN 90 on a Pentium 4 PC that utilizes a 2.4-GHz microprocessor capable of generating a complete chromatogram prediction in 3 to 12 hrs depending on the enantiomer elution time. As a result, the mapping of column performance over all operational space is prohibitively time consuming (several months of simulation), but could be reduced on an IBM "Eclipse" SP4 or an equivalent multinode supercomputer, making
possible global optimization of a CLEC-based separation in less than a week. Here, however, we focus on a more restricted application of the model to system optimization with the aim of illustrating the potential power of the approach.

Figure 4.11 maps the predicted peak resolution $R_s$ for a racemic mixture of valine on the Nucleosil Chiral-1 column as a function of mobile phase Cu(II) concentration and pH under conditions where all other column operating variables are held constant. Here the resolution is defined in the conventional way such that baseline peak separation is achieved at $R \geq 1$. Model results were confirmed through comparison to a set of 16 operating conditions randomly distributed throughout the 2-variable domain (data not shown to avoid graphical clutter). The model results show that $R_s$ depends nonlinearly on pH and Cu(II), exhibiting a maximum value at a mobile phase pH and Cu(II) concentration of ca. 5.0 and 0.5 mM, respectively. At all Cu(II) concentrations, $R_s$ drops off rapidly with decreasing pH due to severe destabilization of the ternary mixed-ligand complex under acidic conditions.

We set as an optimization target those operating conditions that give an $R_s$ of 1.1, thereby ensuring complete baseline separation, and a minimum enantiomer retention time. The operating condition of pH = 3.75 and [Cu(II)] = 4 mM then results in the smallest retention time for the more retained D-enantiomer. Column throughput at this restricted system optimum is more than 3-times that at pH = 5.4 and [Cu(II)] = 0.5 mM (the column manufacturer's suggested operating conditions), illustrating the potential power of this computational optimization strategy.
4.6 Conclusions

Used to separate racemic mixtures of alkaloids, amino acids, barbiturates, β-blockers and other adrenergic drugs, CLEC discriminates enantiomers through differences in the $\Delta G^\circ$ required to transfer each enantiomer from the mobile phase to the stationary phase surface. In this work we show that CLEC separations are not only governed by the equilibrium formation constants for the ternary mixed-ligand complexes formed at the stationary phase surface, but also depend on an additional set of chemical equilibria in solution and at the stationary phase surface.

We have proposed a one-dimensional non-ideal equilibrium-dispersion chromatography model that can be used to predict and interpret enantiomer transport and elution profiles in CLEC columns. The model accounts for all complexation reactions within the solution and stationary phases of a CLEC column. All thermodynamic parameters required to compute chemical equilibria in the column are taken from potentiometric titration data reported in the literature [34,35,39]. The formation constants used are therefore pure thermodynamic parameters that do not include contributions specific to the CLEC column used. A single adjustable parameter, determined by regression to a single elution peak for the pure component, is used to capture column-specific effects on the stereoselectivity of the immobilized ligand. The predictive performance of the model was assessed through comparison to chromatograms for two hydrophobic amino acids separated on a Nucleosil Chiral-1 column. In addition to providing a means of predicting elution profiles, the model provides a sufficiently detailed picture of the separation process to permit interpretation of the mechanism of separation and the associated influence of key column operating variables.
The explicit inclusion of all chemical equilibria makes the model parameter rich. However, the additional parameters are all equilibrium formation constants that, in most systems of interest, can be found in standard thermodynamic databases [34,35]. In cases where required formation constants are not available, we [37,39] and many others [34,35 and references therein] have shown that they can be determined with high precision through analysis of potentiometric titration data using efficient matrix inversion programs such as SUPERQUAD [60]. Inclusion in the model of the complete set of chemical equilibria relations therefore requires no more on-column experiments than are typically required to regress parameters in traditional models of chromatography that utilize either a noncompetitive or a competitive Langmuir isotherm to compute binding equilibria. In both cases, a single elution experiment for each pure component is all that is required. However, by accounting for time and position dependent chemical equilibria within the column, a richer picture of the CLEC separation process can be obtained.
4.7 Tables

Table 4.1 Column characteristics, mass transfer coefficients and some dimensionless parameters characterizing the transport of L-valine injected onto the Nucleosil Chiral-1 column as a 10 μL pulse at a flow rate of 1 mL min\(^{-1}\) at 298.15 K.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol (units)</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column Length</td>
<td>(L \text{ (m)})</td>
<td>0.25</td>
</tr>
<tr>
<td>Column Radius</td>
<td>(R_c \text{ (m)})</td>
<td>(2.0 \times 10^{-3})</td>
</tr>
<tr>
<td>Bead Radius</td>
<td>(R_p \text{ (m)})</td>
<td>(2.5 \times 10^{-6})</td>
</tr>
<tr>
<td>Column Void</td>
<td>(\varepsilon)</td>
<td>0.45 ± 0.01</td>
</tr>
<tr>
<td>Stationary Phase Porosity</td>
<td>(\varepsilon_p)</td>
<td>0.65 ± 0.01</td>
</tr>
<tr>
<td>Kinematic Viscosity of Mobile Phase</td>
<td>(\nu \text{ (m}^2/\text{sec)})</td>
<td>(9.09 \times 10^{-7})</td>
</tr>
<tr>
<td>Column Axial Dispersion Coefficient</td>
<td>(D_L \text{ (m}^2/\text{sec)})</td>
<td>(3.2 \pm 0.2 \times 10^{-8})</td>
</tr>
<tr>
<td>Overall Mass Transfer Coefficient</td>
<td>(K_m \text{ (sec}^{-1})</td>
<td>18.99 ± 0.45</td>
</tr>
<tr>
<td>Pore Diffusion Coefficient</td>
<td>(D_p \text{ (m}^2/\text{sec)})</td>
<td>1.24 ± 0.02 (\times 10^{-11})</td>
</tr>
<tr>
<td>Film Mass Transfer Coefficient</td>
<td>(k_f \text{ (m/sec)})</td>
<td>7.1 ± 0.1 (\times 10^{-4})</td>
</tr>
<tr>
<td>Peclet Number</td>
<td>(Pe = \frac{Lu}{D_L})</td>
<td>1.04 ± 0.07 (\times 10^4)</td>
</tr>
<tr>
<td>Biot Number</td>
<td>(Bi = \frac{k_fR_p}{D_p})</td>
<td>143 ± 2</td>
</tr>
<tr>
<td>Forward Rate Constant</td>
<td>(k_{ads} \text{ (sec}^{-1})</td>
<td>(\geq 7.60 \times 10^4)</td>
</tr>
<tr>
<td>Damkohler Number</td>
<td>(Da = \frac{k_{ads}R_p^2}{D_p})</td>
<td>(\geq 3.83 \times 10^4)</td>
</tr>
<tr>
<td>Reynolds Number</td>
<td>(Re = \frac{2R_pu}{\nu})</td>
<td>7.30 (\times 10^{-3})</td>
</tr>
</tbody>
</table>
Table 4.2  Ternary equilibrium formation constants determined from potentiometric titration measurements \((I_c = 0.1\text{ M KNO}_3, \ T = 298.15 \text{ K})\) and the on-column correction factor \(k_l\) accounting for effects of chiral selector immobilization for various amino-acid•Cu(II)•L-hydroxyproline complexes.

<table>
<thead>
<tr>
<th>System</th>
<th>(\log_{10}\beta) Value</th>
<th>(k_l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{Cu, H, L-Val, D-Val, L-Hydropro})</td>
<td>(15.73 \pm 0.02^{(i)})</td>
<td>(1.3 \times 10^{-3})</td>
</tr>
<tr>
<td></td>
<td>(15.64 \pm 0.02)</td>
<td>(3.4 \times 10^{-2})</td>
</tr>
<tr>
<td>(\text{Cu, H, L-Phe, D-Phe, L-Hydropro})</td>
<td>(15.57 \pm 0.02)</td>
<td>(2.2 \times 10^{-2})</td>
</tr>
<tr>
<td></td>
<td>(15.46 \pm 0.02)</td>
<td>(4.2 \times 10^{-2})</td>
</tr>
</tbody>
</table>

1) Errors reported as standard deviation from the mean for 10 to 15 independently regressed data sets

Table 4.3  Ternary equilibrium formation constants determined from potentiometric titration measurements and \(k_l\) values for the \(L\)-valine•Cu(II)•\(L\)-hydroxyproline complex over the temperature range 298.15 to 333.15 K.

<table>
<thead>
<tr>
<th>Temperature (K)</th>
<th>(\log_{10} \beta_{10101}) Value</th>
<th>(k_l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>298.15</td>
<td>(15.73 \pm 0.02^{(i)})</td>
<td>(1.3 \times 10^{-3})</td>
</tr>
<tr>
<td>310.15</td>
<td>(15.25 \pm 0.02)</td>
<td>0</td>
</tr>
<tr>
<td>318.15</td>
<td>(14.88 \pm 0.01)</td>
<td>(1.5 \times 10^{-2})</td>
</tr>
<tr>
<td>333.15</td>
<td>(14.0 \pm 0.1)</td>
<td>(3.0 \times 10^{-2})</td>
</tr>
</tbody>
</table>

1) Errors reported as standard deviation from the mean for 10 to 15 independently regressed data sets
4.8 Figures

Figure 4.1 General description of an elution chromatography column containing spherical, porous sorbent particles as the stationary phase.
Figure 4.2 Measured first central moment ($\mu_1$) as a function of superficial velocity $u$ of the mobile phase for 10 $\mu$l pulses of 10 mg ml$^{-1}$ Dextran T10 over the superficial velocity range $u = 2.65 \times 10^{-4}$ to $1.33 \times 10^3$ (m s$^{-1}$). The mobile phase contained 0.5 mM CuSO$_4$ (pH = 5.4, T = 298.15 K).
Figure 4.3 Height equivalent of a theoretical plate (HETP) values for 10 \mu l pulses of 1 mM $D$-glucose (filled squares) and 1 mM $L$-valine (filled triangles) loaded onto a Nucleosil Chiral-1 column at interstitial velocities $u_0$ ranging from $2.06 \times 10^{-3}$ to $3.83 \times 10^{-3}$ (m s$^{-1}$). The mobile phase contained 0.5 mM CuSO$_4$ (pH = 5.4, T = 298.15 K).
Figure 4.4 Comparison between experimental (dashed line) and model predicted (solid line) elution profiles for a 10 μL pulse injection of 2 mM $\text{D,L-valine}$ onto the Nucleosil Chiral-1 column at a flow rate of 1 mL min$^{-1}$. The mobile phase contained 1 mM CuSO$_4$ at pH 5.4. The column was maintained at a temperature of 298.15 K and contained 310 mM immobilized $L$-hydroxyproline.
Figure 4.5  Comparison between experimental (dashed line) and model predicted (solid line) elution profiles for a 10 μL pulse injection of 2 mM D, L-phenylalanine onto the Nucleosil Chiral-1 column at a flow rate of 1 mL min⁻¹. The mobile phase contained 2 mM CuSO₄ at pH 5.4. The column was maintained at a temperature of 298.15 K and contained 510 mM immobilized L-hydroxyproline.
Figure 4.6 Comparison as a function of mobile-phase Cu(II) concentration between experimental (filled squares: $L$-valine, filled stars: $D$-valine) and predicted retention times (solid lines) for valine enantiomers (2 mM $D,L$-valine) injected as a 10 μL pulse onto the Nucleosil Chiral-1 column at 1 mL min$^{-1}$. The column was maintained at a temperature of 298.15 K and contained 310 mM immobilized $L$-hydroxyproline. The mobile phase contained CuSO$_4$ at pH 5.4.
Figure 4.7 The pH dependence of equilibrium Cu(II)-containing complexes in an aqueous solution containing 310 mM L-hydroxyproline and 2 mM L-valine at 298.15 K. The copper concentration in the system was set at 184.47 mM, which corresponds to the total concentration of Cu(II) when the mobile phase Cu(II) concentration is 0.5 mM, or at 223.29 mM, the total Cu(II) concentration when the mobile phase Cu(II) concentration is 3 mM. Subscript 'abcde' on concentration $C_{abcde}$ of complex abcde indicates the molecules of Cu$^{2+}$ (a), protons (b), L-amino-acid enantiomer (c), D-amino-acid enantiomer (d), and chiral selector (e) in the complex:

(A) open squares - $C_{10101}$ when [Cu] = 3 mM, open circles - $C_{10100}$ when [Cu] = 3 mM, filled squares - $C_{10101}$ when [Cu] = 0.5 mM, filled circles - $C_{10100}$ when [Cu] = 0.5 mM, filled inverse triangles - $C_{02100}$ when [Cu] = 0.5 mM, filled diamonds - $C_{01100}$ when [Cu] = 0.5 mM;

(4.7B) open circles - $C_{10002}$ [Cu] = 3 mM, open squares - $C_{10001}$ when [Cu] = 3 mM, filled circles - $C_{10002}$ when [Cu] = 0.5 mM, filled squares - $C_{10001}$ when [Cu] = 0.5 mM, filled diamonds - $C_{01001}$ when [Cu] = 0.5 mM.
Figure 4.8 The dependence of enantiomer retention times on pH. Comparison between experimental (D-valine: open stars and L-valine: open squares) and predicted (line) retention times for 2 mM D,L-valine injected as a 10 μL pulse onto the Nucleosil Chiral-1 column. The column was at 298.15 K and contained 310 mM immobilized L-hydroxyproline. The mobile phase contained 0.5 mM CuSO₄ at pH 5.4 and was flowing at 1 ml min⁻¹.
Figure 4.9  The dependence of the elution chromatogram on sample loading concentration for a 10 μL injection of (a) 10 mM \(D,L\) valine, (b) 5 mM \(D,L\) valine and (c) 2 mM \(D,L\) valine onto the Nucleosil Chiral-1 column. Both experimental (dashed lines) and predicted (solid lines) elution profiles are shown. The mobile phase flow rate was maintained at 1 mL min\(^{-1}\), and the mobile phase contained 1.5 mM CuSO\(_4\). The column contained 310 mM immobilized \(L\)-hydroxyproline and was maintained at 298.15 K.
Figure 4.10  Temperature dependence of the retention time for a 10 μL pulse of 0.5 mM L-valine injected onto the Nucleosil-Chiral-1 column at a flow rate of 1 mL min⁻¹. The mobile phase contained 0.5 mM CuSO₄ at pH 5.4 and the stationary phase contained 510 mM L-hydroxyproline. Both experimental (filled squares) and predicted (solid line) retention times are shown.
Figure 4.11  Response surface plot of the predicted resolution ($R_s$) of valine enantiomers on a Nucleosil Chiral-1 column. All simulations were for a 10 μL pulse injection of 1 mM D,L-valine onto the column and the results were validated with 16 experimental chromatograms randomly distributed across the response surface (data not shown to avoid graphical clutter). The column was 0.1 m in length and contained a ligand density of 310 mM L-hydroxyproline. The column temperature and mobile-phase flowrate were maintained at 298.15 K and 1 ml min$^{-1}$, respectively. The resolution $R_s$ was calculated as 

$$R_s = \frac{(t_{e_2} - t_{e_1})}{0.5(W_1 + W_2)}$$

where $t_{e_i}$ is the retention time of enantiomer $i$ and $W_i$ is the corresponding peak width at baseline.
4.9 References


50-67.

[42] Holland, C.D., Liapis, A.I., Computer methods for solving dynamic separation


[44] Haynes, H.W., Sarma, P.N., "Model for application of gas-chromatography to
measurements of diffusion in bidisperse structured catalysts", AIChE J. 19 (1973):
1043-1046.


1984.

[47] Wilson, E.J., Geankopolis, C.J., "Liquid mass transfer at very low reynolds numbers

[48] Perry, R.H., Green, D.W., Maloney, J.O., Perry's chemical engineers' handbook. 7th

[49] Wu, Y.X., Ma, P.S., Liu, Y.Q., Li, S.F., "Diffusion coefficients of L-proline, L-
threonine and L-arginine in aqueous solutions at 25 °c", Fluid Phase Equilib. 186

ligand exchange chiral stationary phase for the liquid chromatographic resolution of

[51] Hyun, M.H., Han, S.C., Whangbo, S.H., "Liquid chromatographic separation of the
enantiomers of beta-amino acids on a ligand exchange chiral stationary phase",

[52] Zheng, Z.X., Lin, J.M., Qu, F., Hobo, T., "Chiral separation with ligand-exchange
micellar electrokinetic chromatography using a D-penicillamine-copper(II) ternary

[53] Doury-Berthod, M., Poitrenaud, C., Tremillon, B., "Ligand-exchange separation of
amino-acids .2. Influence of the eluent composition and of the nature of the ion-


5  Modeling Profiles and Separation of Dopa-Enantiomer Elution Bands During Chiral Ligand Exchange Chromatography

5.1  Introduction

Parkinson's disease is a progressive neurological disorder characterized by akinesia and resting tremor, abnormal posture, bradykinesia, and abnormal rigidity. In advanced stages of the disease, the patient may also experience severe autonomic and sensorimotor dysfunction, cognitive decline, and depression. The predominant pathology of Parkinson's disease is a significant loss of dopaminergic neurons in the substantia nigra. In most instances, the formation of intracellular proteinaceous inclusions throughout the brain is also observed [1]. The loss of dopaminergic cells results in a reduction in the level of the essential neurotransmitter dopamine. This leads to a progressive reduction of cortical motor output and the associated changes in posture and movement symptomatic of Parkinson's disease [2,3]. In the early stages of Parkinson's disease, motor function abnormalities can be readily restored by dopamine replacement therapy using the dopamine precursor L-dopa (3-(3',4'-dihydroxyphenyl)-L-alanine) in conjunction with a peripheral dopa-decarboxylase inhibitor (e.g., benserazide, carbidopa).

L-dopa is prepared by direct enantioselective synthesis or by achiral synthesis of the dopa racemate and separation of the two enantiomers using chiral ligand-exchange chromatography (CLEC) [4]. Introduced by Davankov and coworkers [5], CLEC is based on the formation of

* A version of this chapter has been submitted to the AIChE J. [Reference: Sanaie, N. and Haynes, C.A., Modeling Profiles and Separation of Dopa-Enantiomer Elution Bands During Ligand Exchange Chromatography. AIChE J.]
labile ternary metal complexes, usually at the surface of the stationary phase, between a stereoselective ligand, a transition metal (usually Cu\(^{2+}\)) and one of the enantiomers. Resolution of enantiomers is achieved through a small difference in the stabilities of the two diastereomeric ternary complexes, permitting CLEC to be used to separate racemates of amino acids and their derivatives, including many important classes of alkaloids, barbiturates, \(\beta\)-blockers and other adrenergic drugs [6,7]. CLEC separation of racemates generally depends on a complex set of chemical equilibria within the mobile and stationary phases of the column. As a result, the separation efficiency (resolution) depends strongly on a number of column operating variables, including temperature, pH, ligand density, and the concentrations of the enantiomers and the transition metal in the feed. Although identification of suitable operating conditions for separation of enantiomers by CLEC can be achieved through proper design of experiments, we have recently shown that process optimization can be achieved more rapidly and exhaustively using a new model for enantiomer transport and elution that combines a traditional reaction-diffusion theory of chromatography with multiple chemical equilibria theory. The latter theory is used to define all chemical speciation and adsorption equilibria within the column [8].

