Investigation of copper(II) complexes: I Copper(II) complexes of tryptophan and its analogues as postcolumn interaction components for indirect fluorescence detection of amino-containing analytes II High-performance liquid chromatographic separation of copper(II) azamacrocyclic complexes

Xiaoxuan Shen
University of New Hampshire, Durham

Follow this and additional works at: https://scholars.unh.edu/dissertation

Recommended Citation
https://scholars.unh.edu/dissertation/328

This Dissertation is brought to you for free and open access by the Student Scholarship at University of New Hampshire Scholars' Repository. It has been accepted for inclusion in Doctoral Dissertations by an authorized administrator of University of New Hampshire Scholars' Repository. For more information, please contact nicole.hentz@unh.edu.
Investigation of copper(II) complexes: I Copper(II) complexes of tryptophan and its analogues as postcolumn interaction components for indirect fluorescence detection of amino-containing analytes II High-performance liquid chromatographic separation of copper(II) azamacrocyclic complexes

Abstract
The initial phase of this project investigates the use of copper(II) complexes of tryptophan and its analogues for indirect fluorescence detection in high-performance liquid chromatography. First, indirect photometric and fluorometric detection in high performance liquid chromatography (HPLC) is reviewed, giving the functional definition of indirect detection employed for this project, distinguishing different approaches to indirect photometric and fluorometric detection in HPLC, and explaining the basis of indirect fluorescence detection using copper(II) complexes of tryptophan and its analogues as postcolumn interaction components. Subsequent studies were conducted to develop and apply indirect HPLC detection for detecting specific mono-amino sugars, glucosamine, galactosamine, and mannosamine. Two tryptophan analogues, 5-hydroxy-L-tryptophan and DL-5-methoxytryptophan were evaluated as potential alternatives to L-tryptophan for the detection of these mono-amino sugars. This indirect fluorescence detection method was also evaluated for the analysis of glucosamine in commercial dietary supplements following chromatographic separation.

The second part of this project focuses on separating copper(II)-azamacrocyclic complexes with the use of high-performance liquid chromatography. Interest in these complexes arises from the increasing use of copper radioisotopes in imaging and therapy leading to the synthesis of ligands which form stable copper complexes. The effects of the concentration of the buffer, the pH of the buffered mobile phase, and the concentration of the organic modifier, methanol, on the separations produced were investigated. Separation of these copper complexes by ion-pair HPLC with the use of a mass spectrometry-compatible ion-pair reagent is also presented. The reversed phase chromatographic conditions utilized also allow the pa's and the lipophilicity parameter of the complexes studied to be estimated. The separation and retention of several copper(II)-azamacrocyclic complexes on a graphitic carbon stationary phase, a very hydrophobic reversed-phase stationary phase with the capability of retaining highly polar compounds, was also investigated.

Finally the potential application of the indirect fluorescence detection method described here for detecting some of the ligands of the copper(II)-azamacrocyclic complexes, which do not absorb in the UV-vis range or fluoresce, following separation by liquid chromatography is discussed. Future studies are proposed for estimating the lipophilicity of metal complexes by reversed-phase high-performance liquid chromatography and capillary electrophoresis.

Keywords
Chemistry, Analytical
INVESTIGATION OF COPPER(II) COMPLEXES:
I. COPPER (II) COMPLEXES OF TRYPTOPHAN AND ITS ANALOGUES AS POSTCOLUMN INTERACTION COMPONENTS FOR INDIRECT FLUORESCENCE DETECTION OF AMINO-CONTAINING ANALYTES
II. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF COPPER(II) AZAMACROCYCLIC COMPLEXES

BY

Xiaoxuan Shen
B.S. University of Science and Technology of China, 1994

DISSERTATION

Submitted to the University of New Hampshire in Partial Fulfillment of the Requirement for the Degree of

Doctor of Philosophy in Chemistry

May, 2006
INFORMATION TO USERS

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleed-through, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.
This dissertation has been examined and approved.

Dissertation Director, Sterling A. Tomellini
Professor of Chemistry

Christopher F. Bauer
Professor of Chemistry

Richard A. Hartwick, PhD
Director of Analytical Services
PharmAssist Analytical Laboratory

Howard R. Mayne
Professor of Chemistry

Edward H. Wong
Professor of Chemistry

May 1, 2006
Date
DEDICATION

To my father, Qingshuang Shen, and my mother, Yixi Jiang
for their love
ACKNOWLEDGEMENTS

I would like to thank my advisor Prof. Sterling Tomellini for his tremendous support and invaluable advice during my five years of study at UNH. His remarkable enthusiasm and high standards for scientific research will positively influence my future career. His open mindedness and encouragement to pursue the answers in my research projects enhanced my enjoyment. I also would like to thank Prof. Edward Wong for his advice and in particular, his guidance in my research project of copper(II)-azamacrocyclic complexes.

I am also grateful to the members of my research committee, Prof. Chris Bauer, Dr. Richard Hartwick, and Prof. Howard Mayne for their efforts, assistance and direction. I appreciate Prof. Gary Weisman’s support during my proposal defense and his help with my research project. I also value the assistance given to me by the faculty and students in the Department of Chemistry. It is difficult to name all the people who have helped make this program’s completion possible. But, I would like to thank the following for their assistance during my stay at UNH: Prof. Carolyn Anderson and Dr. Andrew Boswell at the Washington University, Cindi and Peggy in the department office, Amy in the General Laboratory, Bob in the Chemistry Library, Jane and Kristin in the stockroom, Rob and John in the Instrumentation Center, Dr. Phil Ramsay in the Department of Mathematics and Statistics, and Dr. Roger Hsu. I appreciate the teaching assistantship
from the Graduate School. I would also like to gratefully acknowledge the following awards during my research and teaching: Dissertation Fellowship (2005-2006) and Summer TA Fellowship (2003) from the Graduate School, Pratt-Diniak Award (2006), Alvin R. Ingram Award (2005), Clarence L. and M. Garland Grant Award (2003 and 2004), Morrison Award (2004), and Pratt Summer Supplement Award (2002), from the Department of Chemistry. I also acknowledge the funding provided by NIH R01 CA93375 for the project of copper(II)-azamacrocyclic complexes.

Special thanks should go to my friend Dr. Min Yang for his support and help, which enabled me to come to UNH and finish my PhD program. I also appreciate my group colleagues, Oliver, John, Megan, Scott, Ilia, and Ruxin, who make my life and study in this group so enjoyable.

Finally, I would like to thank my wife, Danya, for her unselfish support in making the completion of this research possible. I also thank my father, Qingshuang, who passed away nine years ago, my mother, Yixi, my sister, Xiaoming, my parents-in-law, Miaoqin and Shuying, my son, Zehao (Howie), and my daughter, Annie, for their love.
# TABLE OF CONTENTS

DEDICATION ........................................................................................................ iii

ACKNOWLEDGEMENTS .......................................................................................... iv

LIST OF TABLES ...................................................................................................... xi

LIST OF FIGURES .................................................................................................. xii

ABSTRACT ............................................................................................................... xviii

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. INTRODUCTION</td>
<td>.............................................. 1</td>
</tr>
</tbody>
</table>

PART I. COPPER(II) COMPLEXES OF TRYPTOPHAN AND ITS ANALOGUES AS POSTCOLUMN INTERACTION COMPONENTS FOR INDIRECT DETECTION OF AMINO-CONTAINING ANALYTES ................................................................. 6

2. INDIRECT PHOTOMETRIC AND FLUOROMETRIC DETECTION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY .......................................................... 7

2.1 Introduction ...................................................................................................... 7

2.2 Defining Indirect Detection ............................................................................. 8

2.3 Indirect Detection Resulting From Interactions Occurring Within The Column ................................................................................................................. 10

2.3.1 Indirect Detection Based on Measuring a Change in the Concentration of the Detectable Component in the Eluent ......................................................... 11

2.3.1.1 Basis for Indirect Photometric Detection .................................................. 11

2.3.1.2 Application of Indirect Photometric Detection ......................................... 17

2.3.1.3 Application of Indirect Fluorometric Detection ....................................... 22
2.3.2 Indirect Detection Based on Measuring a Change in a Spectroscopic Property of the Detectable Component in the Eluent ................................................................. 23

2.4. Indirect Detection Resulting From Interactions Occurring Postcolumn .......... 24

2.4.1 Interaction Mode: $A + ML \rightarrow MA + L$ .................................................. 26

2.4.1.1 Measuring a Decrease in Absorbance or Fluorescence Intensity ................. 26

2.4.1.2 Measuring an Increase in Absorbance or Fluorescence Intensity ............... 32

2.4.2 Interaction Mode: $A + ML \rightarrow A' + M'L$ .................................................. 34

2.4.3 Interaction Mode: $A + B \rightarrow A' + B'$ ......................................................... 35

2.4.4 Interaction Mode: $A + B \rightarrow A-B^*$ ......................................................... 38

2.5 Conclusions ........................................................................................................... 39

3. INDIRECT FLUORESCENCE DETECTION OF AMINO SUGARS WITH THE USE OF COPPER(II) COMPLEXES OF TRYPTOPHAN AND ITS ANALOGUES FOLLOWING LIQUID CHROMATOGRAPHIC SEPARATION ........................................ 40

3.1 Introduction ............................................................................................................. 40

3.2 Experimental .......................................................................................................... 43

3.2.1 Reagents ............................................................................................................. 43

3.2.2 Apparatus .......................................................................................................... 45

3.3 Results and Discussions ........................................................................................ 46

3.3.1 Emission Spectra of Tryptophan and Its Analogues ........................................ 46

3.3.2 Evaluation of Fluorescence Quenching of Tryptophan and Its Analogues by Copper ion as a Function of Solution pH ...................................................... 46

3.3.3 Evaluation of the Stability of Potential Postcolumn Reagents in a Basic Sodium Borate Buffer Solution .......................................................... 53

3.3.3.1 Evaluation of the Stability of $\text{Cu(5-HTP)}_2$ in a 32 mM, pH 8.6, Sodium Borate Solution .......................................................... 53

vii
3.3.3.2 Evaluation of the Stability of Cu(5-MTP)_2 in a 32 mM, pH 8.4, Sodium Borate Solution............................................................................................................................55

3.3.4. Chromatographic Detection of the Amino Sugars Using two Different Postcolumn Reagents.................................................................57

3.3.4.1. Chromatographic Detection of the Amino Sugars via Postcolumn Addition of Cu(L-Trp)_2 in Sodium Borate Solution, pH=9.0.................................57

3.3.4.2. Chromatographic Detection Using Cu(5-MTP)_2 in Sodium Borate Solution at pH 8.4..............................................................................................61

3.4 Conclusions........................................................................................................................63

4. LIQUID CHROMATOGRAPHIC DETERMINATION OF GLUCOSAMINE IN COMMERCIAL DIETARY SUPPLEMENTS BY INDIRECT FLUORESCENCE DETECTION..............................................................................................................................65

4.1 Introduction...........................................................................................................................65

4.2 Experimental..........................................................................................................................66

4.2.1 Materials and Reagents....................................................................................................66

4.2.2 Dietary Supplement Samples..........................................................................................67

4.2.3 Apparatus..........................................................................................................................67

4.2.4 Preparation of Glucosamine Standard Solutions..........................................................68

4.2.5 Preparation of Glucosamine-Containing Sample Solutions........................................69

4.2.5.1 Solid Samples (Tablet, Capsule, and Powder)..........................................................69

4.2.5.2 Liquid Sample.................................................................................................................69

4.2.6 Procedure for Reaction of Glucosamine with OPA/MPA............................................69

4.2.7 Procedure for Precolumn Derivatization Reaction of Glucosamine by PITC...........70

4.2.8 Chromatographic Conditions..........................................................................................70

4.2.8.1 Indirect Fluorescence Detection Method.....................................................................70

4.2.8.2 Precolumn Derivatization Method.............................................................................71
5.3.4 Comparison of the Separations with the Use of Two Different Mobile Phases on C8 and C18 Columns..............................................................................................98

5.3.5 Separation of Cu-Azamacrocyclic Complexes by Ion-Pair HPLC Using a C8 Column.....................................................................................................................................103

5.3.6 Estimation of the pKa's of Cu-TETA Using Reversed-Phase Chromatographic Data..................................................................................................................................................107

5.3.7 Estimation of the Lipophilicity of the Complexes by Reversed-Phase HPLC.........................................................................................................................................109

5.4 Conclusion............................................................................................................111

6. SEPARATION OF COPPER(II)-AZAMACROCYCLIC COMPLEXES WITH A GRAPHITIC CARBON STATIONARY PHASE............................................. 113

6.1. Introduction........................................................................................................113

6.2. Experimental........................................................................................................116

6.2.1 Reagents............................................................................................................116

6.2.2 Apparatus..........................................................................................................116

6.2.3 Chromatographic Conditions.............................................................................117

6.2.4 Sample Preparation............................................................................................117

6.3 Results and Discussions.......................................................................................118

6.3.1 Effect of the Concentration of Methanol in the Mobile Phase on Solute Retention.........................................................................................................................118

6.3.2 Effect of the Concentration of Acetonitrile in the Mobile Phase on Solute Retention.........................................................................................................................122

6.3.3 Effect of the Column Temperature on the Retention..................................122

6.3.4 Effect of the Sample Concentration on the Observed Peak Profile..............126

6.4 Conclusions......................................................................................................130

7. SUMMARY AND FUTURE STUDIES....................................................................134

REFERENCES..............................................................................................................138

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2.1</td>
<td>13</td>
</tr>
<tr>
<td>Outline for the selection of a suitable detectable component upon the analyte and the mode of liquid chromatography</td>
<td></td>
</tr>
<tr>
<td>Table 2.2</td>
<td>18</td>
</tr>
<tr>
<td>Examples of the application of indirect photometric detection based on a change of the concentration of the detectable component in the eluent</td>
<td></td>
</tr>
<tr>
<td>Table 2.3</td>
<td>28</td>
</tr>
<tr>
<td>Summary of the applications based on the indirect detection approach: $A + ML \rightarrow MA + L$</td>
<td></td>
</tr>
<tr>
<td>Table 3.1</td>
<td>59</td>
</tr>
<tr>
<td>Statistical summary for detection of three amino sugars following chromatographic separation, as represented in Figure 3.9(1)</td>
<td></td>
</tr>
<tr>
<td>Table 4.1</td>
<td>77</td>
</tr>
<tr>
<td>Comparison of results obtained using Cu(L-Trp)$_2$ and Cu(5-MTP)$_2$</td>
<td></td>
</tr>
<tr>
<td>Table 4.2</td>
<td>83</td>
</tr>
<tr>
<td>Content of glucosamine determined by indirect fluorescence detection method and PITC pre-column derivatization method</td>
<td></td>
</tr>
</tbody>
</table>

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 2.1</td>
<td>27</td>
</tr>
<tr>
<td>Indirect detection resulting from interaction: ( A + ML \rightarrow MA + L ): (a) Measuring a decrease in absorbance or fluorescence intensity, (b) Measuring an increase in absorbance or fluorescence intensity. Solid circle for L indicates the analyte (L) either absorbs UV-vis light or fluoresces. Open circle for L indicates the analytes (L) neither absorbs UV-vis light nor fluoresces.</td>
<td></td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>41</td>
</tr>
<tr>
<td>Molecular structures of the three amino sugars</td>
<td></td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>44</td>
</tr>
<tr>
<td>Molecular structures of tryptophan and its analogue compounds: (a) L-Tryptophan (L-Trp), (b) 5-Hydroxy-L-tryptophan (5-HTP), (c) DL-5-Methoxytryptophan (5-MTP)</td>
<td></td>
</tr>
<tr>
<td>Figure 3.3</td>
<td>47</td>
</tr>
<tr>
<td>Emission spectra of (a) 10 ( \mu )M L-Trp in 32mM pH 9.0 sodium borate solution, (b) 8 ( \mu )M 5-HTP in 32mM pH 8.6 sodium borate solution, (c) 10 ( \mu )M 5-MTP in 32mM pH 8.4 sodium borate solution, excitation wavelength = 280 nm</td>
<td></td>
</tr>
<tr>
<td>Figure 3.4</td>
<td>49</td>
</tr>
<tr>
<td>Fluorescence intensity of 4 ( \mu )M L-Trp and 2 ( \mu )M Cu(L-Trp)(_2) as a function of pH, excitation wavelength = 280nm, emission wavelength =360nm</td>
<td></td>
</tr>
<tr>
<td>Figure 3.5</td>
<td>51</td>
</tr>
<tr>
<td>Fluorescence intensity of 4 ( \mu )M 5-HTP and 2 ( \mu )M Cu(5-HTP)(_2) as a function of pH, excitation wavelength = 280nm, emission wavelength = 340nm</td>
<td></td>
</tr>
<tr>
<td>Figure 3.6</td>
<td>52</td>
</tr>
<tr>
<td>Fluorescence intensity of 4 ( \mu )M 5-MTP and 2 ( \mu )M Cu(5-MTP)(_2) as a function of pH, excitation wavelength = 280nm, emission wavelength = 340nm</td>
<td></td>
</tr>
<tr>
<td>Figure 3.7</td>
<td>54</td>
</tr>
<tr>
<td>Stability study: fluorescence intensity of 8( \mu )M Cu(5-HTP)(_2) in a pH of 8.6 sodium borate buffer solution as a function of time, excitation wavelength = 280 nm, emission wavelength = 360 nm</td>
<td></td>
</tr>
<tr>
<td>Figure 3.8</td>
<td>56</td>
</tr>
<tr>
<td>Emission spectra of 5-HTP released from 8( \mu )M Cu(5-HTP)(_2) in a pH of 8.6 sodium borate solution by adding EDTA after (a) 3 hours, (b) 75 hours, excitation wavelength = 280 nm</td>
<td></td>
</tr>
</tbody>
</table>

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Figure 3.9  Fluorescence detection of amino sugars with two different longpass glass filters. Separation was achieved with the use of Hamilton anion exchange PRP-X100. Mobile phase: 1.6mM sodium borate, pH=9.0, 1ml/min. Postcolumn reagent: $2 \times 10^{-5} \text{M Cu(L-Trp)}_2$ in 40mM sodium borate at pH=9.0, 1ml/min. Detector sensitivity: 0.005$\mu$AFS. Chromatogram 1: longpass glass filter $\lambda_{\text{cutoff}} = 350$nm. Chromatogram 2: longpass glass filter $\lambda_{\text{cutoff}} = 320$nm. Peak (a) D-Glucosamine (2.5nmol); (b) D-Galactosamine (2.5nmol); (c) D-Mannosamine (2.5nmol). Excitation wavelength = 280 nm