In this work, we apply our model to prediction of elution chromatograms for the separation of an \(L,D\)-dopa racemate by CLEC. Owing to its 3',4'-dihydroxyphenyl side-chain, dopa possesses a large number of protonation states and Cu\(^{2+}\) binding sites (Figure 5.1). The chemical equilibria driving the CLEC-based separation of dopa enantiomers are therefore unusually complex, making modeling of this process particularly challenging. The performance of the model in predicting elution chromatograms and separation performance as a function of key column operating variables is reported. The model is then used to better
understand the connection between chemical equilibria within the system and changes in band profiles and band separation resulting from changes in column operating conditions.

5.2 Theory

5.2.1 Model Development

The complete derivation of our model is provided elsewhere [8] and we therefore present here only its key features. The structure of the model is based on results from first and second moment analysis of experimental elution chromatograms that confirm that solute mass transfer within the column is limited by the rate of diffusion within the pores of the stationary phase. As a result, the condition of local equilibrium can be assumed at all times at every radial position within the spherical stationary phase particle. Multiple chemical equilibria theory is used to calculate the concentration of each diastereomeric ternary complex formed at the stationary phase surface along with the chemical speciation profiles within the mobile phase and pore liquid. Speciation is used here in the usual thermodynamic sense and refers to the complete set of equilibrium components and complexes present in the system. For a cylindrical column of length $L$ packed with a stationary phase composed of spherical sorbent particles of radius $R_p$ and porosity $\varepsilon_p$, the continuity equation for transport of any solute $i$ within the interstitial volume of void fraction $\varepsilon$ is given by

$$\frac{\partial c_i}{\partial t} = D \frac{\partial^2 c_i}{\partial z^2} - \frac{u \partial c_i}{\varepsilon \partial z} - \frac{(1 - \varepsilon)}{\varepsilon} \frac{\partial s_i}{\partial t}$$

(5.1)
where $c_i(z,t)$ (mole/m$^3$ bulk fluid) is the total concentration of component $i$ in the mobile phase (assumed to be independent of the radial and angular position within the column cross section), $D_L$ is the axial dispersion coefficient (m$^2$ s$^{-1}$), $u$ is the superficial liquid velocity (m s$^{-1}$), and $z$ is the axial position coordinate. The total concentration $c_i$ of component $i$ includes the moles of free component $i$ in the mobile phase plus the moles of $i$ in all complexes present in the mobile phase. The average solute concentration $s_i(z,t)$ (mole/m$^3$ adsorbent particle) within the stationary phase particles located at column position $z$ at time $t$ is given by

$$s_i(z,t) = \frac{1}{\frac{4}{3} \pi R_p^3} \int_0^{R_p} \left[ \varepsilon_p c_i^* (r,z,t) + (1 - \varepsilon_p) q_i^* (r,z,t) \right] 4\pi r^2 dr$$

(5.2)

where $c_i^* (r,z,t)$ (mole/m$^3$ pore fluid) and $q_i^* (r,z,t)$ (mole/m$^3$ adsorbent particle) are the total concentration of the solute in the intraparticle fluid and adsorbed to the sorbent surface, respectively.

Solution of the continuity equation requires knowledge of the rate of solute uptake $\frac{\partial s_i}{\partial t}$ within the porous sorbent particles, given by

$$\frac{(1 - \varepsilon_p)}{\varepsilon_p} \frac{\partial s_i}{\partial t} = 3\frac{(1 - \varepsilon_p)}{R_p \varepsilon_p} D_p \left( \frac{\partial c_i}{\partial r} \right)_{r=R_p}$$

(5.3)

where $D_p$ is the intraparticle diffusivity of the solute (m$^2$ s$^{-1}$).
Finally, gradients in free solute concentration within the pores of the stationary phase are determined through a corresponding solute mass balance within the bead

\[
\frac{\partial c_i^*}{\partial t} = D_p \left( \frac{\partial^2 c_i^*}{\partial r^2} + 2 \frac{\partial c_i^*}{\partial r} \right) - \frac{l - \epsilon_p}{\epsilon_p} \frac{\partial q_i^*}{\partial t}
\]  

(5.4)

Solution of Equation (5.4) requires a model for chemical equilibria relating \( q_i^*(r, z, t) \) to the chemical speciation of component \( i \) in the adjacent pore fluid.

### 5.2.2 Determination of Chemical Equilibria

Chemical speciation in chiral ligand-exchange chromatography systems is governed by a set of equilibrium formation reactions of the general form

\[
a M^{2+} + b H^+ + c L^- + d L'^- + e C\tilde{S}^- \leftrightharpoons \beta_{abcde} \rightarrow M_{a/b/c/d/e} H \_L \_L' \_C \tilde{S}
\]  

(5.5)

where \( M^{2+} \) is the metal ion (i.e., Cu\(^{2+}\)), \( H^+ \) is the proton, \( L^- \) is the fully deprotonated (free) \( L^- \)-enantiomer, \( L'^- \) is the fully deprotonated \( D \)-enantiomer, \( C\tilde{S}^- \) is the fully deprotonated chiral selector (N-octyl-3-octythio-\( D \)-valine, a derivative of \( D \)-penicillamine, in this application), and \( a, b, \ldots \) are the stoichiometric coefficients for the equilibrium complex. The formation constant \( \beta_{abcde} \) is defined in terms of equilibrium concentrations of the reactants. We therefore designate \( \beta_{abcde} \) as a concentration formation constant to clearly differentiate it from a formation constant based on the activities of the reactants. It is important to recognize that the major thermodynamic databases for bidentate ligand complexes almost exclusively report concentration formation constants, as activity coefficients for the pure and complexed
components are rarely available [9,10]. Thus, in many cases, the required $\beta_{abcde}$ values can be taken from the literature and need not be measured. A negative value for the stoichiometric coefficient $b$ indicates the presence and number of hydroxyl ions in the complex.

In our model, equilibria Equations (5.5) are combined with total mass balance equations for $M^{2+}, H^+, L^-$, and $CS^-$ to obtain a set of nonlinear algebraic equations that may be solved to determine the equilibrium composition at any solution temperature and pH. For example, the total mass balance for the metal ion is given by

$$T_M = \left[M^{2+}\right] + \sum_k a_k \left[M^{2+}_k H_k L_k L'_k CS_k\right] = \left[M^{2+}\right] + \sum_k a_k \beta_{abcde_k} \left[M^{2+}\right] \left[H^+\right]^{b_k} \left[L^-\right]^{c_k} \left[L'_-\right]^{d_k} \left[CS^-\right]^{e_k}$$  

(5.6)

where $T_M$ represents the total concentration of the metal ion (mol L$^{-1}$) in the system and $\beta_{abcde_k}$ is the formation constant for complex $k$. With all model parameters known, including the equilibrium formation constant for each complex present, Equations (5.1) through (5.6) can be solved to predict composition profiles within and exiting the column as a function of time. Boundary conditions and a description of the Crank-Nicolson scheme [11] used to approximate differentials by a central difference in time and an average central difference in space are described in detail elsewhere [8]. The column was meshed in the $z$ dimension to match or exceed the number of column NTUs and the number of radial volume elements within the stationary phase particle was set equal to 5.
5.3 Experimental

5.3.1 Materials and Methods

Dopa enantiomers, copper sulfate (CuSO₄) pentahydrate, potassium nitrate (KNO₃) and copper nitrate (Cu(NO₃)₂) were purchased from Sigma-Aldrich Chemical Canada Ltd. (Oakville, ON). The chemicals were always of the highest available purity (>99%) and were used without further purification. The D-penicillamine derivative (p-methylbenzyl-D-penicillamine) used as a solution-phase analogue of the chiral selector was purchased from Peptides International Inc. (Louisville, Kentucky). Water used in all experiments was first distilled and then treated with a NANOpure® II ultrafiltration system (Barnstead; Dubuque, IW). All solutions for the HPLC system were filtered through 0.22 μm (GV) Durapore filters (Millipore) and degassed prior to use. The Waters HPLC system used in these studies consists of a 717 autosampler, a 486 UV detector, a 410 differential refractor and a 600S controller. A 610 Waters column heater was used to control the column at the desired operating temperature.

5.3.2 Potentiometric Titrations

Protonation constants for L,D-dopa and formation constants for all complexes formed between L-dopa, D-dopa and the Cu²⁺ ion were determined by potentiometric titration for two solvent systems: water and an aqueous solution containing 10% isopropanol. The details of the potentiometric titration protocol have been described previously [12]. Potentiometric titrations were also used to determine protonation constants for the chiral selector, as well as formation constants for binary complexes formed between Cu²⁺ and the chiral selector and for ternary CS, Cu²⁺, L,D-dopa mixed-ligand complexes. In these experiments, a water-soluble analogue (p-methylbenzyl-D-penicillamine, hereafter referred to as p-MBD-penicillamine) of the chiral
selector (N-octyl-3-octylthio-\textit{D}-valine) was used to allow titrations to be conducted in a single phase. The sulfide-protecting methylbenzyl group was included in the analogue to prevent disulfide bond formation which leads to the reduction of Cu(II) to Cu(I) through donation of an electron to copper. Equilibrium formation constants were regressed from potentiometric titration data using the chemometric program \textit{CHEMEQ} described previously [13].

5.3.3 Chromatography Experiments

CLEC studies were performed on a Chirex 3126 column from Phenomenex Inc. (Torrance, CA). The stationary phase consists of porous silica particles with the average particle diameter of 5 \( \mu \text{m} \) and an average pore diameter of 110 Å. The particles are packed into a stainless steel column of length \( L = 150 \ \text{mm} \) and inner diameter 4.6 mm. The N-octyl-3-octylthio-\textit{D}-valine chiral selector is physically immobilized on the silica matrix through hydrophobic interactions between its dioctyl tail and the C18 brush grafted onto the silica matrix (Figure 5.2). The column manufacturer reports the density of the chiral selector immobilized on the Chirex 3126 column to be 50 mM.

Degassed stock solutions (90% water, 10% isopropanol) of each enantiomer (10 mM \( L \) or \( D \)-dopa) were made in 2 mM CuSO\(_4\) such that the \( \frac{[\text{Cu}]}{[\text{dopa}]} \) ratio was 1:5. A stock solution of the racemate (10 mM total dopa) was then prepared by mixing equal volumes of each enantiomer stock solution. All samples were filtered through an Acrodisc 0.2 \( \mu \text{m} \) PVDF syringe filter (Gelman) prior to injection into the column. Experiments were performed at a volumetric flow rate of 1 mL/min. The column was equilibrated with at least 10 column
volumes of mobile phase (90% water, 10% isopropanol) prior to each 10 μL sample injection. Eluent from the column was monitored by UV absorbance at 254 nm.

5.4 Results and Discussion

5.4.1 Column Properties and Solute Transport Characteristics

Table 5.1 reports the geometric properties of the Chirex 3126 column as well as mass transfer coefficients and related parameters describing the transport of (L,D)-dopa within the mobile and stationary phases of the column. The first and second moment analysis method of Ruthven [14] was applied to determine the fraction of external voids (ε) and all solute transport parameters inside the column [15-17]. Results from this analysis were used to compute dimensionless groups that allowed determination of the rate-limiting resistance to solute mass transfer within the column. Both the Damköhler number (Da > 10^4) and the Biot number (Bi ~ 370) are large, indicating that solute diffusion through the pores of the stationary phase limits the overall rate of adsorption to the porous resin. This permits the local equilibrium approximation to be applied throughout the column.

5.4.2 Equilibrium Formation Constants for the Cu^{2+}-Dopa-p-MBD-Penicillamine System

The multiple chemical equilibria theory encoded in our CLEC model requires a set of equilibrium formation constants and reaction stoichiometric coefficients for complexes formed within the mobile phase and stationary phase of the CLEC column. The speciation in aqueous systems containing protons, a transition metal ion and a catecholamine like dopa can be quite
complex, necessitating a careful set of potentiometric and spectroscopic studies to identify the dopa-Cu\(^{2+}\) species formed and to determine all related thermodynamic parameters [18,19].

5.4.2.1. Protonation Constants

Dopa (3-(3', 4'-dihydroxyphenyl)-alanine) is a polyprotonic acid for which it can be difficult to assign measured protonation constants to specific functional groups due to the overlap of protonation states. This problem is generally overcome by defining a set of 'macro'-protonation constants that define the overall protonation state of the compound but are not linked to specific titratable groups. Jameson [20] used linear free-energy relations and kinetic evidence to attempt to assign the order of protonation of dopa, which begins with either one of the phenolato groups of the catechol ring, and proceeds with protonation of the second phenolato group, the \(\alpha\)-amino group, and finally the \(\alpha\)-carboxyl group. However, the protonation states of the phenolato groups and the \(\alpha\)-amino group are partially overlapping, making it difficult to precisely assign the second and third protonation states of dopa.

Measured equilibrium formation constants for all H\(^+\)-dopa species are reported in Table 5.2. All are in good agreement with previous studies, confirming the accuracy of the proton titration experiments carried out in this work [19,21]. The four stepwise macro-protonation constants \((pK_a)\) of dopa may be computed from the reported \(\beta\) values by subtracting \(\beta_{ij100}\) from \(\beta_{i(i+1)100}\); the \(pK_a\) value for species 04100 of dopa in aqueous solution is therefore 2.20. The addition of 10% isopropanol increases the two highest macro-protonation constants \((pK_a\) for species 01100 and \(pK_a\) for species 02100), which we believe correspond to the two phenolato
groups on the catechol, and also increases the $pK_a$ for species 04100, which can be unambiguously assigned to the $\alpha$-carboxyl group.

Addition of 10% isopropanol decreases the $pK_a$ of species 03100, which strongly suggests that this $pK_a$ represents the protonation constant for the $\alpha$-amino group. Addition of an organic alcohol to the mobile phase lowers the dielectric constant of the solvent and therefore increases electrostatic forces between charged groups. Thus, the activity of the proton is increased in the presence of isopropanol, shifting mass action away from the free proton and toward neutral complexes containing the proton. Thus, the $pK_a$ for the two phenolato groups are expected to increase with addition of isopropanol, as is observed with the $pK_a$s of species 01100 and 02100, while the $pK_a$ for the $\alpha$-amino group is predicted to decrease, as is observed for species 03100. In a recent study [17] we observed similar trends for $\alpha$-amino acid protonation constants when methanol served as the organic cosolvent.

Aqueous-phase protonation constants for the chiral selector analogue ($p$-MBD-penicillamine) are similar but not identical to those reported previously for the carboxylic (the $pK_a$ for species 02001 is reported to be 1.91 [22,23]) and $\alpha$-amino ($pK_a$ for species 01001 reported to be 8.10 [23]) groups of penicillamine. The addition of 10% isopropanol decreases the $pK_a$ for species 01001, the protonation constant for the $\alpha$-amino group, and increases the $pK_a$ for the $\alpha$-carboxyl group. As with dopa, the addition of organic cosolvent therefore disfavors the zwitterionic state of $p$-MBD-penicillamine in favor of the neutral forms of each titratable group on the molecule [17,24,25].
5.4.2.2. Equilibrium Formation Constants for Binary and Ternary Complexes

Equilibrium speciation in aqueous solutions containing dopa and transition metals has been the subject of intense study due to its biological importance [18,19,21]. In the presence of a transition metal ion, dopa can form a traditional amino-acid type O,N bidentate complex with Cu$^{2+}$ via its α-carboxyl and α-amino groups, and pyrocatechol type O,O bidentate complexes via its two phenolato groups. UV and ESR spectral studies by Gergely and Kiss [19] of the copper (II), D,L-dopa system indicate that at low pH (< 5) amino-acid type complexes of composition CuH$_2$A and Cu(H$_2$A)$_2$ are preferentially formed, while at higher pH values (> 9) the pyrocatechol type complex Cu(HA)$_2$ and its various deprotonated species are favored. In the intermediate pH range (5 < pH < 9), amino-acid type complexes are favored, but cyclic and open-chain dimeric species are also produced in appreciable amounts, including complexes containing both N,O and O,O bonds.

Speciation within the Cu$^{2+}$-H$^+$-dopa system is also strongly dependent on the Cu$^{2+}$ to dopa ratio. As a result, determination of formation constants for all Cu$^{2+}$-H$^+$-dopa complexes formed in solution is not possible in a single titration experiment, and instead requires regression of titration data recorded at two or more metal-ligand ratios. When the Cu$^{2+}$ to dopa ratio is 1 to 1, standard 1:1 and 1:2 amino-acid type complexes are favoured, but significant amounts of dimetallic complexes (Cu$_2$H$_2$A$_2$ and Cu$_2$A$_2$) can also form, particularly at higher pH, due to dopa behaving as a bridging ligand. When the Cu$^{2+}$ to dopa ratio is 1 to 2, formation of dimetallic complexes is significantly reduced, permitting more accurate regression of formation constants for those complexes containing a single Cu$^{2+}$ ion [26-28].
Equilibrium formation constants for binary Cu\(^{2+}\)-(H\(^+\))-dopa complexes and binary Cu\(^{2+}\)-p-MBD-penicillamine complexes formed in water and in aqueous solutions containing 10% isopropanol are reported in Table 5.3. Together, the formation constants indicate that the addition of 10% isopropanol shifts equilibria towards formation of \emph{bis} binary complexes, thereby reducing the concentration of uncomplexed dopa and p-MBD-penicillamine in the system, particularly at solution pH values above 3.5. The stability of the \emph{mono}-binary Cu\(^{2+}\)-p-MBD-penicillamine complex, the active form of the chiral selector, also increases appreciably.

The overall equilibrium formation constant for the dominant ternary complexes (species 12101 and 12011) also increases with addition of isopropanol (Table 5.4). Stabilization of these neutral ternary complexes occurs not only because of the lower dielectric permittivity of the cosolvent, but also through the lower hydrogen bonding potential of isopropanol, which disfavors solvation of Cu(II) through coordination of the oxygen of isopropanol to one of the four planar or two distal coordination sites of the metal ion. As a result, the displacement of an alcohol ligand by a dopa enantiomer or the chiral selector is favored relative to the displacement of a water molecule complexed with the metal ion.

\textbf{5.4.2.3. Species Distribution Within the Chirex CLEC Column}

Our potentiometric titration data show that up to 30 species can form within the mobile and stationary phases of the Chirex CLEC column during the separation of \(L,D\)-dopa. However, differences in the magnitudes of the formation constants reported in Tables 5.2 to 5.4 indicate that not all of these complexes will be present in the column under normal column operating conditions. The manufacturer recommends operating the Chirex 3126 column at 2 mM Cu\(^{2+}\) with 10 mM racemate in the feed pulse. The operating pH is typically set between
3.5 and 6, with a value between 5 and 5.5 being the most common. Consider then the injection of only L-dopa onto the column under this range of recommended operating conditions. The resulting dominant chemical equilibria are shown in Figure 5.3, which reports the solution of Equations (5.6) within a central volume element of the L-dopa elution band when the Chirex column is operated at 25°C with a 10% isopropanol/90% water mobile-phase containing 2 mM CuSO₄. Although more than two dozen equilibrium complexes are formed under these conditions, only species 04100, 03100, 12101, 11101, 14200, 22200, 12100, 10001 and 01001 are present at significant concentrations. Moreover, at pH 5.5 and below, the concentration of the 12101 ternary complex is much higher than that of the 11101 complex. We have used this knowledge to simplify the model by including in our multiple chemical equilibria equations only those complexes (04100, 03100, 12101, 14200, 22200, 12100, 10001, 01001, and the corresponding complexes for D-dopa) that are present at sufficiently high concentrations to influence the elution behavior of the enantiomers.