Figure 3.10  Detections of amino sugars by new postcolumn reagent. Separation was achieved with the use of Hamilton anion exchange PRP-X100. Mobile phase: 1.6mM sodium borate, pH=9.0, 1ml/min. Postcolumn reagents: $2 \times 10^{-5} \text{M Cu(5-MTP)}_2$ in 40mM sodium borate at pH=8.4, 1ml/min. Detector sensitivity: 0.005$\mu$AFS. Longpass glass filter $\lambda_{\text{cutoff}} = 320$nm. Peak (a) D-Glucosamine (2.5nmol); (b) D-Galactosamine (2.5nmol); (c) D-Mannosamine (2.5nmol). Excitation wavelength = 280 nm

Figure 4.1  Separation of glucosamine from other compounds
Mobile phase: 1.6 mM sodium borate, pH=9.0, 1ml/min;
Postcolumn interaction component: $2 \times 10^{-5} \text{M Cu(L-Trp)}_2$ in 40 mM sodium borate at pH=8.4, flow rate: 1ml/min; Column: Hamilton PRP-X100, anion exchange column; Excitation wavelength = 280 nm. Longpass filter $\lambda_{\text{cutoff}} = 340$ nm.
a: mixture of glucosamine standard (GlcN-HCl: 0.0766mg/ml) and chondroitin-containing sample P (sample P: 2.04mg/ml)
b: glucosamine-containing sample B (sample B: 0.21mg/ml)
c: chondroitin-containing sample P (sample P: 4.08mg/ml)
d: chondroitin-containing sample Q (sample Q: 4.46mg/ml)
Peaks 1 and 2: unknown compounds in chondroitin-containing sample P

Figure 4.2  Calibration curves for the methods using (a) $2 \times 10^{-5} \text{M Cu(L-Trp)}_2$ in 40mM sodium borate at pH 9.0 (1ml/min), (b) $2 \times 10^{-5} \text{M Cu(5-MTP)}_2$ in 40mM sodium borate at pH 8.4 (1ml/min) as the postcolumn interaction component.
Mobile phase: 1.6 mM sodium borate, pH=9.0, 1ml/min; Column: Hamilton PRP-X100, anion exchange column; Excitation wavelength = 280 nm. Longpass filter $\lambda_{\text{cutoff}} = 320$ nm

Figure 4.3  Absorbance for glucosamine-OPA/MPA derivative solution vs. reaction time at room temperature

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Figure 4.4 Representative chromatogram of glucosamine-PITC derivative
Mobile phase: CH\textsubscript{3}OH:H\textsubscript{2}O:CH\textsubscript{3}COOH = 10.00:89.96:0.04
(v/v/v), 1ml/min; Column: XTerra C18 (50x4.6mm, 3µm);
Detector: Waters 484 UV-Vis detector, λ=254nm

Figure 4.5 Calibration curves for the determination of glucosamine in ten
samples based on (a) indirect fluorescence detection method, (b)
PITC pre-column derivatization method. Chromatographic
conditions: (a) Mobile phase: 1.6 mM sodium borate, pH=9.0,
1ml/min; Column: Hamilton PRP-X100, anion exchange column;
Postcolumn interaction component: 2x10\textsuperscript{-5} M Cu(L-Trp)\textsubscript{2} in 40mM
sodium borate at pH 9.0 (1ml/min); Excitation wavelength = 280
nm; Longpass filter λ\textsubscript{cut} = 340 nm. (b) Mobile phase:
CH\textsubscript{3}OH:H\textsubscript{2}O:CH\textsubscript{3}COOH = 10.00:89.96:0.04 (v/v/v), 1ml/min;
Column: XTerra C18 (50x4.6mm, 3µm); Detector: Waters 484
UV-Vis detector, λ=254nm

Figure 4.6 Comparison of indirect fluorescence detection method and PITC
pre-column derivatization method for the determination of
glucosamine in ten samples

Figure 5.1 Structures of (a) Cu(II)-azamacrocyclic complexes: Cu-CB-TE2A,
Cu-CB-DO2A, and Cu-TETA, and (b) the corresponding ligands:
H\textsubscript{2}CB-TE2A, H\textsubscript{2}CB-DO2A, and H\textsubscript{4}TETA

Figure 5.2 Separations of Cu-CB-TE2A, Cu-TETA, and Cu-CB-DO2A with
the use of citric acid buffer at (a) 30 mM, (b) 60 mM, and (c) 150
mM. Mobile phase: citric acid at pH 2.5; Stationary phase:
Betabasic C18. Peaks: 1 = Cu-CB-DO2A (0.10 mg/ml); 2 = Cu-
TETA (0.13 mg/ml); 3 = Cu-CB-TE2A (0.10 mg/ml); 4 = impurity

Figure 5.3 Effect of the pH of the mobile phase on the capacity factor of Cu-
CB-TE2A, Cu-TETA, and Cu-CB-DO2A. Mobile phase: 30 mM
citric acid. Stationary phase: Betabasic C18. Fitted curve of Cu-
TETA is plotted according to Eq. (1), see the text for details

Figure 5.4 Effect of the concentration of methanol in the mobile phase on the
capacity factor of Cu-CB-TE2A, Cu-TETA, and Cu-CB-DO2A.
Mobile phase: 30 mM citric acid at pH 2.5; Stationary phase:
Betabasic C18

Figure 5.5 Plots of the log(k') values of Cu-CB-TE2A, Cu-TETA, and Cu-
CB-DO2A vs. the concentration of methanol and their trends.
Mobile phase: 30 mM citric acid at pH 2.5; Stationary phase:
Betabasic C18
Figure 5.6  Separations of Cu-CB-TE2A, Cu-TETA, and Cu-CB-D02A with the use of different mobile phases and different columns. (a) Mobile phase: 22 mM formic acid (pH 2.5); Column: Betabasic C18; (b) Mobile phase: 22 mM formic acid (pH 2.5); Column: Zorbax SB-C8; (c) Mobile phase: 30 mM citric acid buffer at pH 2.5; Column: Zorbax SB-C8. Peaks: 1 = Cu-CB-D02A (0.10 mg/ml); 2 = Cu-TETA (0.13 mg/ml); 3 = Cu-CB-TE2A (0.10 mg/ml); 4 = impurity

Figure 5.7  Separations of the crude Cu-CB-TE2A (1.0 mg/ml) with the use of different mobile phases. (a) Mobile phase: 6 mM citric acid buffer at pH 6.2; (b) Mobile phase: 6 mM citric acid buffer at pH 2.5; (c) 22 mM formic acid (pH 2.5). Stationary phase: Betabasic C18

Figure 5.8  Ion-pair HPLC separations of (a) Cu-CB-TE2A, Cu-TETA, and Cu-CB-D02A; (b) the crude Cu-CB-TE2A (1.0 mg/ml). Mobile phase: 40 mM triethylammonium acetate (TEAA) at pH 6.3. Stationary phase: Zorbax SB-C8. Peaks: 1 = Cu-CB-D02A (0.10 mg/ml); 2 = Cu-TETA (0.13 mg/ml); 3 = Cu-CB-TE2A (0.10 mg/ml)

Figure 6.1  Structures of (a) copper complexes: Cu-CB-TE2A, Cu-CB-D02A, Cu-CB-TE2LA, Cu-CB-D02A, and (b) the corresponding ligands: $\text{H}_2\text{CB-TE2A}$, $\text{H}_2\text{CB-D02A}$, $\text{H}_2\text{CB-TE2LA}$, $\text{H}_2\text{CB-D02LA}$

Figure 6.2  Plots of log($k'$) vs. methanol concentration for Cu-CB-D02A (0.11mg/ml), Cu-CB-D02LA (0.11mg/ml), Cu-CB-TE2A (0.13mg/ml), and Cu-CB-TE2LA (0.14mg/ml)

Figure 6.3  Separation of Cu-CB-TE2A, Cu-CB-D02A, Cu-CB-TE2LA, and Cu-CB-D02LA under isocratic elution conditions. Mobile phase: 30% (v/v) methanol/water; Column: Hypercarb (100x3mm, 5μm), column temperature: 35 °C; Detection: UV absorbance $\lambda$=280nm; Peaks: 1= Cu-CB-D02A (0.11mg/ml); 2= Cu-CB-D02LA (0.11mg/ml); 3= Cu-CB-TE2A (0.13mg/ml); 4= Cu-CB-TE2LA (0.14mg/ml)

Figure 6.4  Separation of Cu-CB-TE2A, Cu-CB-D02A, Cu-CB-TE2LA, and Cu-CB-D02LA under gradient elution conditions. Mobile phase: A: 60% (v/v) methanol/water, B: water; Gradient profile: 0-3.5min, 30%A-30%A; 3.5-18.5min, 30%A-100%A; Column: Hypercarb (100x3mm, 5μm), column temperature: 25 °C; Detection: UV absorbance $\lambda$=280nm; Peaks: 1= Cu-CB-D02A (0.11mg/ml); 2= Cu-CB-D02LA (0.11mg/ml); 3= Cu-CB-TE2A (0.13mg/ml); 4= Cu-CB-TE2LA (0.14mg/ml)
Figure 6.5  Plots of log(k') vs. acetonitrile concentration for Cu-CB-DO2A (0.11mg/ml), Cu-CB-DO2LA (0.11mg/ml), Cu-CB-TE2A (0.13mg/ml), and Cu-CB-TE2LA (0.14mg/ml)

Figure 6.6  Separation of Cu-CB-TE2A, Cu-CB-DO2A, Cu-CB-TE2LA, and Cu-CB-DO2LA under isocratic elution conditions. Mobile phase: 10% (v/v) acetonitrile/water; Column: Hypercarb (100x3mm, 5μm), column temperature: 25°C; Detection: UV absorbance λ=280nm; Peaks: 1= Cu-CB-DO2A (0.11mg/ml); 2= Cu-CB-DO2LA (0.11mg/ml); 3= Cu-CB-TE2A (0.13mg/ml); 4= Cu-CB-TE2LA (0.14mg/ml)

Figure 6.7  Separation of Cu-CB-TE2A, Cu-CB-DO2A, Cu-CB-TE2LA, and Cu-CB-DO2LA under gradient elution conditions. Mobile phase: A: 50% (v/v) acetonitrile/water, B: water; Gradient profile: 0-3.5min, 15%A-15%A; 3.5-18.5min, 15%A-40%A; Column: Hypercarb (100x3mm, 5μm), column temperature: 25°C; Detection: UV absorbance λ=280nm; Peaks: 1= Cu-CB-DO2A (0.11mg/ml); 2= Cu-CB-DO2LA (0.11mg/ml); 3= Cu-CB-TE2A (0.13mg/ml); 4= Cu-CB-TE2LA (0.14mg/ml)

Figure 6.8  Plot of log k' vs. 1/T for the complexes: Cu-CB-DO2A (0.11mg/ml), Cu-CB-DO2LA (0.11mg/ml), Cu-CB-TE2A (0.13mg/ml), and Cu-CB-TE2LA (0.14mg/ml), with the use of a graphitic carbon stationary phase for mobile phase: (a) methanol/water or (b) acetonitrile/water

Figure 6.9  Separation of Cu-CB-TE2A, Cu-CB-DO2A, Cu-CB-TE2LA, and Cu-CB-DO2LA with a column temperature of 65°C. Mobile phase: 30% (v/v) methanol/water; Column: Hypercarb (100x3mm, 5μm); Detection: UV absorbance λ=280nm; Peaks: 1= Cu-CB-DO2A (0.11mg/ml); 2= Cu-CB-DO2LA (0.11mg/ml); 3= Cu-CB-TE2A (0.13mg/ml); 4= Cu-CB-TE2LA (0.14mg/ml)

Figure 6.10  Peak profiles for Cu-CB-DO2LA (0.11mg/ml) obtained by using different concentrations of methanol in the mobile phase. Column: Hypercarb (100x3mm, 5μm); column temperature: 25°C; Detection: UV absorbance λ=280nm
Figure 6.11 The peak profiles for uracil (0.067mg/ml) obtained by using mobile phases having different concentration of methanol. Column: Hypercarb (100x3mm, 5µm), column temperature: 25 °C; Detection: UV absorbance λ=254nm

Figure 6.12 Peak profiles for a one-tenth dilution of the sample concentration for the complexes with the use of a mobile phase of 10% (v/v) acetonitrile/water. Column: Hypercarb (100x3mm, 5µm), column temperature: 25 °C; Detection: UV absorbance λ=280nm; Peaks: 1= Cu-CB-DO2A (0.011mg/ml); 2= Cu-CB-DO2LA (0.011mg/ml); 3= Cu-CB-TE2A (0.013mg/ml); 4= Cu-CB-TE2LA (0.014mg/ml)
ABSTRACT

INVESTIGATION OF COPPER(II) COMPLEXES:
I. COPPER(II) COMPLEXES OF TRYPTOPHAN AND ITS ANALOGUES AS POSTCOLUMN INTERACTION COMPONENTS FOR INDIRECT FLUORESCENCE DETECTION OF AMINO-CONTAINING ANALYTES
II. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF COPPER(II)-AZAMACROCYCLIC COMPLEXES

by

Xiaoxuan Shen

University of New Hampshire, May, 2006

The initial phase of this project investigates the use of copper(II) complexes of tryptophan and its analogues for indirect fluorescence detection in high-performance liquid chromatography. First, indirect photometric and fluorometric detection in high-performance liquid chromatography (HPLC) is reviewed, giving the functional definition of indirect detection employed for this project, distinguishing different approaches to indirect photometric and fluorometric detection in HPLC, and explaining the basis of indirect fluorescence detection using copper(II) complexes of tryptophan and its analogues as postcolumn interaction components. Subsequent studies were conducted to develop and apply indirect HPLC detection for detecting specific mono-amino sugars, glucosamine, galactosamine, and mannosamine. Two tryptophan analogues, 5-hydroxy-L-tryptophan and DL-5-methoxytryptophan were evaluated as potential alternatives to L-twitter
tryptophan for the detection of these mono-amino sugars. This indirect fluorescence detection method was also evaluated for the analysis of glucosamine in commercial dietary supplements following chromatographic separation.

The second part of this project focuses on separating copper(II)-azamacrocyclic complexes with the use of high-performance liquid chromatography. Interest in these complexes arises from the increasing use of copper radioisotopes in imaging and therapy leading to the synthesis of ligands which form stable copper complexes. The effects of the concentration of the buffer, the pH of the buffered mobile phase, and the concentration of the organic modifier, methanol, on the separations produced were investigated. Separation of these copper complexes by ion-pair HPLC with the use of a mass spectrometry-compatible ion-pair reagent is also presented. The reversed phase chromatographic conditions utilized also allow the pKₐ's and the lipophilicity parameter of the complexes studied to be estimated. The separation and retention of several copper(II)-azamacrocyclic complexes on a graphitic carbon stationary phase, a very hydrophobic reversed-phase stationary phase with the capability of retaining highly polar compounds, was also investigated.

Finally the potential application of the indirect fluorescence detection method described here for detecting some of the ligands of the copper(II)-azamacrocyclic complexes, which do not absorb in the UV-vis range or fluoresce, following separation by liquid chromatography is discussed. Future studies are proposed for estimating the lipophilicity of metal complexes by reversed-phase high-performance liquid chromatography and capillary electrophoresis.
CHAPTER 1
INTRODUCTION

High-performance liquid chromatography (HPLC) is a popular and powerful analytical tool when coupled with an appropriate detector for the analytes being investigated [1]. Two of the most popular and convenient HPLC detectors in use today are ultraviolet-visible (UV-vis) absorbance and fluorescence detectors. For analytes which do not possess a chromophore or a fluorophore, one can be incorporated by reacting with a suitable derivatization reagent, either precolumn or postcolumn. A number of issues are usually associated with the use of such a derivatization method. These issues include: the choice of derivatization reagents, minimizing time-consuming sample treatments, ensuring complete and/or reproducible product formation, minimizing side-product formation and, in the case of postcolumn derivatization, limiting band broadening due to the presence of a postcolumn reaction volume.

While direct detection of analytes or their derivatives remains the most widely practiced approach to spectroscopic detection in HPLC, a number of other approaches have been developed to detect analytes that lack a chromophore or a fluorophore. These methods, which provide an alternative to direct detection, are often characterized as indirect detection methods.

A simple, indirect fluorescence detection method for HPLC, based on the recovery of L-tryptophan (L-Trp) fluorescence, has been applied to the detection of amino-containing compounds such as the aliphatic biogenic polyamines, amino acids,
and aminoglycoside antibiotics [3-5]. For this method, a solution containing the copper(II) complex of L-tryptophan (L-Trp), Cu(L-Trp)$_2$, is added to the eluent postcolumn. Fluorescence of L-Trp is quenched when bound in the Cu(L-Trp)$_2$ complex. In the presence of the eluting analyte molecules, which are capable of complexing with the Cu$^{2+}$, some fraction of the L-Trp is released from the Cu(L-Trp)$_2$ complex. The fluorescence of L-Trp is thus recovered, as shown by:

$$\text{Cu(L-Trp)}_2 + n\text{A} \leftrightarrow \text{Cu(A)}_n + 2 \text{L-Trp}^* \quad (1.1)$$

where A is the analyte molecule, L-Trp* is the fluorescent form of L-Trp and n is the number of the analyte molecules coordinated to the Cu$^{2+}$.