5.4.3 Modeling the Separation of Dopa Enantiomers

The formation constant data reported in Tables 5.2 to 5.4 suggest that it is possible to use p-MBD-penicillamine to separate L,D-dopa racemates in water. However, the Chirex 3126 column cannot be operated in pure water due to the fact that the chiral selector (N-octyl-3-octylthio-D-valine) is immobilized through hydrophobic bonding of the dioctyl tail with the C18 chemistry of the underlying resin. As with all reverse-phase columns, the structure of the C18 brush is sensitive to solvent polarity and collapses in pure water, effectively eliminating proper column function. As a result, all elution chromatograms and modeling results refer to a mobile phase containing 10% isopropanol.
While the $\beta_{12101}$ and $\beta_{12011}$ values reported in Table 5.4 define the stabilities of the two ternary mixed-ligand complexes in solution, they do not account for changes in complex stabilities arising from differences in the chemistry of the chiral selector (N-octyl-3-octythio-$D$-valine) relative to that of the solution-phase analogue ($p$-MBD-penicillamine) used in the titration studies. Nor do they include the effect (e.g. steric packing changes, etc.) of immobilizing the chiral selector onto the surface of the stationary phase. Although generally quite small, these effects influence CLEC elution band profiles in subtle ways [8] that can be accounted for by defining a constant $k_l$

$$\log(\beta_{ijklm}^*) = (1 + k_i) \log(\beta_{ijklm})$$

(5.7)

that may be used to determine the on-column ternary formation constant $\beta_{ijklm}^*$ from the corresponding solution-phase value $\beta_{ijklm}$. Reported in Table 5.4, $k_l$ values were determined by model regression to the elution band profiles for a 10-$\mu$L pulse injection of each pure enantiomer (10 mM) onto the Chirex column operating at a flowrate of 1 mL min$^{-1}$ and a mobile phase pH and CuSO$_4$ concentration of 5.4 and 2 mM, respectively. Each $k_l$ value is a function of temperature only. For both enantiomers, $k_l$ is quite small, providing no more than a 3% correction to the value of $\beta_{ijklm}$. As a result, the elution behavior of dopa enantiomers on the Chirex column can be described with reasonable accuracy using tabulated formation constants obtained from standard thermodynamic databases provided an appropriate solution-phase analogue of the chiral selector can be identified.
Figure 5.4 compares with experiment the predicted chromatogram for a 10 µL pulse injection of a 10-mM dopa racemate onto the Chirex 3126 column at a flow rate of 1 mL min⁻¹, a temperature of 25°C, and a mobile phase pH of 5.4. Unlike the conditions used to regress \( k_i \), the mobile-phase Cu²⁺ concentration is now 3 mM. The predicted chromatogram quantitatively captures the experimental data. Similar agreement is observed at other operating conditions (see below), indicating that the model can be used to map the \( L,D\)-Dopa separation efficiency as a function of column operating conditions.

5.4.3.1. Copper Concentration Effects

The mobile-phase copper concentration is known to have strong influence on the quality of CLEC-based separations [8,29-32]. For the Chirex column operated at pH 5.4, Figure 5.5 reports experimental retention times for both \( L \)- and \( D \)-dopa as a function of Cu²⁺ concentration under otherwise constant column operating conditions. An increase in \([\text{Cu}^{2+}]\) results in a nonlinear decrease in the retention times of both enantiomers, with the decrease in the retention of the more retained enantiomer being considerably more pronounced. Model analysis reveals that the observed decrease in retention time with increasing \([\text{Cu}^{2+}]\) results from changes in chemical equilibria that reduce partitioning of the enantiomer into the stationary phase. Figure 5.6 compares chemical equilibria within a central volume element of the elution band for \( L\)-Dopa when the Chirex column is operated at two different mobile-phase Cu²⁺ concentrations: 1 mM \( \text{CuSO}_4 \) and 3 mM \( \text{CuSO}_4 \). Only those complexes present at concentrations large enough to influence the elution behavior of the enantiomer are shown. At pH 5.4 (the mobile phase pH used to collect the data shown in Figure 5.5), an increase in the Cu²⁺ concentration increases the concentration of activated chiral selector (species 10001) (Figure 5.6b), but also shifts chemical equilibria in the mobile phase toward Cu(II)-containing
L-dopa complexes (Figure 5.6a). Formation of the dominant ternary complex (species 12101) at the stationary phase then requires release of a Cu$^{2+}$ ion into the solution phase

$$\text{CuL}^+ + \text{CuCS}^+ \xrightleftharpoons{\beta_{12101}} \text{CuLCS} + \text{Cu}^{2+},$$

which is thermodynamically disfavored at high Cu$^{2+}$ concentrations. As a result, although it increases the density of active stationary-phase ligand, an increase in Cu$^{2+}$ in the mobile phase reduces formation of the ternary complex, leading to the observed decrease in enantiomer retention time.

The quality of a chromatographic separation is often measured by the resolution, $R$, which is equal to the ratio of the distance between the peak maxima to the mean band width of the two neighboring peaks, $\bar{w}$,

$$R = \frac{\Delta t_R}{\bar{w}} = \frac{2(t_{R,2} - t_{R,1})}{(w_1 + w_2)},$$

where $t_{R,1}$ and $t_{R,2}$ are the retention times of components 1 and 2, and $w_1$ and $w_2$ are the peak widths measured by the baseline intercept. As shown in Figure 5.7, model predictions accurately capture the complex dependence of the resolution on Cu$^{2+}$ concentration.

5.4.3.2. pH Effects

Figure 5.8 compares with experiment predicted retention times for L- and D-dopa as a function of pH. As is generally observed in CLEC separations [33-35] the retention times of
both enantiomers decrease nonlinearly with decreasing pH, with the retention time for the D-enantiomer showing a more pronounced decline. At pH 3.2 and below, the retention times for the two enantiomers become identical and equal to the elution time of the column void; no separation is predicted or observed at these pH values.

Predictions of the dependence of the resolution ($R_s$) on pH agree well with experiment (Figure 5.9), indicating that our model is capable of quantitatively capturing the dependence of the separation performance on primary column operating parameters. It also allows us to understand the underlying molecular phenomena through analysis of changes in the distribution of various complexes in the system following a change in operating conditions (e.g., pH, Cu$^{2+}$ concentration, [Cu$^{2+}$]/[dopa], etc.). For example, in the pH range of 4 to 5.5, increasing the pH results in a corresponding increase in the concentration of the activated chiral selector (species 10001) and a concomitant decrease in the protonated state of the chiral selector (species 01001) (Figure 5.3b). Similarly, the concentrations of various protonated forms of the two enantiomers to be separated diminish with increasing pH, thereby avoiding the thermodynamically unfavorable release of free protons into a proton-rich solution during ternary complex formation. In contrast, at low pH the free enantiomer mainly exists in two highly protonated states, species 03100 and 04100. Formation of ternary complex then must proceed via a reaction between fully or highly protonated forms of the enantiomer and the chiral selector, resulting in a sharp decrease in the concentration of the dominant ternary complexes (12101 and 12011). Finally, because the stability of the hetero-chiral $L$-dopa-Cu$^{2+}$-chiral selector complex is less than that of the homo-chiral $D$-dopa-Cu$^{2+}$-chiral selector complex, the model predicts a stronger impact of the mobile phase pH on the retention time of the $D$-enantiomer, as observed experimentally (Figure 5.8).
5.5 Conclusions

L-dopa has served as a primary treatment of Parkinson's disease for over 30 years. We have shown that the separation behavior of a dopa racemate within a N-octyl-3-octylthio-\textit{D}-valine bearing CLEC column can be accurately predicted by incorporating a complete description of solution and surface chemical equilibria into a traditional one-dimensional reaction-diffusion model of chromatography. The model relies on a set of equilibrium formation constants and reaction stoichiometries that can be taken from standard thermodynamic databases or measured to a high degree of accuracy through regression of potentiometric titration data. Although fairly difficult to solve due to the complex speciation within the system, the model quantitatively captures the dependence of the elution chromatogram on key column operating variables, thereby providing a facile route to interpret the mechanism of separation and to determine optimum column operating conditions.
5.6 Tables

Table 5.1 Column characteristics, mass transfer coefficients and some dimensionless parameters characterizing the transport of L-dopa injected onto the Chirex 3126 column as a 10 μL pulse at a flow rate of 1 mL min⁻¹ at 298.15 K.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol (units)</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column Length</td>
<td>$L$ (m)</td>
<td>0.15</td>
</tr>
<tr>
<td>Column Radius</td>
<td>$R_c$ (m)</td>
<td>$2.3 \times 10^{-3}$</td>
</tr>
<tr>
<td>Bead Radius</td>
<td>$R_p$ (m)</td>
<td>$2.5 \times 10^{-6}$</td>
</tr>
<tr>
<td>Column Void</td>
<td>$\varepsilon$</td>
<td>$0.32 \pm 0.01$</td>
</tr>
<tr>
<td>Stationary Phase Porosity</td>
<td>$\varepsilon_p$</td>
<td>$0.81 \pm 0.01$</td>
</tr>
<tr>
<td>Average Pore Diameter</td>
<td>$P_d$ (m)</td>
<td>$1.1 \times 10^{-8}$</td>
</tr>
<tr>
<td>Kinematic Viscosity of Mobile Phase</td>
<td>$\nu$ (m²/sec)</td>
<td>$9.09 \times 10^{-7}$</td>
</tr>
<tr>
<td>Column Axial Dispersion Coefficient</td>
<td>$D_L$ (m²/sec)</td>
<td>$5.8 \pm 0.2 \times 10^{-8}$</td>
</tr>
<tr>
<td>Overall Mass Transfer Coefficient</td>
<td>$K_m$ (sec⁻¹)</td>
<td>$14.07 \pm 0.35$</td>
</tr>
<tr>
<td>Pore diffusion Coefficient</td>
<td>$D_p$ (m²/sec)</td>
<td>$6.15 \pm 0.2 \times 10^{-12}$</td>
</tr>
<tr>
<td>Film Mass Transfer Coefficient</td>
<td>$k_f$ (m/sec)</td>
<td>$9.05 \pm 0.1 \times 10^{-4}$</td>
</tr>
<tr>
<td>Interstitial Velocity</td>
<td>$u_0$ (m/sec)</td>
<td>$3.14 \times 10^{-3}$</td>
</tr>
<tr>
<td>Peclet Number</td>
<td>$Pe = L \ u / D_L$</td>
<td>$2.60 \pm 0.09 \times 10^3$</td>
</tr>
<tr>
<td>Biot Number</td>
<td>$Bi = k_f R_p / D_p$</td>
<td>$368 \pm 16$</td>
</tr>
<tr>
<td>Forward Rate Constant</td>
<td>$k_{ads}$ (sec⁻¹)</td>
<td>$\geq 7.60 \times 10^4$</td>
</tr>
<tr>
<td>Damkohler Number</td>
<td>$Da = k_{ads} R_p^2 / D_p$</td>
<td>$\geq 7.72 \times 10^4$</td>
</tr>
<tr>
<td>Reynolds Number</td>
<td>$Re = 2 R_p u / \nu$</td>
<td>$5.5 \times 10^{-3}$</td>
</tr>
</tbody>
</table>
Table 5.2 Protonation constants for dopa and \textit{p-MBD-penicillamine} determined from potentiometric titration experiments \((T = 298.15 \text{ K}, 0.1 \text{ M KNO}_3)\) in two solvent systems: water and an aqueous solution containing 10\% isopropanol.

<table>
<thead>
<tr>
<th>System</th>
<th>(\log_{10}\beta)</th>
<th>Aqueous</th>
<th>90% Water, 10% isopropanol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\beta_{01100}^{(0)})</td>
<td>13.3 ± 0.1(^{(0)})</td>
<td>13.4 ± 0.4</td>
</tr>
<tr>
<td>Cu, H, L-dopa, D-dopa, \textit{p-MBD-penicillamine}</td>
<td>(\beta_{02100})</td>
<td>23.18 ± 0.04</td>
<td>23.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>(\beta_{03100})</td>
<td>31.95 ± 0.02</td>
<td>31.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>(\beta_{04100})</td>
<td>34.15 ± 0.03</td>
<td>34.1 ± 0.2</td>
</tr>
<tr>
<td>Cu, H, L-dopa, D-dopa, \textit{p-MBD-penicillamine}</td>
<td>(\beta_{01001})</td>
<td>8.51 ± 0.02</td>
<td>8.42 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>(\beta_{02001})</td>
<td>10.4 ± 0.1</td>
<td>10.5 ± 0.1</td>
</tr>
</tbody>
</table>

I) The subscript '\(abcde\)' on \(\beta_{abcde}\) indicates the molecules of Cu\(^{2+}\) (a), protons (b), L-dopa (c), D-dopa (d) and ligand selector \textit{p-MBD-penicillamine} (e) in species \(abcde\).

II) Errors reported as standard deviation from the mean for 10 to 15 independently regressed data sets.
Table 5.3  Equilibrium formation constants for binary Cu$^{2+}$•(H$^+$)•L-dopa and Cu$^{2+}$•p-MBD-penicillamine complexes determined from potentiometric titration data (T = 298.15 K, 0.1 M KNO$_3$) in two solvent systems: water and an aqueous solution containing 10% isopropanol. The last column indicates the ratio of [L-dopa] to [Cu(II)] in the titration experiment used to regress the corresponding $\beta_{abcd}$ value. The pH range over which the titration was carried out was 3 to 6.5 when the L-dopa to Cu$^{2+}$ ratio was 1, and was 6.5 - 11.5 when the L-dopa to Cu$^{2+}$ ratio was 2.

<table>
<thead>
<tr>
<th>System</th>
<th>$\log_{10}\beta$</th>
<th>Aqueous</th>
<th>90% water, 10% isopropanol</th>
<th>[L-dopa]/[Cu]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>60.4 ± 0.3 (I)</td>
<td>60.5 ± 0.2</td>
<td>1:1</td>
</tr>
<tr>
<td>Cu$^{2+}$, H$^+$, L-dopa, D-dopa, p-MBD-penicillamine</td>
<td>$\beta_{14200}$</td>
<td>54.1 ± 0.4</td>
<td>53.5 ± 0.3</td>
<td>2:1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>45.3 ± 0.4</td>
<td>45.5 ± 0.1</td>
<td>2:1</td>
</tr>
<tr>
<td></td>
<td>$\beta_{1200}$</td>
<td>35.4 ± 0.3</td>
<td>36.2 ± 0.1</td>
<td>2:1</td>
</tr>
<tr>
<td></td>
<td>$\beta_{12200}$</td>
<td>25.3 ± 0.3</td>
<td>25.8 ± 0.1</td>
<td>2:1</td>
</tr>
<tr>
<td></td>
<td>$\beta_{2200}$</td>
<td>30.6 ± 0.1</td>
<td>30.7 ± 0.1</td>
<td>1:1</td>
</tr>
<tr>
<td></td>
<td>$\beta_{22200}$</td>
<td>53.1 ± 0.2</td>
<td>53.4 ± 0.2</td>
<td>1:1</td>
</tr>
<tr>
<td></td>
<td>$\beta_{20200}$</td>
<td>41.9 ± 0.2</td>
<td>42.4 ± 0.1</td>
<td>1:1</td>
</tr>
<tr>
<td>Cu$^{2+}$, H$^+$, L-dopa, D-dopa, p-MBD-penicillamine</td>
<td>$\beta_{10001}$</td>
<td>7.35 ± 0.11</td>
<td>7.49 ± 0.04</td>
<td>2:1</td>
</tr>
<tr>
<td></td>
<td>$\beta_{10002}$</td>
<td>14.64 ± 0.09</td>
<td>14.67 ± 0.08</td>
<td>2:1</td>
</tr>
</tbody>
</table>

I) Errors reported as standard deviation from the mean for 10 to 15 independently regressed data sets
Table 5.4  Ternary equilibrium formation constants for Cu$^{2+}$•L-dopa•p-MBD-penicillamine and Cu$^{2+}$•D-dopa•p-MBD-penicillamine complexes determined from potentiometric titration measurements ($T = 298.15$ K, 0.1M KNO$_3$) in two solvent systems: water and an aqueous solution containing 10% isopropanol. The on-column correction factor $k_l$ accounting for the effects of chiral selector immobilization within the Chirex 3126 column is also reported.

<table>
<thead>
<tr>
<th>System</th>
<th>Log$_{10}\beta$</th>
<th>Aqueous</th>
<th>90% water, 10% isopropanol</th>
<th>$k_l$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\beta_{12101}$</td>
<td>37.6 ± 0.2$^{(1)}$</td>
<td>37.9 ± 0.5</td>
<td>2.1×10$^{-2}$</td>
</tr>
<tr>
<td>Cu$^{2+}$, H', L-dopa, D-dopa,</td>
<td>$\beta_{11101}$</td>
<td>31.8 ± 0.5</td>
<td>31.7 ± 0.7</td>
<td>-------</td>
</tr>
<tr>
<td>p-MBD-penicillamine</td>
<td>$\beta_{12011}$</td>
<td>37.1 ± 0.3</td>
<td>37.6 ± 0.4</td>
<td>3.2×10$^{-2}$</td>
</tr>
<tr>
<td></td>
<td>$\beta_{11011}$</td>
<td>31.6 ± 0.4</td>
<td>31.9 ± 0.5</td>
<td>-------</td>
</tr>
</tbody>
</table>

I)  Errors reported as standard deviation from the mean for 10 to 15 independently regressed data sets
5.7 Figures

Figure 5.1  Structure of 3-(3',4'-dihydroxyphenyl)-L-alanine (L-dopa)

Figure 5.2  Structure of the chiral selector (N-octyl-3-octythio-D-valine) immobilized through hydrophobic bonding with the C18 reversed-phase chemistry of the Chirex 3126 column.
Figure 5.3 The pH dependence of major complexes formed in an aqueous solution containing 10% isopropanol, 2 mM CuSO₄ and 10 mM L-dopa injected as a 10 μL pulse onto the Chirex 3126 column at 1 mL min⁻¹. The column was maintained at 298.15 K and contained 50 mM immobilized N-octyl-3-octylthio-D-valine.