The goal of the first part of my research is investigating the use of copper(II) complexes of tryptophan and two tryptophan analogues for the indirect fluorescence detection of amino-containing analytes in high-performance liquid chromatography. Indirect photometric and fluorometric detection in high-performance liquid chromatography (HPLC) is reviewed in Chapter 2. Importantly, a functional definition of indirect detection employed is established and different approaches to indirect photometric and fluorometric detection in HPLC based on the interactions which occur between the analytes and the detectable components either within the column or postcolumn are distinguished. The basis of indirect fluorescence detection using copper(II) complexes of tryptophan and its analogues as postcolumn interaction components is also explained. Chapter 3 discusses studies which were conducted to develop and apply this approach to indirect detection for detecting specific mono-amino sugars, glucosamine, galactosamine, and mannosamine, following chromatographic separation. The two tryptohan analogues, 5-hydroxy-L-tryptophan (5-HTP) and DL-5-
methoxytryptophan (5-MTP), were evaluated as potential alternatives to L-tryptophan for the detection of these mono-amino sugars. 5-MTP was found to be a suitable alternative to L-Trp for detecting these mono-amino sugars. The analysis of glucosamine in commercial dietary supplements following chromatographic separation using this approach to indirect detection is presented in Chapter 4. The results obtained using indirect fluorescence detection were compared to the results obtained for precolumn derivatization with phenylisothiocyanate (PITC). The indirect fluorescence detection method was found to provide an alternative to precolumn derivatization for determining the concentration of glucosamine in commercial dietary supplements. A study was also conducted to evaluate the stability of the glucosamine-o-phthalaldehyde/3-mercaptopropionic acid derivative to investigate the applicability of the popular precolumn derivatization reagent, o-phthalaldehyde (OPA)/3-mercaptopropionic acid (MPA), for glucosamine analysis.

Research efforts were also focused on separating copper(II)-azamacrocyclic complexes with the use of high-performance liquid chromatography. The use of copper radioisotopes in imaging and targeted radionuclide therapy has resulted in increased interest in ligands which form stable copper complexes, such as copper(II)-azamacrocyclic complexes [6-9]. The effects of the charge and the size of the macrocyclic backbone of the Cu(II)-azamacrocyclic complexes on their biological behavior have been evaluated [10-15]. Here a reversed-phase high-performance liquid chromatographic method was developed to separate several copper(II)-azamacrocyclic complexes. Absorbance at 280 nm was used to monitor the complexes as they eluted from the reversed-phase column. The effects which the concentration of the buffer, the
pH of the buffered mobile phase, and the concentration of the organic modifier, methanol, have on the separation were investigated. Separation of these copper complexes by ion-pair HPLC with the use of a mass spectrometry-compatible ion-pair reagent, triethylammonium acetate (TEAA), was also investigated. The reversed phase chromatographic conditions provided a means to estimate the $pK_a$'s and the lipophilicity parameter of the complexes studied. These studies form the basis of Chapter 5. It was observed that these complexes are weakly retained on octyl and octadecyl bonded stationary phase. This led to additional studies with the goal being to ascertain the retention behavior and separation of four copper(II)-azamacrocyclic complexes using a graphitic carbon stationary phase. The graphitic carbon is a very hydrophobic reversed-phase stationary phase material which can also retain highly polar compounds. The effects of the organic mobile phase modifier (methanol or acetonitrile), the column temperature, and the sample concentration, on the retention of these complexes for a graphitic carbon stationary phase were also investigated. These studies are presented in Chapter 6.

One connection between the first part and the second part of the research presented in this thesis is the potential application of the indirect fluorescence detection method described here for detecting some of the ligands of the copper(II)-azamacrocyclic complexes. Many of these ligands do not absorb in the UV-vis range or fluoresce. This potential application is discussed in Chapter 7. Future studies are also proposed for estimating the lipophilicity of metal complexes by reversed-phase high-performance liquid chromatography and capillary electrophoresis. Compared to the traditional shake-flask method for measuring the octanol-water partition coefficient, reversed-phase high-
performance liquid chromatography (RP-HPLC) and capillary electrophoresis (CE) may provide efficient methods for estimating lipophilicity. A long-term additional benefit of this research will be the development of separation methods which can be applied to the analysis of metal complexes in biological and environmental samples.
PART I. COPPER(II) COMPLEXES OF TRYPTOPHAN AND ITS ANALOGUES AS POSTCOLUMN INTERACTION COMPONENTS FOR INDIRECT FLUORESCENCE DETECTION OF AMINO-CONTAINING ANALYTES.
CHAPTER 2

INDIRECT PHOTOMETRIC AND FLUOROMETRIC DETECTION IN HIGH-
PERFORMANCE LIQUID CHROMATOGRAPHY

2.1 Introduction

High-performance liquid chromatography (HPLC) is a powerful analytical tool when coupled with a suitable detector. Ultraviolet-visible (UV-vis) absorbance and fluorescence detectors are two of the most popular and convenient HPLC detectors in use today. However, as generally practiced, these approaches to analyte detection require that the analyte possess a functional group which either absorbs UV-vis light (i.e., has a chromophore) or fluoresces after being exposed to the appropriate wavelength of UV-vis light (i.e., has a fluorophore). A chromophore or fluorophore may be attached to a non-absorbing or non-fluorescing analyte by reacting with a suitable derivatization reagent. The derivatization reaction is performed either prior to injecting the sample into the chromatographic column (i.e., precolumn) or after the analytes elute from the column but prior to detection (i.e., postcolumn). Many fine examples demonstrating the application of each type of derivatization may be found in the literature [16]. A number of issues must be addressed when developing such a derivatization method, including: problems associated with choosing suitable reagents, minimizing time consuming sample treatments, ensuring complete and/or reproducible product formation, minimizing side-product formation and, in the case of postcolumn derivatization, limiting band broadening due to the presence of a postcolumn reaction volume.
While direct detection of analytes or suitable derivatives remains the most widely practiced approach to spectroscopic detection in HPLC, a number of other approaches have been developed to detect analytes that lack a chromophore or a fluorophore. These methods, which provide an alternative to direct detection, are often characterized as \textit{indirect} methods of photometric or fluorometric detection.

The general topic of indirect photometric detection was included in the biannual reviews of column liquid chromatography published in Analytical Chemistry from 1986 to 1994 [17-21]. Several reviews with abundant references on indirect detection, including indirect photometric detection and applications in liquid chromatography, have been published [22-30]. Here, the several ways in which indirect detection has been defined, especially as related to indirect photometric and fluorometric detection, are reviewed and a broader definition is introduced. Based on this broader definition, a number of approaches which have been used for indirect photometric and fluorometric detection in HPLC are reviewed and classified according to the types of interactions which occur between the analyte and the detectable component.

\section*{2.2 Defining Indirect Detection}

"Indirect detection" has been defined in many different ways and, in practice, a large number of different approaches have been applied. For example, Small and Miller [31] described the use of indirect photometric detection in ion chromatography. The detection of a UV-Vis-transparent analyte is accomplished by adding light-absorbing ionic species into the mobile phase. The presence of the analyte is monitored by measuring a decrease in the light absorbed by the eluent as the analytes elute from the
column. This approach to indirect detection was extended to other chromatographic systems such as reversed-phase chromatography [32-35] and reversed-phase ion-pairing chromatography [36,37]. Jurkiewicz and Dasgupta similarly defined indirect detection as a technique which monitors deficiencies in eluent constituents rather than the emergence of sample components [38]. Yeung indicated the key feature of indirect detection methods is that the indirect response measured is due to the absence of a mobile-phase component rather than the presence of the analyte [26]. The indirect detection methods highlighted in that review included measuring changes in refractive index, conductivity, light absorption (indirect photometry), polarimetry, and fluorescence [26].

Takeuchi and co-workers [24,30,39] defined indirect detection as a method whereby the analytes are visualized by measuring variations in the background signal resulting from the interactions between the analytes and the detectable component or by the detection of a detectable species produced by postcolumn interaction. Postcolumn ion replacement and postcolumn enzyme reactions are included as methods of indirect detection under this definition [24,30]. Verchère and Dona [27] considered indirect detection methods as involving exchange processes where the exchange specifically replaces one species by another without modification of the internal bonds of either. They included some forms of titration and "re-extraction" as examples of indirect detection. The detection of carbohydrates via postcolumn reaction with alkaline cupriammonium to form ternary complexes with enhanced absorbance at 300 nm is also an example of indirect detection under this definition [27].

The many definitions of indirect detection as well as inconvenient cross-referencing among these methods may limit their application. For this reason, a
somewhat broader, and simpler, functional definition of indirect detection will be
employed for this review. Any method of detection in which the presence of an analyte
as it elutes from the column is not measured directly, but instead is inferred by measuring
a response due to some other component, will be included here as an indirect method of
detection. This brief review will be limited to UV-vis absorption or fluorescence
methods since these are the most popular optical detection methods used in HPLC. Here,
indirect photometric and fluorometric detection in HPLC will be distinguished further by
whether the interactions between the analytes and the detectable components occur
within the column or postcolumn.

2.3 Indirect Detection Resulting From Interactions Occurring Within The Column

Interactions which occur between the analyte and the detectable component,
within the column, may change either the concentration of the detectable component in
the eluent or the spectral properties of that detectable component. Monitoring changes in
the photometric or fluorometric signal of the detectable component as it elutes from the
column provides an opportunity to detect the analyte indirectly. Most of the indirect
methods of detection covered in the literature describe monitoring changes in the
concentration of a detectable component due to the versatility of such an approach. In
some cases, however, analyte detection may be also achieved by monitoring changes in
the spectral properties of the detectable component (e.g., a shift in the wavelength of
maximum absorbance) rather than changes in the concentration of the component.
2.3.1 Indirect Detection Based on Measuring a Change in the Concentration of the Detectable Component in the Eluent

The methods covered in the following three sections are further characterized either as indirect photometric detection or indirect fluorometric detection depending on whether the property being detected is UV-vis-absorption or fluorescence. In general, the basis for indirect photometric detection described here is also applicable to indirect fluorometric detection.

2.3.1.1 Basis for Indirect Photometric Detection

The indirect photometric detection methods described in this section are based on the following principles: 1) The detectable component is continuously introduced into the column with the mobile phase; 2) Equilibrium is established for the detectable component between the mobile and the stationary phases; 3) The equilibrium concentration of the detectable component in the mobile phase provides a constant background level for detection; 4) The presence of the analyte affects the distribution equilibria within the column which results in a positive or negative change in the detector’s response for the detectable component as the analyte elutes. Using this form of indirect detection, injecting a sample results in a peak or peaks being observed for each of the injected analytes as well as one or several additional peaks that are characteristic of the system, called “system peaks”. Several studies have been conducted to evaluate the origin of these system peaks [40-52]. The detectable component has also been referred to as a “probe” [36,53-58], “marker” [59,60], or “visualization agent” [24,61-63]. Most of the detectable components employed to date absorb UV light, though others which absorb in
the visible region, such as methylene blue [64,65], ethyl violet [66], thymol blue [67],
methyl red [68], and chlorophyll (a & b) [69] have also been employed. Selection of a
suitable detectable component depends primarily upon the analyte and the mode of liquid
chromatography providing the separation. A general outline is presented in Table 2.1.

Ionic analytes have been analyzed by ion-exchange and ion-pairing
chromatography with the use of ionic detectable components. In ion-exchange
chromatography, the ionic detectable components serve a dual role, affecting the
retention of the analytes and providing detection for ionic analytes having the same
charge polarity as the ionic detectable components. For anionic analytes, the detectable
components used for indirect detection have included: phthalate [31,59,60,70-80],
naphthalene sulfonate derivatives [79], 2,4-dihydroxybenzoate [80], p-hydroxybenzoate
[81], pyromellitate [82,83], trimesate [84,85], nitrate [31,86-88], iodide [63],
hexacyanoferrate (II) and hexacyanoferrate (III) [89], anthraquinone-disulfonate [90],
sulfosalicylate, and 1,2-dihydroxybenzene-3,5-disulfonate [91]. Detection for otherwise
undetectable analytes which are cations has been achieved with the use of Cu(II) [31,92],
Ce(III) [92-94], aromatic monoamines [70,95,96], and 3-hydroxytyramine [97]. Anions
and cations can also be detected simultaneously with the use of a detectable component
which is a cation-anion pair following separation by cation and anion exchange columns
arranged in series [31,98], or a mixed-bed ion-exchange column[99].

Indirect detection has been accomplished in ion-pairing chromatography by
adding a detectable ion-pairing (or ion-interaction) reagent to the mobile-phase. The ion-
pairing reagent consists of a hydrophobic ion, which enhances the retention of the ionic
analyte, and a relatively hydrophilic counter ion. Three types of detectable, ion-pairing
Table 2.1. Outline for the selection of a suitable detectable component upon the analyte and the mode of liquid chromatography

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Mode of liquid chromatography</th>
<th>Detectable component</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ionic</td>
<td>Ion-exchange</td>
<td>UV-vis-absorbing ionic species with the same sign of the charge of analyte</td>
</tr>
<tr>
<td>Ionic</td>
<td>Ion-pairing</td>
<td>(1) UV-vis-absorbing hydrophobic ion and non-UV-vis-absorbing hydrophilic counter ion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2) non-UV-vis-absorbing hydrophobic ion and UV-vis-absorbing hydrophilic counter ion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3) UV-vis-absorbing hydrophobic ion and hydrophilic counter ion</td>
</tr>
<tr>
<td>Non-ionic</td>
<td>Reversed-phase</td>
<td>Ionic or non-ionic UV-vis-absorbing compounds</td>
</tr>
</tbody>
</table>
reagents have been reported. These types of reagents are distinguished based on whether the hydrophobic or the hydrophilic ions serve as the detectable species. The first type of ion-pairing reagent consists of a UV-absorbing hydrophobic ion and a non-UV-absorbing hydrophilic counter ion. UV-absorbing hydrophobic ions include: 1-phenethyl-2-picolinium [36,37,100,101], the naphthalenesulfonates [36,37,101-104], protriptyline [101,104], cetylpyridinium [105], iron(II) 1,10-phenanthroline [106,107], ruthenium(II) 1,10-phenanthroline [108-110], and ruthenium(II) 2,2’-bipyridine [108]. This type of ion pairing reagent provides a means for monitoring the elution of ionic analytes possessing the same charge polarity as the detectable species or the opposite charge polarity of the detectable species. The second type of ion-pairing reagent consists of a non-UV-absorbing hydrophobic ion and a more hydrophilic, UV-absorbing counter ion. UV-absorbing counter ions which have been employed include: thymolsulphonate [111], 3-nitrophthalate [112], phthalate [113], salicylate [113,114], sulfanilate [55], 3-hydroxybenzoate [56], benzoate [115], 1-naphthalenesulphonate[115], naphthalene-1,5-disulfonate [115,116], methylpyridine [56,117], papaveraldine[118], and chromate [119]. The mechanism for detecting ionic analytes using this type of ion-pairing reagent is similar to that observed for ion-exchange chromatography, where the detectable species provides detection for analytes having the same charge polarity as the detectable species. The third type of ion pairing reagent consists of a hydrophobic ion and a hydrophilic counter ion, both of which absorb light at the same wavelength, such as: 1-phenethyl-2-picolinium and 3-hydroxybenzoate [53,101], or 1-phenethyl-2-picolinium and 6-hydonaphthalene-2-sulphonate [101]. It has been suggested that the use of this type of
ion pairing reagent provides improved detection sensitivity for compounds which have considerably lower retention than that of the system peak[53,101].

Separation of non-ionic analytes, also called "nonelectrolytes" [29,120] or "neutral analytes" [36,56,58], is often achieved with reversed-phase chromatography. Indirect detection for otherwise undetectable analytes has been achieved by adding components to the mobile phase which absorb either ultraviolet or visible light. Both ionic and non-ionic compounds have been used as mobile phase additives to provide indirect detection of non-ionic analytes. Among the additives which have been used as detectable components are: naphthalene-2-sulfonate [36,58], 1-phenethyl-2-picolinium [36], 1-methylpyridine [56], nicotinamide [33,120], sulfanilamide [120], isoniazid[120], salicylamide [33,35], arbutin (hydroquinone-β-D-glucopyranoside) [33], cholecalciferol [35], benzamide [32,121], n-propyl-p-aminobenzoate[32], theobromine [121], benzene, nitrobenzene, benzonitrile, benzaldehyde, benzyl alcohol [34], methyl blue[64], chlorophyll (a & b) [69], and theophylline [122].

In addition to the specific analytes and the mode of liquid chromatography employed, other factors that may affect the chromatographic separation and the detection sensitivity must be considered when selecting detectable components. These factors include: the molar absorptivity and the concentration of the detectable components in the mobile phase, the pH of the mobile phase, and for ion exchange chromatography, the exchange capacity of the stationary phase. For example, the concentration of the detectable component in the mobile phase and the molar absorptivity at the wavelength of detection determine the background absorbance for indirect photometric detection. Barber and Carr [123] found that for the detector which they employed, the signal-to-
noise ratio began to decrease when the background absorbance was above about 1.0 AU (absorbance unit). Above this background absorbance, there was no gain in the signal level for the analytes. Small and Miller [31] suggested the absorbance level of the background should range from 0.2 to 0.8. Generally, a relatively low concentration of the detectable component in the eluent has been employed to avoid a high background absorbance level. Conversely, there is a lower limit for the concentration of the detectable component in the eluent. A low concentration of the detectable component may result in increased elution time of the analyte and thus increased band broadening [31], assuming the detectable component affects the retention of the analyte. For these reasons, the concentration of the detectable component in the mobile phase and the wavelength of detection must not be considered independently [123]. For detectable components which affect the analyte’s retention, choosing a different wavelength of detection, where the molar absorptivity of the detectable component is small, a low level of background absorbance for a high concentration of detectable component in the eluent may be achieved, while the desired displacing power of the eluent ion is still maintained. The pH of the mobile phase is another factor which often must be considered. The pH of the mobile phase may affect the degree of ionization and the absorbance properties of the detectable component [124], potentially affecting the displacing power of the eluent ion in ion-exchange chromatography or the level of background absorbance. In ion-pairing and reversed-phase chromatography, the affinity which the detectable component has for the stationary phase, as reflected by its capacity factor, is also critical. Studies have shown that an analyte is detected with maximum sensitivity when its capacity factor is close to that of the detectable component [22,33,53,56,100,101,111]. Variables that may
affect the capacity factor of the probe, such as the concentration and nature of the organic modifier and the mobile phase buffer, have been studied [105,125]. Studies demonstrating the optimization of the indirect photometric detection system have also been presented in the literature [59,105,126,127].