(3A) Open squares - complex 03100, filled triangles - 04100, filled squares - 12101, open stars - 11101, open triangles - 14200, open circles - 22200, and filled circles - 12100

(3B) Filled squares - 10001, and filled circles - 01001
Figure 5.4 Comparison between experimental (dashed line) and model (solid line) elution profiles for a 10 µL pulse injection of 10 mM D,L-dopa onto the Chirex 3126 column. The aqueous mobile phase (1 mL min⁻¹) contained 10% isopropanol and 3 mM CuSO₄ at pH 5.4. The column was maintained at 298.15 K and contained 50 mM immobilized N-octyl-3-octylthio-D-valine.

![Graph showing elution profiles](image-url)
Figure 5.5  Comparison as a function of mobile-phase Cu(II) concentration between experimental (filled squares: L-dopa, filled triangles: D-dopa) and predicted (solid lines) retention times for dopa enantiomers (10 mM D,L-dopa) injected as a 10 μL pulse onto the Chirex 3126 column at 1 mL min⁻¹. The column was maintained at 298.15 K and contained 50 mM immobilized N-octyl-3-octylthio-D-valine. The mobile phase contained 10% isopropanol at pH 5.4.
Figure 5.6  The pH dependence of the dominant equilibrium complexes in an aqueous mixture at 298.15 K containing 10 mM L-dopa, 50 mM N-octyl-3-octylthio-D-valine and 10% isopropanol. The copper concentration in the system was set at 47.24 mM, corresponding to the total concentration of Cu(II) when the mobile phase Cu(II) concentration is 1 mM, or at 51.68 mM, the total Cu(II) when the mobile phase Cu(II) concentration is 3 mM. The open and filled symbols represent complexes when [Cu(II)] is 1 mM and 3 mM, respectively, in the mobile phase.

(6A): Squares - complex 12101, circles - 11101, stars - 14200, diamonds - 22200, triangles - 12100, inverse triangles - 04100, pentagons - 03100

(6B): Squares - 01001, circles - 10001.
Figure 5.7 Comparison between the experimental (filled circles) and predicted (solid lines) resolution of dopa enantiomers as a function of mobile phase Cu(II) concentration. The data apply to a 10 μL pulse of 10 mM L,D-dopa injected onto the Chirex 3126 column at a flow rate of 1 mL min⁻¹. The column was maintained at 298.15 K and contained 50 mM immobilized N-octyl-3-octylthio-D-valine. The mobile-phase contained 10% isopropanol at pH 5.4.
Figure 5.8  The dependence of retention time on pH. Comparison between experimental (L-dopa: filled stars and D-dopa: filled circles) and predicted (line) retention times for injection of 10 mM D,L-dopa as a 10 μL pulse onto the Chirex 3126 column operated at 298.15 K. The column contained 50 mM N-octyl-3-octylthio-D-valine. The aqueous mobile phase contained 10% isopropanol and 2 mM CuSO₄. The flow rate was 1 ml min⁻¹.
Figure 5.9 Comparison between experimental (filled squares) and model (solid line) results for the resolution $R_S$ of dopa enantiomers on the Chirex 3126 column. A 10 µL pulse of 10 mM $L,D$-dopa was injected onto the column (298.15 K) at a flow rate of 1 ml min$^{-1}$. The aqueous mobile phase contained 10% isopropanol and 2 mM CuSO$_4$. 
5.8 References


6 Conclusions and Suggestions for Future Work

The modeling of chromatographic separations has matured considerably since the inception of the technology more than 50 years ago. Resistances to solute mass transfer are now well understood, and a number of reliable theories for quantifying rates of solute and eluent mass transfer within packed-bed columns are now available for characterizing column loading under conditions where the solutes in the mobile phase are noninteracting/noncomplexing and solute binding to the stationary phase falls within the linear isotherm regime [1-4]. Current efforts to improve modeling of complex chromatographic separations therefore largely focus on accounting for changes in chromatographic behavior resulting from adsorption in the nonlinear regime, and many empirical and semi-fundamental models have been derived for this purpose [5-7]. In most cases, these models are applied only to the target solute of interest in order to characterize its binding and elution behavior in a background solution containing contaminating and potentially competing analytes. As the transport and binding properties of the contaminating analytes are not well known, model parameters for the target are generally regressed from a limited set of chromatographic data.

Considerably less attention has been given to understanding the impact on solute elution band profiles of the ability of the solutes to be separated to complex with each other in the mobile phase and within the pore liquid of the stationary phase, presumably because of the paucity of thermodynamic data characterizing the chemical equilibria within these systems and the computational challenge in explicitly accounting for both surface and solution-phase equilibria. However, our ability to handle more computationally intensive problems has improved dramatically over the past two decades. More importantly, standard thermodynamic
databases [8,9] now include many of the equilibrium data and thermodynamic parameters required to compute solution-phase equilibria in chromatographic separations, and entries into these databases are increasing at a rapid rate. In cases where the required data are not available, reliable methods for their accurate and rapid determination are available. This thesis exploits these advances to address issues related to how fundamental thermodynamic data may be combined with chromatographic theory to understand, predict and optimize chromatographic separations in cases where chemical complexation occurs in the liquid phase. While solution-phase chemical complexation can occur in essentially any mode of liquid chromatography, it is normally encountered in certain modes, including ion chromatography [10,11], reverse-micellar extraction chromatography [12,13], and the various forms of ligand exchange chromatography [14,15] including CLEC [16,17].

A model of chiral ligand-exchange chromatography (CLEC) is presented that combines the nonideal equilibrium-dispersion equation for solute transport with equations describing all chemical equilibria within the column. The model connects elution band profiles to the time and space resolved formation of diastereomeric complexes in both the mobile and stationary phases, thereby providing insights into the overall separation mechanism. The stoichiometries and formation constants for all equilibrium complexes formed in the mobile phase are taken from standard thermodynamic databases and independent potentiometric titration experiments. Formation constants for complexes formed with the stationary phase ligand are determined from potentiometric titration data for a water-soluble analogue of the stationary-phase ligand. Together this set of pure thermodynamic parameters can be used to calculate the equilibrium composition of the system at any operating condition. The model includes a temperature-
dependent pure-component parameter, determined by regression to a single elution band for the pure component, that corrects for subtle effects associated with immobilizing the chiral selector onto the stationary phase. Model performance is assessed through comparison with chromatograms for two hydrophobic amino acid racemates loaded on the Nucleosil Chiral-1 CLEC column, and for dopa racemate loaded onto the Chirex 3126 column. The model is also applied to a restricted optimization of column operating conditions to assess its predictive power. In all cases, model predictions compare well with experiment while also providing a molecular understanding of the separation process. In particular, the model permits one to predict and interpret the dependence of enantiomer retention times and elution band profiles on the temperature, solvent composition, pH, Cu(II) concentration, and enantiomer concentration within the mobile phase. The model therefore demonstrates how tabulated pure thermodynamic constants may be combined with chromatographic theory to provide a molecular understanding of the separation mechanism in CLEC columns and the associated influence of key operating variables on column performance.

As noted above, the fundamental thermodynamic approach taken in this model should be applicable to a number of common modes of chromatography where components in the mobile phase interact strongly with each other. A particularly interesting application is in the modeling and optimization of isoelectric chromatofocusing (ICF). Pioneered by Sluyterman et al. [18], ICF is a powerful chromatographic technique for resolving and fractionating complex protein mixtures. Capable of focusing protein and peptide analytes into tight elution bands, ICF typically utilizes a weak anion exchange column that has been equilibrated with a high pH buffer prior to loading of the protein mixture. A pH gradient is then generated within the column by introducing a low pH elution buffer either isocratically or as a gradient. Each
negatively charged protein bound to the column in the high-pH loading buffer then desorbs and elutes when the pH of the mobile phase approaches the pI of the protein. The elution band of the protein is strongly self-sharpening since any peak dispersion toward the column exit places the protein back in a high pH environment where adsorption is favored. Similarly, dispersion behind the peak maximum is disfavored by the associated low pH environment, in which the protein becomes positively charged and is thereby electrostatically repelled by the stationary phase. As the mechanism of separation is based on protein pI, proton equilibria in the mobile phase and at the stationary phase surface must be accounted for to accurately predict elution band profiles. The model presented in this thesis might therefore useful for interpreting, predicting and optimizing ICF chromatograms following a simple extension to incorporate pKa's of amino acid residues and the use of Henderson-Hasselbach equation [19] to predict the pIs of the eluting proteins. One can also envision using this model to address long-standing questions related to the pH difference between the mobile and stationary phases that is known to contribute to nonideal elution behavior in ion-exchange chromatography [20,21].

Further work on the molecular-thermodynamic modeling strategy presented in this thesis is warranted in light of its performance in interpreting and predicting CLEC-based separations and its potential applicability to a number of other important separation technologies. Of particular importance is the implementation of new codes and computer hardware capable of dramatically reducing computation times. The prediction of an elution chromatogram for a given column operating condition currently takes between 3 and 12 hours, limiting the utility of the model in column optimization studies and as a process simulator. Some strategies for reducing computation times are outlined in chapter 4 of this thesis and
include conversion to a vector-oriented scalable parallel code suitable for compiling and running on a multinode supercomputer.

The molecular thermodynamic model developed in this work utilizes concentration equilibrium formation constants regressed from potentiometric titration data at a constant ionic strength. Although the results from this work suggest that the influence of changes in background ionic strength on CLEC column performance are small, the overall thermodynamic strategy proposed in this thesis would certainly benefit from the ability to compute ion and solute activity coefficients and therefore the dependence of the standard (i.e., activity-based) equilibrium formation constants on ionic strength and composition. This is a challenging concept, although recent work by Alberty [22] provides a potential strategy for measuring activity coefficients of amino acids and other small-molecule enantiomers and for computing their dependence on temperature and ionic strength.
6.1 References


Appendix I

Measurement of the Ligand Density by Breakthrough Analysis

An estimate of the density of the immobilized chiral selector was provided by the manufacturer of each column used in this study. However, a method was sought to verify these ligand density values since neither manufacturer was confident in their estimate.

First moment analysis applied to frontal-loading data for the solute provides a convenient method for determining solute partitioning behavior within a chromatography column. In the frontal-loading experiment, a mobile phase containing solute (e.g. one of the enantiomers) at a fixed concentration \( c^0 \) is continuously fed through the column until complete breakthrough is observed such that the solute concentration in the eluent is \( c^0 \). The average elution volume \( V_e \) (m\(^3\) bulk fluid) is then computed from the maximum in the first derivative of the breakthrough curve. Partitioning of a solute into the stationary phase slows its rate of migration through the column since no flow along the column axis is observed within the stationary phase. To account for this effect, \( V_e \) (m\(^3\) bulk fluid) is typically expressed as the sum of two terms:

\[
V_e = V_o + \frac{S_i}{c_i} V_s
\]

A(I).1

where \( V_o \) (m\(^3\) interstitial fluid) is the column void volume, \( V_s \) (m\(^3\) stationary phase) is the volume of stationary phase particles, \( c_i \) (mole/m\(^3\) interstitial fluid) is the concentration of the analyte in the interstitial fluid, and \( s_i \) (mole/m\(^3\) stationary phase) is the corresponding concentration in the stationary phase. The first term on the right hand side of equation A(I).1 accounts for the volume of fluid that must be displaced from the column before breakthrough
of a nonbinding, nonpenetrating solute would be observed. Therefore we can substitute \( V_0 \) with \( V \varepsilon \), where \( V \) (m\(^3\)) is the column volume and \( \varepsilon \) is the void fraction. The second term accounts for the additional volume of mobile phase that must pass through the column to elute a solute that can partition into the stationary phase, where the ratio \( \frac{s_i}{c_i} \) defines the solute distribution coefficient.

As described in chapter 4 (see Table 4.1), solute mass transfer is limited by convective flow at \( Re < 2 \times 10^{-5} \), which corresponds to solvent superficial velocities less than \( 3.6 \times 10^{-6} \) m s\(^{-1}\). Equilibrium is then achieved everywhere in the column, so that the concentration of solute \( i \) within the pores of stationary phase \( c_i^* \) (mole/m\(^3\) pore fluid) is equal to \( c_i \). To fix ideas, let us now assume that solute binding to the stationary phase is well described by the one-component Langmuir isotherm equation, so that

\[
s_i = \varepsilon_p c_i + (1 - \varepsilon_p)q_i
\]

A(I).2

and

\[
q_i = q_{\text{max}} \frac{K_L c_i}{1 + K_L c_i}
\]

A(I).3
where $\varepsilon_p$ is the stationary phase porosity, $K_L$ (m$^3$ pore fluid/mole) is the Langmuir equilibrium binding constant, and $q_{\text{max}}$ (mole/m$^3$ stationary phase) is the equilibrium binding capacity of the column. Equation A(I).1 can then be written as:

$$V_e = \varepsilon V + \varepsilon_p (1 - \varepsilon)V + \frac{q_{\text{max}} K_L (1 - \varepsilon_p)(1 - \varepsilon)V}{1 + K_L c_i}$$  

or in double reciprocal form

$$\frac{V}{V_e - V(\varepsilon + \varepsilon_p (1 - \varepsilon))} = \frac{1}{(1 - \varepsilon)(1 - \varepsilon_p)q_{\text{max}} K_L} + \frac{c_i}{(1 - \varepsilon)(1 - \varepsilon_p)q_{\text{max}}}$$  

Equation A(I).5 explicitly relates $V_e$ to the feed concentration $c_i$. Under appropriate flow conditions, one can therefore determine $q_{\text{max}}$ from the slope of a plot of $\frac{V}{V_e - V(\varepsilon + \varepsilon_p (1 - \varepsilon))}$ versus $c_i$.

The same strategy can be used to determine the binding capacity of a CLEC column provided all reaction equilibria involving the solute are taken into account. In this case, the Langmuir adsorption isotherm must be replaced with a complete model of chemical equilibria at the surface of the stationary phase. Consider the case where the frontal-loading experiment is performed by continuously feeding the $L$ enantiomer of a racemate into a CLEC column containing a constant concentration of Cu(II) in the mobile phase. At pH $\leq 7$, the mass balance for the total density of immobilized chiral selector (CS) is then given by
$CS_{total} = [CS \cdot H] + [CS \cdot Cu] + [CS \cdot Cu \cdot L] + 2[CS_2 Cu]$  

$= \beta_{01001}[CS][H] + \beta_{10001}[CS][Cu] + \beta_{10101}[CS][Cu][L] + 2\beta_{10002}[CS]^2[Cu]$  

where $\beta_{abcde}$ is the equilibrium formation constant for the given complex; the subscripts define from left to right the number of metal ions (a), protons (b), L-enantiomer molecules (c), D-enantiomer molecules (d), and immobilized chiral selector molecules (e) in the complex.

In ligand exchange, binding of the chiral selector to the enantiomer is mediated by a Cu(II) ion. Thus, prior to injection of the enantiomer, the concentration of "active" chiral selector available for occupancy by the enantiomer is given by

$CS^o_{active} = CS_{total} - [CS \cdot H]_o - [CS_2 \cdot Cu]_o = [CS \cdot Cu]_o$  

where the subscript o indicates the enantiomer-free system. If enantiomer L is introduced at a low enough concentration, $CS^o_{active}$, the total concentration of active ligand, remains approximately equal to $CS^o_{active}$. The equilibrium concentration of unoccupied active ligand is then given by

$[CS \cdot Cu] = \frac{CS^o_{active}}{1 + \frac{\beta_{10101}}{\beta_{10001}}[L]} = \frac{CS^o_{active}}{1 + K_G[L]}$  

211
where \([L]\) is the concentration of free \(L\)-enantiomer. The adsorbed amount of enantiomer \(L\) is likewise equal to

\[
q_L = [CS \cdot Cu \cdot L] = \beta_{10101}^{10101} [CS][Cu][L] = \frac{\beta_{10001}^{10101} [CS \cdot Cu][L]}{\beta_{10001}^{10001}} = \frac{CS^o_{active} K_G[L]}{(1 + K_G[L])}
\]

Equation A(I).9 can be combined with equations A(I).1 and A(I).2

\[
\frac{V}{V_e - V(\varepsilon + \varepsilon_p(1 - \varepsilon))} = \frac{1}{(1 - \varepsilon)(1 - \varepsilon_p)CS^o_{active} K_G} + \frac{c_i}{(1 - \varepsilon)(1 - \varepsilon_p)CS^o_{active}}
\]

A(I).10

to allow determination of \(CS^o_{active}\) from the slope of a linear plot of \(\frac{V}{V_e - V(\varepsilon + \varepsilon_p(1 - \varepsilon))}\) versus \(c_i\) for frontal loading data where \(c_i\) is small. An example of this analysis is illustrated in Figure A(I)-1, which plots frontal loading data for \(L\)-valine on the Nucleosil Chiral 1 column (\(L\)-hydroxyproline is the chiral selector). The porosity and void fraction of this column were determined previously (see Chapter 4) to be 0.65 and 0.45, respectively. From the slope of the linear fit, \(CS^o_{active}\) is estimated to be 57 ± 3.5 mM.
The total density of immobilized ligand $CS_{total}$ can now be determined from the ligand mass balance (equation A(I).6) in the absence of any analyte. For a given Cu(II) concentration and pH in the mobile phase, one guesses a value for $CS_{total}$ and solves for $[CS \cdot H^+], [CS_2Cu]$ and $[CS \cdot Cu]$. A Newton-Raphson iteration is applied until the calculated value of $[CS \cdot Cu]$ is equal to that determined experimentally. For the Nucleosil Chiral 1 column characterized in figure A(I)-1, this yields a $CS_{total}$ value of $310 \pm 16$ mM, which is within experimental error of the ligand density provided by the column manufacturer.
Figure A(I).1 Elution volume data for L-valine as a function of feed concentration obtained from breakthrough analysis carried out on the Nucleosil-Chiral 1 column. Experimental data are shown with filled squares along with the linear fit (equation A(I).10) to the data. The mobile phase contained 0.5 mM CuSO₄ at pH 5.4. Experiments were carried out at a flow rate of 0.2 ml min⁻¹.

\[
Y = 0.091X + 0.78
\]

\[
R^2 = 0.99
\]
Appendix II

CLEC Model Solution Method

This appendix describes the discretization methods applied in the finite difference routine used to solve the column and bead continuity equations. The manner in which boundary conditions are applied is also described. Finally, the source code and a representative input file for the CLEC model program are provided along with embedded comments for each subroutine.

Fully Implicit Discretization Method Applied to the Column Continuity Equation

The column continuity equation (equation 4.1) is solved by segregating it into terms describing the total flux $J$ of solute (subscript $i$ has been eliminated to avoid confusion) crossing the interface of a column volume and the rate of solute uptake $S$ (source term) into the stationary phase

$$\frac{\partial c}{\partial t} + \frac{\partial J}{\partial z} = S$$

A(II).1

where

$$J = \frac{u}{\varepsilon} c - D_c \frac{\partial c}{\partial z}$$

A(II).2

$$S = -\frac{(1-\varepsilon)}{\varepsilon} \frac{\partial s}{\partial t}$$
Figure A(II).1 shows a grid point P in a column that has been discretized along the column axis $z$ and also in time. Spatial position $i$ and temporal position $j$ within the resulting two-dimensional grid contains point P, which is surrounded by a control volume. Solute mass transfer through grid point P proceeds from point W to point E; S to N defines the corresponding time vector. All neighbours are positioned equally in space and time, allowing the control volume to be positioned exactly midway between neighbouring grid points; $i$ is the column position index and $j$ is the time index. Integration of equation A(II).1 over the control volume then gives

$$c_{i,j+1} \frac{\Delta z}{\Delta t} + [J_e - J_w] = S\Delta z + c_{i,j} \frac{\Delta z}{\Delta t}$$

A(II).3
Difficulties solving this convective-diffusive equation (i.e. negative coefficients in the tridiagonal matrix) can be avoided by defining a dimensionless solute flux $J^*$

$$J^* = \frac{J \Delta z}{D_L}$$  \hspace{1cm} \text{(II).4}$$

and Peclet number

$$Pe = \frac{u \Delta z}{\varepsilon D_L}$$  \hspace{1cm} \text{(II).5}$$

The solute flux can then be expressed as

$$J^* = Pe \cdot c - \frac{\partial c}{\partial \left( \frac{z}{\Delta z} \right)}$$  \hspace{1cm} \text{(II).6}$$

The value of $c$, the concentration at the interface of control volume, will then be some weighted average of $c_i$ and $c_{i+1}$, while the gradient $\partial c/\partial (z/\Delta z)$ will be some multiple of $(c_{i+1} - c_i)$. This concept was first proposed by Patankar (1980) [1], who expressed equation (II).6 as
\[ J^* = Pe \left[ \alpha c_i + (1 - \alpha) c_{i+1} \right] - \beta (c_{i+1} - c_i) \]  

A(II).7

where \( \alpha \) and \( \beta \) are dimensionless multipliers that depend on \( Pe \). In this manner \( J^* \) can now be expressed as

\[ J^* = B c_i - A c_{i+1} \]  

A(II).8

\( A \) and \( B \) are dimensionless coefficients that are functions of the Peclet number. The coefficient \( A \) is associated with the grid point \( i+1 \), which is ahead of the interface, while \( B \) is associated with the grid point \( i \), which is behind the interface. Solution of the continuity equation then requires an understanding of the dependence of coefficients \( A \) and \( B \) on \( Pe \). If \( c_i \) and \( c_{i+1} \) are equal, the diffusive flux must be zero and \( J^* \) is simply the convective flux of the solute. Under these conditions,

\[ J^* = Pe \cdot c_i = Pe \cdot c_{i+1} \]  

A(II).9

and the combination of equations A(II).8 and A(II).9 gives

\[ B = A + Pe \cdot c \]  

A(II).10

A second useful property of \( A \) and \( B \) is a kind of symmetry where:

\[ A(-Pe) = B(Pe) \]
\[ B(-Pe) = A(Pe) \]  

A(II).11
The main implication of the two properties is that the functions $A(Pe)$ and $B(Pe)$ are both fully specified once the function $A(Pe)$ is known for positive values of $Pe$. This follows since, for $Pe < 0$,

\[ A(Pe) = B(Pe) - Pe \]
\[ = A(-Pe) - Pe \]
\[ = A(|Pe|) - Pe \]

A new operator $\|A, B\|$ can be defined to denote the greater of $A$ and $B$. Then, for all values of $Pe$, positive or negative, we can write

\[ A(Pe) = A(|Pe|) + \|Pe, 0\| \]
\[ B(Pe) = A(|Pe|) + \|Pe, 0\| \]

The flux relationships for grid points $e$ and $w$ can then be written as
Substitution of $J_w^*$ and $J_e^*$ into the continuity equation yields

\[
\left( c_{i,j+1} - c_{i,j} \right) \frac{\Delta z}{\Delta t} = \left( \frac{D_L}{\Delta z} \left( A(\left| Pe_w \right|) + \left| u_{int} \right| 0 \right) \right) c_{i+1,j+1} + \left( \frac{D_L}{\Delta z} \left( A(\left| Pe_e \right|) + \left| u_{int} \right| 0 \right) \right) c_{i,j+1} + \frac{D_L}{\Delta z} \left( A(\left| Pe_w \right|) + \left| u_{int} \right| 0 \right) c_{i-1,j+1} = S \Delta z
\]

where $u_{int}$, the interstitial fluid velocity, is equal to $u/\varepsilon$. Solution of equation A(II.14) requires knowledge of $A(|Pe|)$. When both convection and diffusion terms are present, Patankar [1] has suggested that $A(|Pe|)$ can be approximated by the following power law scheme

\[
A(\left| Pe \right|) = \left\| 0, (1 - 0.1 \left| Pe \right|)^5 \right\|
\]

In an analytical chromatographic column operating under plug flow conditions (i.e., flow rates < 2 mL min\(^{-1}\)), the Peclet number is independent of grid location so that

\[
Pe_w = Pe_e = \frac{u \Delta z}{\varepsilon D_L}
\]

and

\[
A(\left| Pe_e \right|) = A(\left| Pe_w \right|) = PeDum
\]
Substitution into equation A(II).15 then results in a simplified discretized form of the column continuity equation

\[
-\frac{D_L}{\Delta z} (PeDum) c_{i+1,j+1} + \left[ \frac{2D_L}{\Delta z} PeDum + u_{\text{int}} + \frac{\Delta z}{\Delta t} \right] c_{i,j+1} - \left[ \frac{D_L}{\Delta z} PeDum + u_{\text{int}} \right] c_{i-1,j+1} = S\Delta z + c_{ij} \frac{\Delta z}{\Delta t}
\]

A(II).18

A(II).18 represents a set of linear algebraic equations for \(i=2,3, \ldots, m-1, m\), where \(m\) is the number of grid points in the \(z\) direction of the column, of the general form

\[
a(i) c_{i-1,j+1} + b(i) c_{i,j+1} + c(i) c_{i+1,j+1} = d(i) \quad i=2,3,4, \ldots, m-1
\]

A(II).19

where the coefficients are given by

\[
a(i) = -\left[ \frac{D_L}{\Delta z} PeDum + u_{\text{int}} \right]
\]

\[
b(i) = \left[ \frac{2D_L}{\Delta z} PeDum + u_{\text{int}} + \frac{\Delta z}{\Delta t} \right]
\]

\[
c(i) = -\left[ \frac{D_L}{\Delta z} PeDum \right]
\]

\[
d(i) = c_{ij} \frac{\Delta z}{\Delta t} + S \Delta z
\]

This set of equations can be solved with Thomas back-substitution algorithm [2] and the set of boundary conditions described below.
Column Boundary Conditions

Column boundary conditions must be applied to the general discretized form of the column continuity to complete the tridiagonal matrix required to solve equations A(II).19. The boundary conditions used are

1) \[ c = c_{in} = 0 \quad t = 0, \quad 0 \leq z \leq L \]

2) \[ D_L \frac{\partial c}{\partial z} \bigg|_{z=0} = \frac{u}{\varepsilon} \left( c_{i=0} - c_{\text{feed}} \right) \quad t > 0, \quad z = 0 \quad \text{A(II).20} \]

3) \[ \frac{\partial c}{\partial z} \bigg|_{z=L} = 0 \quad t \geq 0, \quad z = L \]

where \( c_{in} \) is the solute concentration in the column prior to sample injection, and \( c_{\text{feed}} \) is the solute concentration in the injected volume.

Discretizing the boundary condition (2) at the column inlet \((i=1)\) results in

\[
\frac{c_{2,i+1} - c_{0,i+1}}{2\Delta z} = \frac{u}{\varepsilon D_L} \left( c_{1,i+1} - c_{\text{feed}} \right) \Rightarrow c_{0,i+1} = c_{2,i+1} - \frac{2u\Delta z}{\varepsilon D_L} \left( c_{1,i+1} - c_{\text{feed}} \right) \quad \text{A(II).21}
\]
which can be substituted for $c_{0,j+1}$ in the discretized column continuity equation written for $i = 1$ to give

\[
-D \frac{D_L}{\Delta z} (Pe Dum) c_{2,j+1} + \left[ \frac{2D_L}{\Delta z} Pe Dum + u_{int} + \frac{\Delta z}{\Delta t} \right] c_{1,j+1} = 
\]

\[
\left[ \frac{D_L}{\Delta z} Pe Dum + u_{int} \right] c_{2,j+1} - \frac{2u\Delta z}{eD_L} (c_{1,j+1} - c_{feed}) = S\Delta z + c_{1,j} \frac{\Delta z}{\Delta t}
\]

Therefore at $i = 1$ the continuity equation will be

\[
\left[ -2 \frac{D_L}{\Delta z} (Pe Dum) - u_{int} \right] c_{2,j+1} + \left[ \frac{2D_L}{\Delta z} Pe Dum + u_{int} + \frac{\Delta z}{\Delta t} + 2 u_{int} (Pe Dum) + 2u_{int}^2 \frac{\Delta z}{D_L} \right] c_{1,j+1} = 
\]

\[
S \Delta z + c_{1,j} \frac{\Delta z}{\Delta t} + \left[ \frac{D_L}{\Delta z} Pe Dum + u_{int} \right] (c_{feed}) 2u_{int} \frac{\Delta z}{D_L}
\]

A(II).23

The elements of tridiagonal matrix at $i = 1$ are then given by
\( a(1) = 0 \)

\[
\begin{align*}
  b(1) &= \left[ \frac{2D_L}{\Delta z} \text{PeDum} + u_{\text{int}} + \frac{\Delta z}{\Delta t} + 2u_{\text{int}}(\text{PeDum}) + 2u_{\text{int}}^2 \frac{\Delta z}{D_L} \right] \\
  c(1) &= \left[ -2 \frac{D_L}{\Delta z} \text{PeDum} \frac{u}{c} \right] \\
  d(1) &= S\Delta z + c_{i,j} \frac{\Delta z}{\Delta t} + \left[ \frac{D_L}{\Delta z} \text{PeDum} + u_{\text{int}} \right] \left( c_{\text{feed}} \right)2P\epsilon
\end{align*}
\]

A similar approach is taken for the boundary condition at \( i = m \), which leads to

\[
\frac{\partial c}{\partial z}_{z=L} = 0 \Rightarrow \frac{c_{m-1,j+1} - c_{m+1,j+1}}{2\Delta z} = 0 \Rightarrow c_{m-1,j+1} = c_{m+1,j+1}
\]

Substituting for \( c_{m+1,j+1} \) in the discretized column continuity equation written for \( (i = m) \) gives

\[
\left[ \frac{2D_L}{\Delta z} \text{PeDum} + u_{\text{int}} + \frac{\Delta z}{\Delta t} \right] c_{m,j+1} - \left[ \frac{2D_L}{\Delta z} \text{PeDum} + u_{\text{int}} \right] c_{m-1,j+1} = S\Delta z + c_{m,j} \frac{\Delta z}{\Delta t}
\]

providing the following coefficients for the tridiagonal matrix at \( i = m \)
\[a(m) = - \left[ 2 \frac{D_L}{\Delta z} PeDum + u_{int} \right]\]

\[b(m) = \left[ \frac{2D_L}{\Delta z} PeDum + u_{int} + \frac{\Delta z}{\Delta t} \right]\]

\[c(m) = 0\]

\[d(m) = S\Delta z + c \frac{\Delta z}{\Delta t}\]
Discretization of the Bead Continuity Equation

Solving the column continuity equation requires an estimate of the rate of solute uptake into the stationary phase, given by Equation (4.2). This equation depends on the rate of solute binding to the stationary phase \( \frac{\partial q}{\partial t} \), which can be subjected to the chain rule to give

\[
\frac{\partial q}{\partial t} = \frac{\partial q}{\partial c} \times \frac{\partial c}{\partial t} \tag{A(II).28}
\]

The rate of solute uptake into the stationary phase is then given by

\[
\left( \frac{1 - e_p}{e_p} \right) \frac{\partial q}{\partial c} + 1 \right) \frac{\partial c}{\partial t} = D_p \left( \frac{\partial^2 c}{\partial r^2} + \frac{2}{r} \frac{\partial c}{\partial r} \right) \tag{A(II).29}
\]

In this work, the Crank-Nicholson finite difference method is used to solve the discretized form of equation A(II).29, a parabolic PDE:

\[
\left(1 - e_p \right) \frac{\partial q}{\partial c} + 1 \right) \frac{c_{i,j+1} - c_{i,j}}{\Delta t} = D_p \left( \frac{c_{i+1,j+1} - 2c_{i,j+1} + c_{i-1,j+1}}{\Delta r^2} + \frac{c_{i+1,j} - 2c_{i,j} + c_{i-1,j}}{\Delta r^2} \right) + \\
\left( \frac{e_{i+1,j+1} - c_{i+1,j+1}}{2\Delta r} \right) + \left( \frac{e_{i+1,j} - c_{i+1,j}}{2\Delta r} \right) \tag{A(II).30}
\]
As noted by equation A(II).30, the Crank-Nicolson method involves taking the derivative half way between the beginning and the end of the time space. It is hence an average between a fully implicit and fully explicit model of PDE's. Multiplying both sides of equation A(II).30 by \( \frac{2\Delta r^2}{D_p} \) gives:

\[
\left(1 - \frac{\Delta r}{r_i}\right)c_{i-1,j+1} - \frac{\Delta r^2}{D_p \Delta t} \left(1 + \frac{1 - \varepsilon_p \partial q}{\varepsilon_p \partial c}\right)c_{i,j+1} + \left(1 + \frac{\Delta r}{r_i}\right)c_{i+1,j+1} = \\
2 \left(1 - \frac{\Delta r}{r_i}\right)c_{i-1,j} + \frac{\Delta r^2}{D_p \Delta t} \left(1 + \frac{1 - \varepsilon_p \partial q}{\varepsilon_p \partial c}\right)c_{i,j} - \left(1 + \frac{\Delta r}{r_i}\right)c_{i+1,j}
\]

Equation A(II).31 represents a set of linear equations whose coefficients can be expressed in a tridiagonal matrix for \( i = 2, 3, ..., m_p - 1 \) (\( m_p \): number of radial volume elements in the meshed stationary phase bead) as follows.
\[ a(i) = \left(1 - \frac{\Delta r}{r_i}\right) \]

\[ b(i) = -2 \left(1 + \frac{\Delta r^2}{D_p \Delta t} \left(1 + \frac{1 - \varepsilon_p \partial q}{\varepsilon_p \partial c}\right)\right) \]

\[ c(i) = \left(1 + \frac{\Delta r}{r_i}\right) \]

\[ d(i) = -\left(1 - \frac{\Delta r}{r_i}\right)c_{i-1,j} + 2 \left(1 - \frac{\Delta r^2}{D_p \Delta t} \left(1 + \frac{1 - \varepsilon_p \partial q}{\varepsilon_p \partial c}\right)\right)c_{i,j} - \left(1 + \frac{\Delta r}{r_i}\right)c_{i+1,j} \]

The boundary condition for the stationary phase bead at \( r = 0 \) is

\[ \frac{\partial c^*}{\partial r} = 0 \quad r = 0, t \geq 0 \]

Therefore, at \( r = 0 \) the bead continuity equation at \( i=1 \) can be written as

\[
\left(1 - \frac{\varepsilon_p \partial q}{\varepsilon_p \partial c}\right) + 1 \left(1 + \frac{\partial c_{i,j}}{\partial t}\right) = \frac{3D_p}{2} \left[ \frac{c_{2,j+1} - 2c_{1,j+1} + c_{0,j+1}}{\Delta r^2} + \frac{c_{2,j} - 2c_{1,j} + c_{0,j}}{\Delta r^2} \right]
\]

This boundary condition also imposes the following constraints on the concentration of the solute in the stationary phase pores

\[ c_{0,j} = c_{2,j} \]
\[ c_{0,j+1} = c_{2,j+1} \]

A(II).32
A(II).33
A(II).34
A(II).35
Multiplying both sides of Equation A(II).34 by \( \frac{2\Delta r^2}{3D_p} \) and substituting for \( c_{0,j}^* \) and \( c_{0,j+1}^* \) then gives

\[
2c_{2,j+1}^* - 2 \left( 1 + \frac{1}{3D_p \Delta t} \left( 1 + \frac{1-\varepsilon_p}{\varepsilon_p} \right) \frac{\partial q^*}{\partial c^*} \right) c_{1,j+1}^* = -2c_{2,j}^* + 2 \left( 1 - \frac{\Delta r^2}{3D_p \Delta t} \left( 1 + \frac{1-\varepsilon_p}{\varepsilon_p} \right) \frac{\partial q^*}{\partial c^*} \right) c_{1,j}^*
\]

A(II).36

The coefficient of the tridiagonal matrix at \( i=1 \) are then given by

\[
a(1) = 0
\]

\[
b(1) = - \left( 1 + \frac{1}{3D_p \Delta t} \left( 1 + \frac{1-\varepsilon_p}{\varepsilon_p} \right) \frac{\partial q^*}{\partial c^*} \right)
\]

A(II).37

\[
c(1) = 1
\]

\[
d(1) = -c_{2,j}^* + \left( 1 - \frac{\Delta r^2}{3D_p \Delta t} \left( 1 + \frac{1-\varepsilon_p}{\varepsilon_p} \right) \frac{\partial q^*}{\partial c^*} \right) c_{1,j}^*
\]

Assuming that film mass transfer is fast compared to pore diffusion, the boundary condition for the bead at \( r = R_p \ (i = m_p) \), where \( R_p \) is the bead radius, is as follows
\[
\begin{align*}
c^*_{r=k_p} = c_{\text{out}} \frac{\varepsilon}{(1 - \varepsilon)} \frac{1}{\varepsilon_p} \\
\Rightarrow c^*_{m_p,i+1} = c_{\text{out}} \frac{\varepsilon}{(1 - \varepsilon)} \frac{1}{\varepsilon_p}
\end{align*}
\]

where \( c_{\text{out}} \) (mole/m\(^3\) bulk liquid) is the concentration of analyte in the volume element in the corresponding time step. We can therefore define the tridiagonal coefficients at point \( i = m_p \) as

\[
\begin{align*}
a(m_p) &= 0 \\
b(m_p) &= 1 \\
c(m_p) &= 0 \\
d(m_p) &= c_{\text{out}} \frac{\varepsilon}{(1 - \varepsilon)} \frac{1}{\varepsilon_p}
\end{align*}
\]
Description of CLEC Simulation and Related Programs

Numerical Algorithm

The column simulation program 'CLEC' works as follows. Model parameters and initial guesses are read into the main program from the input file 'Input.txt'. The total amount of copper in the system prior to injection of sample is then calculated by calling the multiple chemical equilibrium subroutine 'Eqsimu' after fixing the total ligand density, the pH and the concentration of copper in the mobile phase during column equilibration. The total copper in the column is then calculated, thereby fully defining the system to permit solution of the analyte continuity equation for each time step until the injected peak is fully eluted from the column. Within a given column volume element, the rate of solute uptake in each volume element of the stationary phase is calculated by the subroutine 'Bead'. Detailed comments related to the solution algorithm are included in the body of the source code 'CLEC' with an exclamation mark in front of each comment line.

Here, a basic overview of the solution algorithm is provided in a numbered format to assist in understanding the program.