2.3.1.2 Application of Indirect Photometric Detection

Over the years several reviews have been published which highlight a variety of applications of indirect photometric detection in HPLC. Crommen [22] reviewed the application of indirect photometric detection in the analysis of amino acids and dipeptides in 1984. Indirect photometric detection of inorganic anions in HPLC was included in a review which was published in 1984 by Haddad [23]. Schill and Arvidsson [25] in 1989 reviewed the application of indirect photometric detection methods for biomedical analysis by HPLC. A review with 118 references was published covering the indirect photometric detection of nonelectrolytes in HPLC for the 15 years prior to 1998 [29]. The application of indirect photometric detection in HPLC for analyzing real samples including foods, body fluids, environmental and pharmaceutical samples was summarized in 1995 by Hayakawa and Yamamoto [28]. A summary of some examples of applications of the indirect photometric detection for analyzing real samples using HPLC which were not included in Hayakawa and Yamamoto’s review is presented here in Table 2.2 along with information on the analytes being detected, the mobile phase containing the detectable components, column, linear range, wavelength of detection, and limit of detection/quantitation.
Table 2.2. Examples of the application of indirect photometric detection based on a change of the concentration of the detectable component in the eluent

<table>
<thead>
<tr>
<th>Sample</th>
<th>Analyte</th>
<th>Mobile phase</th>
<th>Column</th>
<th>λ (nm)</th>
<th>Linear range</th>
<th>LOD/LOQ b</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pharmaceutical tablets</td>
<td>Captopril</td>
<td>0.1M potassium hydrogenphthalate (pH 6.0)/methanol/water = 25/150/825 (v/v/v)</td>
<td>Vydac anion-exchange column</td>
<td>280</td>
<td>0.08-1.05mg/ml</td>
<td>0.70ng (in 25μl)</td>
<td>60</td>
</tr>
<tr>
<td>Ertapenem sodium bulk drug</td>
<td>Acetate</td>
<td>1.0mM p-hydroxybenzoic acid (pH 9.5)/methanol=99/1 (v/v)</td>
<td>PRP-X100</td>
<td>305</td>
<td>0.0023-0.4663mg/ml</td>
<td>0.002mg/ml (LOQ)</td>
<td>81</td>
</tr>
<tr>
<td>Bulk drug</td>
<td>Alendronate</td>
<td>1mM trimesic acid (pH 5.5)</td>
<td>PRP-X100</td>
<td>254</td>
<td>0.1-2mg/ml</td>
<td>50ng-250ng (in 10μl)</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>Etidronate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Clodronate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2-Phosphono-pyrrolidine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alendronate dimer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pharmaceutical tablets, solutions, and powder</td>
<td>Citrate</td>
<td>0.875mM trimesic acid (pH 10.0)</td>
<td>PRP-X100</td>
<td>280</td>
<td>1-12μg (in 20μl)</td>
<td>0.26μg/ml (LOQ)</td>
<td>85</td>
</tr>
<tr>
<td>Pharmaceutical tablets</td>
<td>Etidronate disodium</td>
<td>7.2mM nitric acid</td>
<td>IC-Pak HR anion-exchange</td>
<td>240</td>
<td>0.2-0.6mg/ml</td>
<td>0.001mg/ml</td>
<td>86</td>
</tr>
<tr>
<td>Pharmaceutical tablets</td>
<td>Alendronate sodium</td>
<td>1.6-12mM nitric acid</td>
<td>IC-Pak HR anion-exchange</td>
<td>235-245</td>
<td>0.02-0.08mg/ml</td>
<td>0.001mg/ml</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>Etidronate disodium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Clodronate disodium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Note:** LOD/LOQ stands for Limit of Detection/Limit of Quantitation.
<table>
<thead>
<tr>
<th>Category</th>
<th>Substance</th>
<th>Concentration/Composition</th>
<th>Stationary Phase</th>
<th>Mobile Phase</th>
<th>Peak Width</th>
<th>0.1 Mole %</th>
<th>0.14mM</th>
<th>0.42ng</th>
<th>3.2mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouthwash</td>
<td>Ethanol</td>
<td>67.8uM sulfanilamide, 0.4mM nicotinamide, 0.28mM</td>
<td>Hypersil ODS</td>
<td>260</td>
<td>0.1%-0.24%</td>
<td>0.15%-0.36% (w/v)</td>
<td></td>
<td>1.57%-2.7%</td>
<td></td>
</tr>
<tr>
<td>Disinfectant solution</td>
<td>Glycerol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>120</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Isopropanol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injection for veterinary use</td>
<td>Propylene glycol N,N-dimethyl acetamide</td>
<td>67.8uM sulfanilamide, 0.4mM nicotinamide, 0.28mM</td>
<td>Hypersil ODS</td>
<td>260</td>
<td>0.21%-0.36% (w/v)</td>
<td>0.028%-0.048% (w/v)</td>
<td></td>
<td></td>
<td>120</td>
</tr>
<tr>
<td>Topiramate drug substance</td>
<td>Sulfamate</td>
<td>5.8mM p-hydroxybenzoic acid and 2.5% methanol (pH 9.4)</td>
<td>PRP-X100</td>
<td>310</td>
<td>0.25-6.3 mole %</td>
<td>0.25-18.8 mole %</td>
<td></td>
<td></td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>Sulfate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pharmaceutical tablets</td>
<td>Meprobamate</td>
<td>1mM benzoic acid in a mixture of 0.05M phosphoric acid (pH 1.9) and methanol (70:30, v/v)</td>
<td>Lichrospher RP-18</td>
<td>273</td>
<td>0.5-8mg/ml</td>
<td></td>
<td></td>
<td></td>
<td>130</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.49uM cinnamic acid in a mixture of phosphate buffer (pH 4.8) and methanol (60:40, v/v)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>130</td>
</tr>
<tr>
<td>Foods</td>
<td>Cyclamate</td>
<td>10-30uM Methyl Red in 0.02M phosphate buffer (pH 7.0)/methanol =3/2 (v/v)</td>
<td>Inertsil ODS-3</td>
<td>433</td>
<td></td>
<td>0.14mM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Foods</td>
<td>Free, bound and total sulphites</td>
<td>0.15g/L potassium hydrogenphthalate (pH 5.7)</td>
<td>Supelcosil LC-SAX</td>
<td>280</td>
<td>0.028%-0.048% (w/v)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>77</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td>Bromide</td>
<td>0.01M citrate-buffer-acetonitrile (80:20, v/v) containing 0.1mM papaveridine perchlorate and 2.6-4.5M tetrabutylammonium hydroxid</td>
<td>μBondapak C18</td>
<td>325</td>
<td>1.25nmol</td>
<td>3.2mg/l</td>
<td>3.2μM</td>
<td>3.9μM</td>
<td>118</td>
</tr>
<tr>
<td>Serum samples Tap water</td>
<td>Chloride</td>
<td>1mM sodium iodide and 0.3mM tartaric acid (pH 3.3)</td>
<td>Octadecylsilica</td>
<td>225</td>
<td>0.1-1.0mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bromide</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nitrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Note: Concentrations are given in units of molality (M), percent (%), milligrams per milliliter (mg/ml), and parts per million (ppm) as appropriate.*
<table>
<thead>
<tr>
<th>Source</th>
<th>Water Type</th>
<th>Chloride</th>
<th>Nitrate</th>
<th>Sulphate</th>
<th>pH (Acid)</th>
<th>pH (Base)</th>
<th>Acetonitrile</th>
<th>Coating</th>
<th>Elution</th>
<th>Retention</th>
<th>Elution</th>
<th>Retention</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drinking water</td>
<td></td>
<td>2mM 3-nitrophthalic acid (pH 4.0) containing 18% (v/v) acetonitrile</td>
<td></td>
<td>Hypersil-5-ODS coated with cetyltrimethylammonium bromide</td>
<td>325</td>
<td>20-2500ppm</td>
<td>325</td>
<td>20-2500ppm</td>
<td>—</td>
<td>112</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tap water</td>
<td></td>
<td>1mM sodium salicylate (pH 5.8) containing 5% acetonitrile</td>
<td></td>
<td>Develosil ODS-3K coated with cetyltrimethylammonium bromide</td>
<td>230</td>
<td>0.02-1mM</td>
<td>0.02mM</td>
<td>0.02mM</td>
<td>114</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sediment samples</td>
<td>Sulfate</td>
<td>1.6mM sulfobenzoic acid and 0.2mM trimesic acid (pH 5.5)</td>
<td></td>
<td>Nucleosil SB anion exchange</td>
<td>258</td>
<td>1-100nmol</td>
<td>600pmol</td>
<td>600pmol</td>
<td>124</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tap water</td>
<td>Nitrate</td>
<td>1mM potassium hydrogenphthalate (pH 3.95)</td>
<td></td>
<td>Partisil 10 SAX</td>
<td>265</td>
<td>3.67-29.2μg/ml</td>
<td>0.1μg/ml</td>
<td>0.1μg/ml</td>
<td>131</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tap water</td>
<td>Drinking fountain</td>
<td>0.4mM 5-sulfoisophthalic acid (pH 4.5)</td>
<td></td>
<td>Nucleosil SB anion exchange</td>
<td>240</td>
<td>0.3-25ppm</td>
<td>20-50pmol</td>
<td>20-50pmol</td>
<td>132</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pore water</td>
<td>Sulfate</td>
<td>0.5mM potassium hydrogenphthalate-0.015 %triethanolamine-3% methanol (pH 7.9)</td>
<td></td>
<td>Eclipse XDR-C18 coated with cetylpyridinium chloride</td>
<td>265</td>
<td>19-430μM</td>
<td>19μM</td>
<td>19μM</td>
<td>78</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atmospheric waters</td>
<td>Sulfate</td>
<td>0.5mM salicylic acid pH 5.5 containing 5% (v/v) acetonitrile</td>
<td></td>
<td>Supelcosil LC-18 coated with cetylpyridinium chloride</td>
<td>293</td>
<td>1-3μg/ml</td>
<td>0.06μg/ml</td>
<td>0.06μg/ml</td>
<td>133</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysimeter leaching test samples</td>
<td>Acetic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.07μg/ml</td>
<td>0.07μg/ml</td>
<td>133</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lactic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.09μg/ml</td>
<td>0.09μg/ml</td>
<td>133</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Propionic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.10μg/ml</td>
<td>0.10μg/ml</td>
<td>133</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pyruvic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gold process</td>
<td>Chloride</td>
<td>0.5mM 1,3,5-benzene-tricarboxylic acid (pH 7) containing 2.5% (v/v) n-butanol</td>
<td>Supelcosil LC-18 coated with cetylpyridinium chloride</td>
<td>254</td>
<td>2-20μg/ml</td>
<td>0.02μg/ml</td>
<td>133</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>----------</td>
<td>--------------------------------------------------------------------------------</td>
<td>---------------------------------------------------</td>
<td>------</td>
<td>----------</td>
<td>----------</td>
<td>------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>effluents</td>
<td>Cyanate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sulphate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thiosulphate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thiocyanate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liquid and solid</td>
<td>Fluoride</td>
<td>2mM potassium hydrogenphthalate (pH 5.0)</td>
<td>PRP-X100</td>
<td>272</td>
<td>0.03-10μg/ml</td>
<td>0.03μg/ml</td>
<td>135</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chemical wastes</td>
<td>Phosphate</td>
<td></td>
<td></td>
<td></td>
<td>0.06-16μg/ml</td>
<td>0.06μg/ml</td>
<td>136</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chloride</td>
<td></td>
<td></td>
<td></td>
<td>0.05-15μg/ml</td>
<td>0.05μg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bromide</td>
<td></td>
<td></td>
<td></td>
<td>0.1-30μg/ml</td>
<td>0.1μg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Iodide</td>
<td></td>
<td></td>
<td></td>
<td>0.7-100μg/ml</td>
<td>0.7μg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sulfate</td>
<td></td>
<td></td>
<td></td>
<td>0.07-50μg/ml</td>
<td>0.07μg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residues of low</td>
<td>Nitrite</td>
<td>4mM benzyltributylammonium chloride/5mM phosphate buffer (pH 4.6)/0.25M hexane-sulfonate</td>
<td>Lichrospher RP-18</td>
<td>222</td>
<td>0.05-10mM</td>
<td>2.8ppm</td>
<td>137</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>explosives</td>
<td>Nitrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.8ppm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chlorate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>21.3ppm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sulfate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>28.4ppm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a λ: wavelength of detection  