1. The numerical solution starts by solving Equation A(II).1 for the very first time step assuming \( \frac{ds}{dt} = 0 \). This results in an estimated solute concentration \( c(r,z) \) for each volume element along the column in each volume element that can be applied to the solution of equation A(II).29 to determine the rate of solute uptake into the stationary phase.
2. Calculation of the concentration distribution throughout the bead \( (c_i^*(r,z,t), q_i^*(r,z,t), \) and \( s(z,t) \)) requires knowledge of \( \frac{dq_i^*}{dc_i} \), which is calculated by multiple chemical equilibria through following set of iterations. For the very first time step of the bead continuity equation (note that the time step for the bead is much smaller than the time step for the column), \( \frac{dq_i^*}{dc_i} \) is set equal to zero. Solving equation A(II).29 with \( \frac{dq_i^*}{dc_i} = 0 \) yields values for \( c_i^*(r,z,t) \) and \( q_i^*(r,z,t) \) that allow one to calculate a new estimate
\[
\frac{dq_i^*}{dc_i} = \frac{q_{i \text{ new}} - q_{i \text{ old}}}{c_{i \text{ new}} - c_{i \text{ old}}}
\]
at each radial position of the bead.

3. Step 2 is repeated using the updated values of \( \frac{dq_i^*}{dc_i} \) and a standard Newton-Raphson scheme until no further changes in \( \frac{dq_i^*}{dc_i} \) are observed (i.e. \( \frac{1 \times 10^{-3}}{} \)).

\[
\begin{bmatrix}
\frac{\partial q_i^*}{\partial c_i} \\
\frac{\partial q_i^*}{\partial c_i}
\end{bmatrix}
\leq 1 \times 10^{-3}.
\]

This sets the values of \( c_i^* \) and \( q_i^* \) for the designated time step. The time increment in the bead is then advanced and Steps 2 and 3 are repeated until the time step for the column control volume is reached.

4. The \( s(z,t) \) term (Equation A(II).2) is then calculated by numerical integration of concentration profiles over the bead volume using a third-order Simpson's rule. Since
both transport within and uptake by the bead takes place over the column time step of $\Delta t$, one can simply calculate $\frac{ds}{dt}$ at each $z$ position from changes in the $S$ term over the column time step $\Delta t$ (i.e. $\frac{ds(z,t)}{dt} = \frac{s(z,t + \Delta t) - s(z,t)}{\Delta t}$).

5. These calculations are carried out (step 1-5 repeated with updated values of $c$ and $ds/dt$) until all injected species are completely eluted from the column.
Input File <Valine.txt>

The input file Input.txt (e.g. 'Valine.txt') holds all the required information for the program. This includes the number of mesh points for the column and bead continuity equations, mass transfer coefficients for the solute, the injection volume, the concentration of the solute(s) in the injected sample, the copper concentration (or any other transition metal) in the mobile phase, and the mobile-phase flow rate. It also contains total concentrations of all the components that exist in the column prior to injection, and the concentrations of each component entering the column in the injection.

The number of the components and complexes that form in the system, as well as the equilibrium formation constant and stoichiometric coefficients for each complex formed are also included.

Output File

The output file contains all information from input file followed by two or more columns of output data. The first column contains the elution time in seconds (sec) and the second and higher columns each report a solute concentration at the column outlet in mM.

Compiling and Running the Program

The simulation program CLEC was edited, compiled and run in Fortran 90 on a Pentium 4 PC using Microsoft Fortran PowerStation compiling and execution software. The program terminates when the elution of both enantiomers is complete.
Comments on the Input File

**mcc**: Number of grid points in column length

**mb**: Number of grid points in bead radius (number of radial volume elements)

**nb**: Number of grid points for bead time interval

**L(m)**: Column length

**R(m)**: Particle (stationary phase) radius

**Rc(m)**: Column radius

**TM2 (Sec)**: Elution time until which the simulation should be carried out.

**C_o(mM)**: Initial concentration of analyte in the column

**C_in(mM)**: Inlet concentration of the analyte which is equal to the analyte concentration in the injected sample

**SK_f(m²/sec)**: Film mass transfer coefficient

**D_L (m²/sec)**: Axial dispersion coefficient

**ε**: Column Void

**Q (ml/min)**: flow rate

**D_p(m²/sec)**: Pore diffusion coefficient

**ε_p**: Stationary phase porosity

**pH**: pH of the mobile phase

**pH2**: pH of the mobile phase prior to injection of sample

**V_inj (μl)**: Sample injection volume

**C_m (mM)**: Copper concentration in the mobile phase

**C_tot-Li (mM)**: Total concentration of the ligand immobilized on the column

**Nspc**: Number of components in the system

**Ncomp**: Number of complexes in the system

**Name** of the components in the system in order that they appear in complex indices
This data entry column is followed by a \([N\text{comp} \times N\text{spc}+1]\) matrix containing the logarithmic values of each required equilibrium formation constant in the first column, and the stoichiometric coefficients of the components in each complex in the remaining columns. A \([N\text{comp}]\) vector appears at the end of the matrix which holds the short-hand chemical formula of the complex.

A second matrix follows the first and contains the equilibrium formation constant and the corresponding stoichiometric coefficients for each complex formed in the mobile phase.
Example of the `<Valine.txt>` Input File

<table>
<thead>
<tr>
<th>mcc</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>mb</td>
<td>5</td>
</tr>
<tr>
<td>nb</td>
<td>5</td>
</tr>
<tr>
<td>L(m)</td>
<td>2.5d-1</td>
</tr>
<tr>
<td>R(m)</td>
<td>2.5d-6</td>
</tr>
<tr>
<td>R_c(m)</td>
<td>2.0d-3</td>
</tr>
<tr>
<td>TM2(sec)</td>
<td>2.5d3</td>
</tr>
<tr>
<td>C_0(mol/m^3)</td>
<td>0.d0</td>
</tr>
<tr>
<td>C_in(mol/m^3)</td>
<td>4.926d-1</td>
</tr>
<tr>
<td>Sk_f(m/s)</td>
<td>7.05d-4</td>
</tr>
<tr>
<td>D_L(m^2/s)</td>
<td>1.1d-7</td>
</tr>
<tr>
<td>e(unitless)</td>
<td>4.5d-1</td>
</tr>
<tr>
<td>Q (ml/min)</td>
<td>1.0d0</td>
</tr>
<tr>
<td>D_p(m^2/sec)</td>
<td>1.6d-10</td>
</tr>
<tr>
<td>e_p(unitless)</td>
<td>6.5d-1</td>
</tr>
<tr>
<td>pH</td>
<td>5.0d0</td>
</tr>
<tr>
<td>pH2</td>
<td>5.0d0</td>
</tr>
<tr>
<td>V_inj(sec)</td>
<td>1.d1</td>
</tr>
<tr>
<td>Cm</td>
<td>2.d0</td>
</tr>
<tr>
<td>Ctotli</td>
<td>3.55d1</td>
</tr>
<tr>
<td>Nspc</td>
<td>4</td>
</tr>
<tr>
<td>Ncomp</td>
<td>9</td>
</tr>
</tbody>
</table>

Proton
L-val
L-hydro
Cu++

<table>
<thead>
<tr>
<th>-13.78</th>
<th>-1</th>
<th>0</th>
<th>0</th>
<th>0</th>
<th>C-1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.48</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>C1100</td>
</tr>
<tr>
<td>11.77</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>C2100</td>
</tr>
<tr>
<td>8.05</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>C0101</td>
</tr>
<tr>
<td>14.79</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>C0201</td>
</tr>
<tr>
<td>9.46</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>C1010</td>
</tr>
<tr>
<td>8.47</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>C0011</td>
</tr>
<tr>
<td>15.58</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>C0021</td>
</tr>
<tr>
<td>15.89</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>C0111</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>-13.78</th>
<th>-1</th>
<th>0</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.48</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>11.77</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>8.05</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>14.79</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>
Main Source Code Program <CLEC.f90>

!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
! MODELING CHIRAL LIGAND EXCHANGE CHROMATOGRAPHY
! written by : Nooshafarin Sanaie  Jan 2001
!This program solves differential equation for transport through the column for
! a pulse input
! 
dC/dt=DL*d^2C/dz^2-U/e*dC/dz-(1-e)/e*dS/dt
!
! S: Substituted from the integral calculated in the beads
! Therefore:
! dC/dt=DL*d^2C/dz^2-U/e*dC/dZ-(1-e)/e*dS/dt
!
!!! b.cs (1) (dC/dZ)Z=L=0  t>=0 and Z=L
!!! (2) [DL(dC/dZ)]Z=0=U/e*(C-Cinlet)  t0>t>0 and Z=0
!!! C=Cin=0  t>t0  and Z=0
!
! Fully implicit method and finite difference method were used to solve the
differential equation.
! Power law scheme were used to account for effect of diffusion and convection
!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
! Rp = Radius of particles (m)
! TM2 = Period of time at which transport and diffusion take place (Sec)
! n = #Grid points in time (for column)
! m = #Grid points in column length
! mb = #Grid points in radial direction (XR)
! nb = #Grid points in time (for beads)
! X1 = Column length
! XL = Vector which contains grid points in L direction (m)
! XT = Vector which contains grid points in time direction (sec)
! C2 = Vector of concentration through the column (mM)
! Cinl = Vector that contains final concentration in the bead(mole/m3 pore liquid)
! Cin = Liquid concentration in the inlet of the column (mol/m3)
! C0 = (C0=0)Initial concentration through the column (mol/m3)
! Cb = Concentration of Liquid (mol/m3)throughout beads at different times
! Cb(i,j): ith bead time interval and in location j through the bead
! C0b = Concentration throughout beads before adsorption in each step takes place
! Cfree = Vector which contains free concentration of each species(mol/m3 or mmolar)
! Ccomp = Vector of each complex concentration (mol/m3)
! Ctot = Vector of total concentration of each species (mM)
! Cm = Copper concentration in the solvent (mM)
! Ctotli = Total ligand concentration
! Beta = Vector of Log values for formation constant of each complex
! Betal = Vector of formation constants of each complex
! t0 = Injection time(sec)
! SKf = Film mass transfer coefficient (m/s)
!DL = Axial dispersion coefficient (m²/s)
!Nspc = # of species
!Ncomp = # of complexes
!Name = Vector which carries label of each complex
!Outpar = Vector which carries label and stiochiometric values for each complex
!e = Column Void
!ep = Particle porosity
!Dp = Pore Diffusion (m²/sec)
!DpH = pH value in the mobile phase
!U = Superficial Lumen velocity (m/s)
!Flag = Indicator which guide the program to the proper bead boundary condition
  ! if flag = 0 Nothing has been adsorbed on the bead, prior to injection
  ! if flag = 1 Adsorption took place and there is a concentration distribution
!Snew = Vector which stores the S values at time t at each position through the column
  ! (S values are sum of the concentrations in two phases throughout the bead)
!Sold = Vector which stores the s values at time t at each position through the column
!Ds = Derivative of s with respect to time
!Vinj = Sample Injection Volume (Micro-lit)
!Qflow = flow rate (ml/min)
!A,B,C,D : Tridiagonal matrix elements

Use PORTLIB
Use MSFLIB
Implicit real*8(a-h,o-z)
Common /re/mb,nb,R,SKf,Dp,e,ep,SKL
Dimension C2(5000),XL(5000),A(5000),B(5000),
 & C(5000),D(5000),C1f(5001),Cbead(5000,601),Qbead(1501,601),C0bi(601),Q4old(601),
 & &
c2new(5000),Q2(601),Q1f(601),ds(5000),Snew(5000),Sold(5000),DqDcBead(5000,601),DQ
DC(601),
 & & Qbeadla(1500,601),Q4(601),Q5(5000),Q5old(5000)

!Elapsed time = Simulation time (a built in function)

Real(8) elapsed_time

Parameter (MXC=15,MXS=5)
Common /para/QR(MXC,MXS),Betal(MXC),Beta(MXC),DpH
Common /para2/QR2(MXC,MXS),Betal2(MXC),Beta2(MXC),DpH2
common/para1/accm
Common ctotCu,Cm,Ctotli,CtotHli,Culi,cms
Character*8 Name(MXS),Outpar(MXC)

External Q
Integer(2) n1
flag=0
n1=1

239
! Read The required data form the 'Valine' file

OPEN (18, FILE= 'Valine.txt')

Read(18,6) m
Read(18,6) mb
Read(18,6) nb
Read(18,5) X1
Read(18,5) R
Read(18,5) Rc
Read(18,5) TM2
Read(18,5) C0
Read(18,5) Cin
Read(18,5) SKf
Read(18,5) DL
Read(18,5) e
Read(18,5) Qflow
Read(18,5) Dp
Read(18,5) ep
Read(18,5) DpH
Read(18,5) DpH2
Read(18,5) Vinj
Read(18,5) Cm
Read(18,5) Ctotli

5 format(13x,d10.4)
6 format(5x,I6)

write(*,5)X1,R,Rc,TM2,C0,Cin,SKf,DL,e,Qflow,Dp,ep,DpH,DpH2,Vinj,Cm,Ctotli

Read(18,6) Nspc
Read(18,6) Ncomp

do i=1,Nspc
  Read(18,7) Name(i)
  write(*,7) Name(i)
endo

1 format(2x,i1,3x,f7.4)
7 format(3x,A7)
8 format(1x,f7.2,4x,f3.0,4x,f2.0,4x,f3.0,4x,f3.0,4x,f3.0)
9 format(6x,f2.0,6x,f2.0,6x,f2.0,6x,f2.0,6x,f2.0,6x,f15.5)
10 format(2x,A7,2x,A7,2x,A7,2x,A7)
17 format(1x,A7)

write(*,*)Nspc,Ncomp

240
! First read complete set of formation constant and complex name
! Calculate the formation constant from their log values

do i=1,Ncomp
   read(18,*) Beta(i),(QR(i,j),j=1,Nspc),outpar(i)
   write(*,8) Beta(i),(QR(i,j),j=1,Nspc)
   Betal(i)=1.d1**Beta(i)
enddo

! Read the second set of formation constant, which is related
! to the mobile phase complexes

do ll=ll,Ncomp-4
   read(18,*) Beta2(ll11),(QR2(ll11,ll21),ll21=1,Nspc-1)
   write(*,8) Beta2(ll11),(QR2(ll11,ll21),ll21=1,Nspc-1)
   Betal2(ll11)=1.d1**Beta2(ll11)
enddo

! Ctotli= total ligand concentration (mM)
! Ctotcu= total copper concentration (Mobile + solid) phase (mM)
! CtotHli= concentration of protonated ligand on the solid phase (mM)
! CuLi= concentration of the activated ligand (Li-Cu)(mM)

! Call Eqsimu which calculates concentration of the activated ligand on the
! solid phase based on the pH of the solution.
.Call Eqsimu(Ctotli,Cm,DpH,CtotCu,CuLi,CtotHli,cmsnew)

! Conversion of solid phase copper concentration to corresponding mobile phase
! Concentration (Unit Conversion)

   cm=cmsnew*(1-ep)*(1-e)/e

write(*,*) Culi,CtotCu
fl=CuLi
elapsed_time=TIMEF()

! Pie Value
Fi=4*atan(1.d0)

! Caluculate column cross section (area)

   Area=Rc**2*Fi
Vcolumn=xL*Area

!Q (ml/min)
!Superficial velocity(m/sec)

U=Qflow*1.xd-6/(Area*60)

!Calculate injection time t0 (sec) from the injection volume and
!flowrate

t0=Vinj*1.xd-3*60/Qflow

! Define mesh size and number of increments in the column direction
! Define time step for the column

mm=m-1
Dx=XL/mm
Dt=0.01*DX*6/6/U
Dt1=20*dt
Tbead=Dt

write(*,*) m,mb,nb
write(*,*) Area,U,dt
write(*,*) t0,dt,dx

!Set initial concentrations in the bead and through the column

do i=1,m
  do j=1,mb
    XL(i)=(i-1)*Dx
    Cbead(i,j)=0
    Qbead(i,j)=0
    Qbeadla(i,j)=0
    C2(i)=0
  enddo

! Initially there is nothing diffused or adsorbed in the beads
! ds(i) is equal to zero for the first time increment (assuming nothing got adsorbed)
! From the second time interval and after calling the bead subroutine there is a
! corresponding number obtained from bead subroutine for ds(i).

  sold(i)=0
  snew(i)=0
  ds(i)=0
  Q5(i)=0
  Q5old(i)=0
  enddo
enddo

! Apply the power law scheme

   Uint = U/e
   Pe = Uint*Dx/DL
   PeDum = Fpower(Pe)

write(*,*) Uint, PeDum, Pe

! Definition of the pulse input and time step proportional to it.

dt0 = dt
itflag1 = 0
itflag2 = 0

Xt0 = 0
Xt = 0
j = 1

! j: Counter for the time step
! Continue simulation till time TM2 is reached

do while (xt < TM2)

   XT = Xt0 + Dt
   j = j + 1

   if (itflag2.eq.0) then
      if (itflag1.eq.0) then
         if (Xt > DABS(t0)) then
            dt = dabs(t0 - XT0)
            xt = xt0 + dt
            itflag1 = 1
         endif
      else
         dt = dt1
      endif
      if (itflag2.eq.0) itflag2 = 1
   endif
endif

! Solve bead continuity equation only if the analyte concentration is larger than ! C2(i) > 1.d-15
! C2 = vector of analyte concentration along the column at time j

243
! C2new=corrected analyte concentration taking into account
! the adsorption at the bead surface [C2new=C2-Q5*(1-ep)*(1-e)/e]

! Q5=adsorbed analyte concentration on the bead surface at time j
! Q5old=adsorbed analyte concentration on the bead surface at time j-1

    do i=1,m
        if(C2(i).gt.1d-15) then
            call Equilib(C2(i),C2new(i),Q5(i),Q5old(i),4,9)
        enddo

! For the very first time step only solve column continuity equation and Skip the
! bead Continuity equation
! From the second time step start to solve bead continuity and
! calculate uptake by the beads

    if(j.gt.1) then
        if (j.gt.2) flag=1

        ! At each position i along the column, concentration distribution inside
! each bead and slope of the isotherm is calculated and stored in a vector
! for the next time step
! 
! DqDC(k)=slope of the isotherm at position k inside the bead
! DqDCbead(i,k)=slope of the isotherm at position i in column
! and at position k inside the bead
! Q4old(k)=adsorbed analyte concentration at position k inside the bead
! Qbead(i,k)=adsorbed analyte concentration at position i in column and
! at position k of bead

        do k=1,mb
            C0bi(k)=Cbead(i,k)
            Q2(k)=Qbead(i,k)
            DqDc(k)=DqDCbead(i,k)
            Q4old(k)=Qbeadla(i,k)
        enddo

! At any position i inside the column solve bead continuity equation
! Calculate Slope of the isotherm and the average bead concentration
! S term

        call Bead(Tbead,C0bi,DqDc,C2new(i),Q5(i),Q2,Q4old,Q4,C1f,Q1f,snew(i),Nspc,Ncomp,j)

! Calculate Ds/Dt term for continuity equation

        ds(i)=(Snew(i)-Sold(i))/dt

244


\begin{verbatim}
sold(i)=snew(i)

    do k=1,mb
      Cbead(i,k)=Clf(k)
      Qbead(i,k)=Qlf(k)
      DQDCbead(i,k)=DqDc(k)
      Qbeadla(i,k)=Q4(k)
      enddo
    endif

else

  ! If analyte concentration is smaller than C2(i)<1.d-15, set it to zero
  c2(i)=0.d0
endif
enddo

! At the very first time step there is nothing happening in the bead
! No diffusion or adsorption inside the bead

if(j.eq.1) then
  do i=1,m
    do k=1,mb
      DqDebead(i,k)=1
      Qbead(i,k)=0
    enddo
  enddo
endif

! Defining step input to the column

11 do i=1,mm
  if(i.eq.1)then
    if(XT.gt.t0) then
      Cin=0
    endif
  endif

! Defining tridiagonal matrix elements (See Appendix for details)

A(i)=0.
B(i)=2*Uint*PeDum+2*Dx/DL*Uint**2+2*DL/Dx*PeDum+Uint+Dx/Dt
C(i)=-2*DL/Dx*PeDum-Uint
\end{verbatim}
\[ D(i) = 2 \cdot Pe \cdot Cin \cdot (DL/Dx \cdot PeDum + Uint) + C2(i) \cdot Dx/Dt - (1-e)/e \cdot (ds(i)) \cdot Dx \]

goto 15

else

\[ A(i) = -DL/Dx \cdot PeDum - Uint \]
\[ B(i) = 2 \cdot DL/Dx \cdot PeDum + Uint + Dx/Dt \]
\[ C(i) = -DL/Dx \cdot PeDum \]
\[ D(i) = C2(i) \cdot Dx/Dt - (1-e)/e \cdot (ds(i)) \cdot Dx \]

endif

15 enddo

\[ A(m) = -2 \cdot DL/Dx \cdot PeDum - Uint \]
\[ B(m) = 2 \cdot DL/Dx \cdot PeDum + Uint + Dx/Dt \]
\[ C(m) = 0 \]
\[ D(m) = C2(m) \cdot Dx/Dt - (1-e)/e \cdot (ds(m)) \cdot Dx \]

! Solve set algebraic equations (In the form of tridiagonal matrix)

call TDMA(A,B,C,D,C2,m)

do i=1,m
    if (c2(i).lt.1.d-15) then
        c2(i)=0
        ds(i)=0
    endif
enddo

4 format(28x,41f23.10)
3 Format(1x, 'Time=', f10.5, ' Sec')

do i=1,m
    if (c2(i).lt.0) then
        write(*,*) c2(i), i
        stop
    endif
enddo

! If output from the column is smaller than 1.d-3 then print it for
! every 30th one (Output file won't get very large )
! If output of the column is larger than 1d-3 then write every single
! calculated value
If(C2(m).lt.1d-3)then
  iter=iter+1
  n1=iter/30
  z1=iter-n1*30
  if(z1.eq.0)then
    write(*,*)XT,C2(m)
  endif
else
  write(*,*)XT,C2(m)
endif

doi=1,m
  q5old(i)=q5(i)
enddo

xtO=xt

endo
close (18)

!! At the end of program write the overall calculation time
  elapsed_time=TIMEF()
  write(*,*) 'Elapsed time ', elapsed_time, ' Seconds'

stop
.
.
End

!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
!!
! Subroutine Bead(TM,C0b,Couti,Q2,Coutf,C1f,Q1f,flag,Nspc,Ncomp,l1)

!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
!!
! This Subroutine calls bead continuity equation subroutine and calculates the S term !
! due to diffusion and adsorption inside the bead. Also calculates slope of the Isotherm!
! DqDc terms and its distribution inside the bead, which is required for bead continuity !
equation
!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
!!
! R = Radius of particle (m)
! TM = Period of time at which diffusion takes place (sec)
! which was chosen to be the time increment of the column
! mb = #Grid points in radial direction (XR)
! nb = #Grid points in time
! XR = Vector which contains grid points in R direction (m)
!XT  =  Vector which contains grid points in time direction (sec)

!C1  =  Vector of concentration through the bead (mol/m^3 pore)

!Q1  =  Concentration of adsorbed species on the bead (mol/m^3)

!Couti = Liquid concentration in the lumen (mol/m^3) at time t=0

!Cout = Vector which contains lumen concentration at each time increment (mol/m^3 lumen)

!C0  =  (C0=0) Initial concentration through the bead (mol/m^3)

!SKf  =  Film mass transfer coefficient (m/s)

!Dp  =  Diffusion through the pores (m^2/s)

!e  =  Bed porosity

!ep  =  Particle porosity

!Iter  =  Counter for the iteration

!Qnew = Vector which stores the solid phase (adsorbed amount) concentration distribution

!S  =  Vector which stores average bead concentration over the bead volume

!C1f  =  Vector that contains final concentration at all the location through bead

!Ratio = vector that stores relative changes for the slope of the isotherm

A,B,C,D : Tridiagonal matrix elements

Subroutine Bead(TM,C0b,DqDc,Couti,Qouti,Q2,Q4old,Q4,C1f,Q1f,s1,Nspc,Ncomp,l2)

Implicit real*8(a-h,o-z)

Common /re/mb,nb,R,SKf,Dp,e,ep,SKL

Parameter (MXC=15,MXS=5)

Dimension CComp(MXC)

Dimension c1(601),Q1(601),XR(601),XT(601),Q2(601),Q4old(601),&
& Q1f(601),DqDc(601),Q3n(601),Q4(601),Q40(601),&
& C1f(601),C0b(601),s(5000),Ratio(601),&
& Y1I(601),Y2I(601),DQDnew(601),C1old(601),Q2old(601),C1new(601),Qnew(601)

External Q3

External Q

Common /para/QR(MXC,MXS),Betal(MXC),Beta(MXC),DpH

Common /para2/QR2(MXC,MXS),Betal2(MXC),Beta2(MXC),DpH2

8 format(1x,f7.2,4x,f3.0,4x,f2.0,4x,f3.0,4x,f3.0,4x,f3.0,4x,f3.0)

! Define the time step and radial step for bead differential equation

n1=nb+1

mm=mb-1

mb1=mb-1

nm=nb-1

DR=R/mm

DT=TM/nm
! Set-up the initial value in the bead for pore liquid and adsorbed
! concentrations

do i=1,mb
    C1old(i)=C0b(i)
    Q2old(i)=Q2(i)
    Q40(i)=Q4old(i)
    XR(i)=(i-1)*DR
enddo

! In this subroutine for bead it is assumed during dt (column time increment)
! concentration of the volume element (surrounding each grid, couti) is constant, and
! remains constant during time (dt). Therefore boundary condition for bead
! remains constant and only analyte diffuses through bead and get adsorbed inside the
! bead

! Sum1= average concentration for bead pore concentration!
! (Integral value of ep*Ci(r,z,t) throughout the bead
! Sum2= average concentration for the adsorbed amount inside the bead
! Integral value of (1-ep)*Qi(r,z,t) throughout the bead

Sum1=0.
Sum2=0.

do j=1,nm

XT(j)=(j)*DT

! Update initial values after the first time step
if (j.gt.1)then
do i=1,mb
    clold(i)=Cl(i)
    Q2old(i)=Q1(i)
enddo
endif

! Solving bead continuity equation for the first time along with column
! continuity DqDc=0, nothing get adsorbed
if (j.eq.1.and.l2.eq.3) then
    Do i=1,mb
        DqDc(i)=0
    enddo
! Solve bead continuity equation over time interval of TM. Update and store
! liquid pore concentrations in C1f vector

call Bead1(TM,C1old,DqDc,Couti,C1f)

! Knowing pore concentration distribution calculate the adsorbed concentrations
! and store them in Qnew vector. Then calculate the new slope of the isotherm.

    do i=1,mb
        Qnew(i)=Q(C1f(i),C1new(i),Nsps,Ncomps,CCOMP,q2old(i))
        if(c1new(i).eq.c1old(i)) write(*,*) i
        DqDcnew(i)=(Qnew(i)-Q2old(i))/(C1f(i)-C1old(i))
        enddo
    iter=1

else
    ! If it is not the first time that bead continuity equation is solved, then
    ! update the slope of the isotherm. Set the new guess for the slope as the
    ! old value.
    do i=1,mb
        DqDcnew(i)=DqDc(i)
        iter=0
        enddo
endif

! Iteration for the Slope of the isotherm.
! Solve bead continuity equation with some old value of DqDC
! Update pore liquid and solid phase concentration
! Calculate new DqDC with these new concentrations and repeat
! this procedure until no further changes observed in DqDC

flg7=1
do while (flg7.eq.1)
    iter=iter+1
    call bead1(TM,C1old,DqDcnew,couti,C1f)
    do i=1,mb
        Q3n(i)=Q3(C1f(i),3.5,CCOMP,q2old(i))
        DqDc(i)=DqDcnew(i)
    enddo
    if(i.ne.mb) then
        ! To prevent division by zero
        if(C1f(i).eq.c1old(i)) then
            DqDcnew(i)=DqDc(i)
            flg7=0
        enddo
    endif
endwhile
else
    DqDcnew(i)=(Q3n(i)-Q2old(i))/(C1f(i)-C1old(i))
endif

if(DqDc(i).eq.0) then
    Ratio(i)=DqDcnew(i)
else
    ! Relative changes of the slope of the isotherm
    Ratio(i)=DABS((DqDc(i)-DqDcnew(i))/DqDc(i))
endif
endif
enddo

! Convergence Criteria: Max value of relative changes for the slope of
! isotherm becomes smaller than 1.d-3

rt1=maxval(Ratio)

if(rt1.lt.1.d-3) then
    do i=1,mb
        DqDc(i)=DqDcnew(i)
        flg7=0
        C1(i)=C1f(i)
        Q1(i)=Q3n(i)
    enddo
else
    ! Iteration larger than 100 stop the program
    if(iter.GT.100) then
        write(*,*) 'war'
        stop
    ENDIF
!Update pore bead and solid phase concentration and repeat iteration
    do i=1,mb
        c1(i)=c1old(i)
        Q1(i)=Q2old(i)
    enddo
endif
enddo

! Define solid phase and pore liquid concentration at the pore mouth
do i=1,mb
    if(i.eq.mb) then
        Q1(i)=Qouti
        C1(i)=Couti*e/(1-e)/ep
endif

! Use Simpson's rule to calculate the integrals
! Define Variables for integral calculation

\[ Y1I(i) = C1(i) \times XR(i)^2 \]
\[ Y2I(i) = (Q1(i)) \times XR(i)^2 \]
enddo

Sum1 = 0
Sum2 = 0
sumevn1 = 0
sumodd1 = 0
sumevn2 = 0
sumodd2 = 0
do i = 2, mm, 2
  Sumevn1 = Sumevn1 + Y1I(i)
  Sumevn2 = Sumevn2 + Y2I(i)
  Sumodd1 = Sumodd1 + Y1I(i+1)
  Sumodd2 = Sumodd2 + Y2I(i+1)
enddo

sum1 = DR * (Y1I(1) + 4 * sumevn1 + 2 * sumodd1 - Y1I(mb)) / 3
sum2 = DR * (Y2I(1) + 4 * sumevn2 + 2 * sumodd2 - Y2I(mb)) / 3

! After each bead time interval the integral value over the volume of the bead is calculated.
! The value of the integral the very last time step is stored in S1 and returned to the main
! program.

s(j) = 3 * (ep * sum1 + (1 - ep) * sum2) / R**3
4 format(32x, 21e14.6)
3 Format(1x, fl0.5)

if(C1(i) .gt. Couti) then
  7 format(1x, 'Warning: Pore liquid concentration is higher than the &
  & lumen concentration', /, 2x, i4, 4f15.8)
endif

do i = 1, mb
  Q40(i) = Q4(i)
endo

16 enddo

! S1 carries out the final value of intergral at the end of time interval.

s1 = s(nm)
! Store the final distributions of adsorbed analyte concentration and pore liquid concentration
!

20 do i=1,mb
   C1f(i)=C1(i)
   Q1f(i)=Q3n(i)
endo

Return
End

!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
! Subroutine Bead1(TM,C0b,DqDc,Couti,C1f,t)
!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
! This Subroutine solves differential equation for diffusion through the bead
! Bead:
! dC/dt=dp[d^2C/dr^2+2/rdC/dr]-(1-ep)/ep*dQ/dt
! i.e. C=0 at t=0 0<r<R
! We assume that beads are in equilibrium with the liquid film surrounding them
! Crank Nicolson method and finite difference method were used to solve the differential
! equation.
!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
!
! R = Radius of particles (m)
! TM = Period of time at which diffusion takes place (sec)
! It was chosen to be the time increment of the column
! m = #Grid points in radial direction (XR)
! n = #Grid points in time
! XR = Vector which contains grid points in R direction (m)
! XT = Vector which contains grid points in time direction (sec)
! C1 = Vector of concentration through the bead (mol/m^3 pore)
! Q1 = Concentration of adsorbed species on the bead(mol/m^3)
! Cout = Liquid concentration in the lumen (mol/m^3) at time t=0
! Cout = Vector which contains lumen concentration at each time increment (mol/m^3 lumen)
! C0 = (C0=0)Initial concentration through the bead (mol/m^3)
! SKf = Film mass transfer coefficient (m/s)
! Dp = Diffusion through the pores(m^2/s)
! e = Bed porosity
! ep = Particle porosity
! S = Vector which stores sum of concentration in both phases
! C1f = Vector that contains final concentration at all the location through bead
! A,B,C,D : Tridiagonal matrix elements
! D1 = Density of the ligand in the bead (mol/m^3)

Subroutine Bead1(TM,C0b,DqDc,Couti,C1f)
Implicit real*8(a-h,o-z)
Common /re/mb,nb,R,SKf,Dp,e,ep,SKL
Parameter (M XC=15, M XS=5)
Dimension c1(601),XR(601),A(601),B(601),C(601),D(601),DqDc(601),C1f(601),C0b(601)

nl=nb+1
mm=mb-1
mb1=mb+1
nm=nb-1
DR=R/mm
DT=TM/nm

do i=1,mb
   XR(i)=(i-1)*DR
   Cl(i)=C0b(i)
endo
doi=1,mb
   XR(i)=(i-1)*DR
   Cl(i)=C0b(i)
endo

! This routine solves the bead continuity equation and returns the concentration
! distribution to the main body of program. For details on this coefficients see
! the appendix

A(1)=0.
B(1)=-(1+DR**2/(3*Dp*DT)*(1+(1-ep)/ep*DqDc(1)))
C(1)=1.
D(1)=(1+DR**2/(3*Dp*DT)*(1+(1-ep)/ep*DqDc(1)))

! This routine solves the bead continuity equation and returns the concentration
! distribution to the main body of program. For details on this coefficients see
! the appendix

A(1)=0.
B(1)=-(1+DR**2/(3*Dp*DT)*(1+(1-ep)/ep*DqDc(1)))
C(1)=1.
D(1)=(1+DR**2/(3*Dp*DT)*(1+(1-ep)/ep*DqDc(1)))*Cl(1)-Cl(2)

do i=2,mm
   A(i)=(1-DR/XR(i))
   B(i)=-2*(1+DR**2/(Dp*DT)*(1+(1-ep)/ep*DqDc(i)))
   C(i)=(1+DR/XR(i))
   D(i)=-(1-DR/XR(i))*C(i-1)+2*(1-DR**2/(Dp*DT)*(1+(1-ep)/ep*DqDc(i)))*Cl(i) &
      & -1*(1+DR/XR(i))*C(i+1)
endo

A(mb)=0.
B(mb)=1.d0
C(mb)=0.
D(mb)=Couti*e/(1-e)/ep

call TDMA(A,B,C,D,C1,mb)
20 do i=1,mb
   Clf(i)=Cl(i)
   if (cl(i).lt.0) then
      do k=1,mb
         write(*,*) cl(k),k,Couti,cl(mb)
      enddo
      stop
   endif
endo

Return
End

This function defines the multiple chemical equilibria in the liquid phase.

double precision function Q3(Cl,Nspc,Ncomp,CCOMP,qold)
Implicit real*8(a-h,o-z)
Parameter (MXC=15,MXS=5)

Common /para/QR(MXC,MXS),Betal(MXC),Beta(MXC),DpH
Common /par2/QR2(MXC,MXS),Betal2(MXC),Beta2(MXC),DpH2
Common /re/mb,nb,R,SKf,Dp,e,ep,SKL
common/para1/accm
Common ctotCu,Cm,Ctotli,CtotHLi,culi,Cms

Dimension CComp(MXC),Ctot(MXS),X(3),Coeff(MXS,MXS),cfree(5), cfreeold(5)

! C1 corresponding to concentration distribution throughout the bead, is equal to sum
! over concentration of all complexes which contain that enantiomer.

! Using multiple chemical equilibria to find concentration of the free species
! in the mobile phase, at each radial position of the bead.

! Species 2 refers to the first Enantiomer
! Species 1 refers to the proton
! Species 3 refers to copper

! Some initial guesses for species (1,2,...) based on the concentration in the liquid
! phase
! Total proton = Free proton in the mobile phase + Proton from ligand
flga=0
Qdumold=Qold*(1-ep)/ep
if(cl.ne.0) then
DpH3 = DpH2
CfreeH = 1.3 * 1.1 ** (DpH3)

Knowing that Multiple Chemical Equilibrium governs in both solid phase and liquid phase, calculate free species (Cu, Proton, AA) concentration from the mobile phase equilibrium and use those values to calculate the corresponding solid phase concentration.

Defining total concentration for the liquid phase:

\[ C_{tot}(3) = C_m * e / (1 - e) / e_p \]
\[ C_{tot}(2) = C_l \]
\[ C_{tot}(1) = (C_{free}H + c_1) \]

\[ \text{call } \text{SIMU2}(C_{tot}, N_{comp}, N_sp, C_{Comp}, C_{free}, c_{freeold}, \text{coeff}, QR2, Betal2) \]

\( X(1) = \) Absorbed amount of the enantiomer or mixed ternary complex concentration (Li-Cu-AA)
\( X_{Li} = \) Free Ligand concentration (mM)

\( C_{form1} : \) Ternary formation const (Li-Cu-AA)
\( C_{form2} : \) Binary formation const (Li-Cu)
\( C_{form3} : \) Ligand Protonation (Li-H)
\( C_{form4} : \) Ligand Bis-Binary complex (Li2Cu)

\[ c_{form1} = 1.3 * 1.1 ** 16.18 \]
\[ c_{form2} = 1.3 * 1.1 ** 8.47 \]
\[ C_{form3} = 1.3 * 1.1 ** 9.46 \]
\[ C_{form4} = 1.3 * 1.1 ** 15.58 \]

Solve the quadratic equation to calculate free ligand concentration:
\( C_{Litot} = [Li] + [LiH] + [LiCu] + [Li.Cu.AA] + [Li2Cu] \)
Refer to Appendix to see details of quadratic equation.

\[ A = 2 * C_{form4} * c_{free(3)} \]
\[ B = (1 + c_{form3} * C_{free(1)} + C_{free(3)} * c_{form2} + c_{free(2)} * C_{free(3)} * c_{form1}) \]
\[ C = -c_{totli} \]

\[ x_{li} = (-B + (B ** 2 - 4 * A * C) ** 0.5) / (2 * A) \]
After finding free ligand concentration calculate concentration of ternary complex and store it in Q3.
\[ x(1) = C_{\text{form1}} \times c_{\text{free}(3)} \times c_{\text{free}(2)} \times x_{\text{li}} \]

\[ Q_3 = x(1) \]

else
  \[ Q_3 = 0 \]
end if
return
end

!!! This function defines the adsorption isotherm (Full Multiple Chemical Eq.)
! where it considers all the complexes that form in the system
! (both in solid and liquid phase). This equilibrium is only used at the bead
! surface where total analyte with the (C1) concentration partitions into pore
! liquid and solid phase.

Subroutine Equilb(C1, c1new, Q1, q1old, Nspc, Ncomp)
Implicit real*8(a-h,o-z)
Parameter (MXC=15, MXS=5)

Common /para/QR(MXC,MXS),Beta(MXC),Beta(MXC),DpH
Common /para2/QR2(MXC,MXS),Beta2(MXC),Beta2(MXC),DpH2
Common /re/mb,nb,R,SKf,Dp,e,ep,SKL
Common/para1/accm
Common ctotCu,Cm,Ctotli,CtotHLi,culi

Dimension CComp(MXC),Ctot(MXS),Coeff(MXS,MXS),cfree(MXS),Cfreeold(MXS)

if(c1.ne.0) then

  DpH3 = -1*DpH
  cfreeH = 1d3*1d1**(DpH3)

! Total copper concentration in each volume element remains constant
! Total analyte concentration is defined taking into account both phases

  Ctot(4) = Ctotcu*(1-ep)*(1-e)/e
  Ctot(3) = Ctotli*(1-ep)*(1-e)/e
  Ctot(2) = C1+q1old*(1-ep)*(1-e)/e
  Ctot(1) = CfreeH+c1+CtotHLi*(1-ep)*(1-e)/e

  call SIMU2(Ctot,Ncomp,Nspc,CComp,Cfree,cfreeold,coeff,QR,Betal)

! New concentration at the pore mouth is defined (taking into account the adsorbed amount)
Q1 = CComp(Ncomp) \times e / (1 - e) / (1 - e) / p
C1_{\text{new}} = c_{\text{tot}}(2) - Q1 \times (1 - e) / e

\text{if (c1_{\text{new}} < 0) stop}
\else
   \text{flgl} = 1
   \text{Q1} = 0
   \text{c1_{\text{new}}} = \text{c1}
\text{endif}

\text{return}
\text{end}

!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
! This function defines the adsorption isotherm (Multiple Chemical Eq.)
!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!

double precision function Q(C1, C1_{\text{new}}, Nspc, Ncomp, CCOMP, qold)
Implicit real*8(a-h,o-z)
Parameter (MXC=15, MXS=5)
!Common /re/mb,nb,R,SKf,DP,e,EP,Qmax,SKL
Common /para/QR(MXC,MXS),Beta1(MXC),Beta2(MXC),DpH
Common /para2/QR2(MXC,MXS),Beta12(MXC),Beta22(MXC),DpH2
Common /re/mb,nb,R,SKf,DP,e,EP,SKL
common/para1/acm
common/para2/acm
common/c1_{\text{tot}}Cu,Cm,Ctotli,CtotHli,culi
Dimension CComp(MXC), Ctot(MXS), Coeff(MXS,MXS), cfree(MXS), Cfreeold(Mxs)
!common/blck1/cfree(MXS), Cfreeold(MXS)

\text{if(c1 \neq 0) then}

! C1, which gives concentration distribution throughout the bead, is equal to sum
! of all complexes which contain enantiomer.

!Using multiple chemical equilibria to find concentration of the free species
!in the mobile phase, at each radial position of the bead.

! p1: Ligand index, ligand index is the second last column NSPC-1
p1 = nspc-1

! Some initial guesses for species (1, 2, ..) based on the concentration in the liquid
! phase
! Total proton = Free proton in the mobile phase + Proton from ligand

\[ DpH_1 = -DpH \]

\[ C_{\text{free}H} = 1d3 \times 1.1^{**}(DpH_1) \]

\[ C_{\text{tot}(4)} = C_{\text{tot}cu} \times (1 - \varepsilon p) / \varepsilon p \]
\[ C_{\text{tot}(3)} = C_{\text{tot}li} \times (1 - \varepsilon p) / \varepsilon p \]
\[ C_{\text{tot}(2)} = C_1 + (1 - \varepsilon p) / \varepsilon p \times qold \]
\[ C_{\text{tot}(1)} = C_{\text{free}H} + C_1 + C_{\text{tot}Hli} \times (1 - \varepsilon p) / \varepsilon p \]

! Find the free concentration of each enantiomer and proton at each radial position
! Assuming that total concentration in the liquid phase is given from solving
! differential equation of the bead. Call Multiple chemical Eq. to find the proper
! free concentration of the enantiomer, which satisfies this conditions.
! Sum of Concentration for each species in the liquid phase (from differential Eq.)
! is equal to corresponding value from multiple Chemical Eq.

call SIMU2(Ctot,Ncomp,Nspc,CComp,Cfree,cfreeold,coeff,QR,Betal)
cfreecu=Ctotcu-ccomp(8)-ccomp(7)

\[ Q = C_{\text{Comp}}(9) \times \varepsilon p / (1 - \varepsilon p) \]
\[ C_{1\text{new}} = C_{\text{tot}(2)} - Q \times (1 - \varepsilon p) / \varepsilon p \]

else

flgl=1
Q=0
endif

return
end

!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
! This Subroutine calculate complex distribution (find free concentration of each species)
! by inputting the total amount of each species and formation constant of each complex.
!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
! NSPC : number of components in the system
! NCOM : number of complex that forms in the system
! Beta(Ncomp) : Vector (Dimension, NCOMP) which contains Log formation constants of complexes
! QR(NCOMP,NSPC) : Matrix which contains the stoichiometric coefficients
! Cfree(Nspc) : vector which contains free concentration of each component
Subroutine SIMU2(Ctot,Ncomp,Nspc,CComp,Cfree,cfreeold,coeff,QR,Betal)
Implicit real*8(a-h,o-z)
Parameter (MXC=15,MXS=5)
! Common /para/QR(MXC,MXS),Betal(MXC),Beta(MXC),DpH
common/para1/acem
! Common /para2/QR2(MXC,MXS),Betal2(MXC),Beta2(MXC),DpH
Common /re/mb,nb,R,SKf,Dp,e,ep,SKL
Dimension
Cfree(MXS),Coeff(MXS,MXS),Dx(MXS),S1(MXS),CComp(MXC),Ctot(MXS),cfreeold(MXS),&
& P1(MXC),P(MXC),Betal(MXC),QR(MXC,MXS)
!
accm=l.d-40
Tolma=l.d-6
!
Calculate the jacobian Matrix
! Note that pH is changing along in the pores.
! Coeff: Jacobian matrix

do i=1,Nspc
! Initial guesses for free concentrations
   Cfree(i)=1.d-3*Ctot(i)
endo

do iter=1,5000

do j=1,Ncomp
   p(j)=Betal(j)
p1(j)=0
   do i=1,Nspc
      p(j)=p(j)*cfree(i)**QR(j,i)
      if(QR(j,i).ne.0.d0) p1(j)=p1(j)+QR(j,i)
   enddo
endo

do k=1,Nspc
   do l=k,Nspc
coeff(k,l)=0
Dx(k)=0

do j=1,Ncomp
   Coeff(k,l)=coeff(k,l)+QR(j,k)*QR(j,l)*p(j)*1.d-3**(p1(j)-1)
   Dx(k)=DX(k)+QR(j,k)*P(j)*1.d-3**(p1(j)-1)
endo

if (k.eq.l) then
   coeff(k,l)=coeff(k,l)+cfree(k)
else
   coeff(l,k)=coeff(k,l)
dendif

Dx(k)=Ctot(k)-cfree(k)-Dx(k)
endo
endo

call ludec(coeff,Dx,Nspc,MXS,0)

fract=1.d0

do ix=1,nspc
   if (dabs(dxGx)).lt.Accm) goto 130
   if(fract*dxGx).gt.(-1.d0)) goto 130
   fract=-0.6d0/dx(ix)
130 enddo

!If a free concentration is going negative, divide the shifts
Amxy=0.d0

do ix=1,Nspc
   Amxy=Dmax1(dabs(dx(ix)),amxy)
   cfree(ix)=cfree(ix)*(1.d0+dx(ix)*fract)
   if(cfree(ix).le.accm) then
      cfree(ix)=accm
   endif
endo

if(amxy.lt.tolma) then

! Sum2 takes care of the conversion factor of concentration for the beta values
   do i=1,Ncomp
      so=1
      sum2=0
      do j=1,Nspc
         s1(j)=cfree(j)**QR(i,j) !Pi (Ck)** aj over species
         if(s1(j).ne.0.d0) then

261
if(Qr(i,j).gt.0) sum2=Qr(i,j)+sum2
if(Qr(i,j).lt.0) sum2=-1
so=so*s1(j)
endif
enddo

ccomp(i)=so*Betal(i)*1d-3**(sum2-1)
if (Ccomp(i).gt.10000) ccomp(i)=0
enddo
do i1=1,Nspc
cfreeold(i1)=cfree(i1)
enddo

return
endif
enddo
end

!!!!!Subroutine TDMA to calculate coefficients!!!!!
! Function F for interpolation
!
!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
Double precision Function F(z)
Implicit real*8(a-h,o-z)
common/BLKA/x(101),y(101),n,nm
common/BLKB/q(100),r(101),s(100)
if(z.lt.x(l)) then
  i=l
  write(5,10) z
10 format(/'warning - ','d10.3,' is outside interpolation range'/)
elseif(z.gt.x(n)) then
  i=nm
  write(5,10) z
else
  i=1
  j=n
! Simple bisection method
20 k=(i+j)/2
  if(z.lt.x(k)) j=k
  if(z.ge.x(k)) i=k
  if(j.gt.i+1) goto 20
endif
  dx=z-x(i)
  F=y(i)+dx*(q(i)+dx*(r(i)+dx*s(i)))
  return
end
!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
!This function applies the power law scheme (Pantankar 1980)
!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!

Real*8 function Fpower(Pe)
Implicit real*8(A-H,O-Z)
  Fpower=DMAX1(0.d0,(1.d0-1.d-1*Dabs(Pe))*5.d0)***5.d0)
end
!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
!This subroutine solves the matrix B=A*X
!If mode=1, the matrix in already decomposed!
!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
subroutine LUDEC (a,b,n,max,mode)
Implicit real*8 (A-H,O-z)
dimension a(max,max),b(n)
Common /paral/acm
if(mode.eq.1) goto 100

263
do j=1,n
  do i=1,j-1
    sum=a(i,j)
    do k=1,i-1
      sum=sum-a(i,k)*a(k,j)
    enddo
    a(i,j)=sum
  enddo
  do i=j,n
    sum=a(i,j)
    do k=1,j-1
      sum=sum-a(i,k)*a(k,j)
    enddo
    a(i,j)=sum
  enddo
  if (a(i,j).eq.0.0) A(i,j)= accm
  endif
enddo
100 do i=1,n
  sum=b(i)
  do j=1,i-1
    sum=sum-a(i,j)*b(j)
  enddo
  b(i)=sum
enddo
do i=n,1,-1
  sum=b(i)
  do j=i+1,n
    sum=sum-a(i,j)*b(j)
  enddo
  b(i)=sum/a(i,i)
  !write(*,*) b(i)
enddo
return
end

! This subroutine Calculates concentration of the active ligand (LiCu), prior to
! sample injection. At constant pH, knowing mobile phase concentration, calculate the
! bound value
Subroutine Eqsimu(Ctotli,Cm,pH,CtotCu,CuLi,CtotHli,cmsnew)
Use MSFLIB
Implicit real*8(a-h,o-z)
Parameter (MXC=15,MXS=5)
Dimension Ctot(MXS),Cfree(MXS),CComp(MXC)
Common /para3/QR3(MXC,MXS),Betal3(MXC),Beta3(MXC),DpH

common/para1/accm
Character*8 Name(MXS),Outpar(MXC)
Integer(2) n1
e=0.45
ep=0.65

n1=1

OPEN (UNIT=18,FILE='Hydropro.txt')

Read(18,6) Nspc
Read(18,6) Ncomp
do i=1,Nspc
  Read(18,7) Name(i)
  write(*,7) Name(i)
enddo

4 format(2x,i1,3x,f7.4)
5 format(f6.2,6x,f2.0,6x,f1.0,6x,f1.0,6x,f1.0,6x,f1.0)
6 format(5x,i6)
61 format(5x,f3.1)
7 format(3x,A7)
8 format(1x,f7.2,4x,f3.0,4x,f2.0,4x,f3.0,4x,f3.0,4x,f3.0)
9 format(6x,f2.0,6x,f2.0,6x,f2.0,6x,f2.0,6x,f2.0,6x,f15.5)
10 format(2x,A7,2x,A7,2x,A7,2x,A7)
11 format(1x,A7)
write(*,* )Nspc,Ncomp,pH

flags=0
do i=1,Ncomp
  read(18,*) Beta3(i),(QR3(i,j),j=1,Nspc),Outpar(i)
write(*,8) Beta3(i),(QR3(i,j),j=1,Nspc)
  Betal3(i)=1.d1**(Beta3(i)
enddo

! Total concentrations in mM
do i=1,Nspc
read(18,*) Ctot(i)
write(*,*) Ctot(i)
enddo

Cms=(Cm)*e/(1-e)/(1-ep)

Do while (flags.eq.0)

call SIMU3(Ctot,Ncomp,Nspc,CComp,Cfree,pH)

Cf=Cms*1.0001
Cb=Cms*0.9999

If (Cfree(NSPC).gt.Cb.and.Cfree(NSPC).lt.Cf) then
flags=1

CtotCu=Ctot(Nspc)
else
Ctot(Nspc)=Ctot(Nspc)+0.0008
endif

enddo
Cmsnew=cfree(Nspc)
CuLi=CComp(NSPC)
CtotHli=CCOMP(2)
Ctotli=Ctot(2)

return
end

!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!
! This Subroutine calculate complex distribution (find free concentration of each species)
! by inputting the total amount of each species and formation constant of each complex.
!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!

! NSPC : number of species in the system
! NCOM : number of complex that forms in the system
! Beta(Ncomp) : Vector (Dimension, COMP) which contains Log formation constants of complexes
! QR(NCOMP,NSPC) : Matrix which contains the stoichiometric coefficients
! Cfree(Nspc) : vector which contains free concentration of each component
! Ctot(NSpc) : vector which contains total concentration of each species
! Coeff : Jacobian matrix
!QR(Ncomp,Nspc): Matrix which contains the stoichiometric coefficients
! Betal(Ncomp) : Vector that contains formation constants for each complex.
! CComp(Ncomp) : Vector which contains complexes concentrations
! Name(Nspc) : Name of the species
! Outpar(Ncomp): Vector that contains all the stoichiometic numbers for each complex
! Dx(Nspc) : Vector of concentrations shift between two steps

Subroutine SIMU3(Ctot,Ncomp,Nspc,CComp,Cfree,pH)
Implicit real*8(a-h,o-z)
Parameter (MXC=15,MXS=5)

Common /para3/QR3(MXC,MXS),Betal3(MXC),Beta3(MXC),DpH
common/paral/accm
Dimension Cfree(MXS),Coeff(MXS,MXS),Dx(MXS),S1(MXS),CComp(MXC),Ctot(MXS)

accm=1.d-40
Tolma=1.d-6

! Calculate the jacobian Matrix
! Assuming that the pH is known only concentrations from the second species
! need to be calculated

! Initial guesses for free concentrations

do i=2,Nspc
Cfree(i)=1.d-3*Ctot(i)
enddo

do iter=1,5000

! Concentration of proton in mM by knowing the pH
Cfree(1)=1.d3*(1.d1**(pH))

! Make the Upper diagonal part of the jacobian Matrix

do k=1,Nspc
  do l=k,Nspc
    sum=0
    sum1=0
    dx(k)=0
    do i=1,Ncomp
      so=1
      sum2=0
      do j=1,Nspc
        s1(j)=Cfree(j)**QR3(i,j) !Pi (Ck)** aj over species
        if(s1(j).ne.0.d0) then
          sum=sum+s1(j)
          sum1=sum1+s1(j)**2
          dx(k)=dx(k)+s1(j)**2
          s1(j)=s1(j)/sum
        enddo
        do j=1,Nspc
          s1(j)=s1(j)**2
        enddo
      enddo
    enddo
    dx(k)=dx(k)/sum1
  enddo
enddo
if(QR3(i,j).gt.0) sum2=QR3(i,j)+sum2
    so=so*s1(j)
  endif
enddo

! sum over complexes
  sum=sum+Beta3(i)*QR3(i,l)*QR3(i,k)*so*1d-3**(sum2-1)
  sum1=sum1+Beta3(i)*QR3(i,k)*so*1d-3**(sum2-1)
enddo
Coeff(k,l)=sum
  Dx(k)=Ctot(k)-cfree(k)-sum1
enddo
enddo

! Add free concentrations to the diagonal elements

do i=1,Nspc
  Coeff(i,i)=cfree(i)+coeff(i,i)
enddo

! Fill the symmetric part (Lower diagonal part)

do ix=2,Nspc
  do jx=1,ix-1
    Coeff(ix,jx)=Coeff(jx,ix)
  enddo
enddo

! Eliminate the first species from the matrix

do i=2,Nspc
  dx(i-1)=dx(i)
  do j=2,Nspc
    coeff(i-1,j-1)=coeff(i,j)
  enddo
enddo

! call ludec(coeff,Dx,Nspc-1,MXS,0)

do i=Nspc,2,-1
  dx(i)=dx(i-1)
enddo

fract=1.d0

do ix=2,Nspc
  if (dabs(dx(ix)).lt.Accm) goto 130

268
!If a free concentration is going negative, divide the shifts
if(frac*dx(ix).gt.(-1.d0)) goto 130
    fract=-0.6d0/dx(ix)
130 enddo

Amxy=0.d0
do ix=2,Nspc
    Amxy=Dmax1(dabs(dx(ix)),amxy)
    cfree(ix)=cfree(ix)*(1.d0+dx(ix)*fract)
    if(cfree(ix).le.accm) cfree(ix)=accm
endo

if(amxy.lt.tolma) then

! Sum2 takes care of the conversion factor of concentration for the beta values
    do i=1,Ncomp
        so=1
            sum2=0
            do j=1,Nspc
                s1(j)=cfree(j)**QR3(i,j) !Pi (Ck)** aj over species
                if(s1(j).ne.0.d0) then
                    if(Qr3(i,j).gt.0) sum2=Qr3(i,j)+sum2
                    if(Qr3(i,j).lt.0) sum2=-1
                endif
            enddo
            so=so*s1(j)
        endif
    enddo
    ccomp(i)=so*Betaw(i)*1d-3**(sum2-1)
endo
return
endif
endo
end
References