b LOD: limit of detection; LOQ: limit of quantitation. (The values listed are LOD unless otherwise specified)
2.3.1.3 Application of Indirect Fluorometric Detection

If the detectable component, which is added to the mobile phase, fluoresces under the conditions employed, indirect detection can also be accomplished using a fluorescence detector. Su et al [138] added aniline to the mobile phase to allow the elution of both fluorescent and non-fluorescent polynuclear aromatic hydrocarbons to be detected. Mho and Yeung [139] detected iodate and chloride ions using a mobile phase containing sodium salicylate as the fluorescent agent. They used a double-beam fluorescence detector to increase the stability of the fluorescence signal measured. Sodium salicylate was also used as the detectable component in the mobile phase to provide indirect fluorometric detection for inorganic anions such as acetate and nitrate [140,141]. Non-electrolytes, such as the alkanols, were detected by introducing 2,7-dichlorofluorescein [141] or anthracene [142] into the mobile phase as the detectable fluorescent component. Gallo and Walters [143] investigated 2-amino-5-methylbenzenesulfonic acid as a detectable, mobile-phase component for indirect fluorometric detection of aliphatic amines such as: butylamine, isobutylamine, and cyclohexylamine. Cerium (III) ion, which has been used for indirect photometric detection by measuring the absorbance level of the eluent at 254 nm [92-94], has also been used for indirect fluorometric detection of sodium, ammonium and potassium ions in urine by exciting at 247 nm and observing the fluorescence at 350 nm [144]. One of the primary advantages of using this fluorometric method for indirect detection is that a UV-absorbing matrix will not interfere with monitoring the fluorescence signal if the matrix does not absorb at the excitation or emission wavelength of the detectable component. Rigas and Pietrzyk [145] added fluorescent ruthenium(II) 1,10-
phenanthroline and ruthenium(II) 2,2'-bipyridine complexes to the mobile phase to provide detection of inorganic and organic anions separated with a Hamilton PRP-1 column. Fung and Tam [146] performed an optimization study to improve the detection of inorganic anions when using ruthenium(II) 1,10-phenanthroline as the mobile phase additive. Diode-laser-based indirect fluorometric detection of n-alkyl alcohols was undertaken with the use of a near infrared dye, IR 125, which was added to the mobile phase [147]. Indirect fluorometric detection of eleven EPA priority phenols following their separation by reversed-phase capillary HPLC has been reported [148]. The detectable component employed in this case was sodium 1-naphthalenesulfonate which has a maximum fluorescence emission wavelength of 665 nm when excited at 280nm.

A novel approach to indirect fluorescence detection was described by Jurkiewicz and Dasgupta [38]. They added either 4-amino-1-naphthalenesulfonic acid or 6,7-dihydroxy-2-naphthalenesulfonate to the mobile phase in concentrations at which they are strongly self-quenched. The concentration of the detectable components in the mobile phase decreases as the analytes elute from the column, resulting in an increase in the fluorescence signal. By monitoring this signal, indirect fluorometric detection of fluoride, chloride, bromide, iodine, thiocyanate, and sulfate was achieved. This approach to indirect detection may be useful in applications where it is possible to operate at a relatively low level of background fluorescence.

2.3.2 Indirect Detection Based on Measuring a Change in a Spectroscopic Property of the Detectable Component in the Eluent

Another approach to indirect detection is based on monitoring a change in a spectroscopic property of the detectable component in the eluent. The spectral change
results from an interaction between the detectable component and the non-detectable analyte in the eluent. For example, the absorbance of phenolphthalein decreases when it forms an inclusion complex with cyclodextrin [149]. Indirect photometric detection of several cyclodextrins was achieved by adding phenolphthalein to the mobile phase as the detectable component [150,151]. However, it was proposed that the mechanism of detection was more complex than this since cyclodextrins disturb the partitioning of phenolphthalein in the column which also contributes to indirect detection by producing a change in the concentration of the detectable component in the mobile phase [150]. Since the absorbance of iodine and the fluorescence intensity of 2-p-toluidinyl-6-naphthalenesulfonate are also enhanced by the presence of cyclodextrins, iodine [152] and 2-p-toluidinyl-6-naphthalenesulfonate [153,154] have also been added to the mobile phase as detectable components for indirect photometric and fluorometric detection of the cyclodextrins, respectively. The inclusion complexes formed between the cyclodextrins and the detectable component are due to hydrophobic interactions, hydrogen bonding and/or non-specific van der Waals forces [152], distinguishing this detection approach from chemical derivatization, which involves the formation of chemical bond(s) between the analyte and the derivatization reagent. For this reason the methods discussed in this section are considered to be forms of indirect detection. Indirect detection of cyclodextrins can also be achieved by introducing the detectable components postcolumn, a topic to be discussed in a later section.

2.4. Indirect Detection Resulting From Interactions Occurring Postcolumn

This approach to indirect detection is easily confused with direct detection following postcolumn derivatization. Direct detection of analytes in HPLC may be
achieved by postcolumn chemical derivatization of the analyte. Postcolumn chemical derivatization is accomplished by introducing a reagent, containing the detectable feature, which reacts with the analyte as it elutes from the column. The key distinguishing feature of postcolumn chemical derivatization is that the added reagent undergoes a chemical reaction with the analyte to produce a third compound that is detectable. The derivatization reaction is given by the equation:

\[ A + B \rightarrow C \]

where \( A \) is the analyte, \( B \) is the derivatization reagent, and \( C \) is the product, which may also be called "derivatized \( A \)".

By contrast, indirect detection resulting from postcolumn interactions does not involve detecting the analyte or a derivatized version of the analyte directly. Instead the presence of the analyte is inferred by monitoring the presence or absence of a detectable species in the eluent. The presence or absence of the detectable species occurs after the postcolumn interactions take place between the analyte and a component introduced either pre- or postcolumn. Thus, postcolumn interaction does not indicate the component which interacts with the analyte must be introduced postcolumn, but instead that the interaction occurs postcolumn. In the following sections some examples will be given where the interactions between the analyte and a component, which is introduced precolumn, actually occur postcolumn. Such interactions may be induced by a catalyst, such as enzyme, which is presented postcolumn. In this review, post-column interactions are classified based on the different approaches which are used to produce or eliminate the species which is ultimately monitored.
2.4.1 Interaction Mode: A + ML → MA + L

Here, A is the analyte and ML is a metal-ligand complex where M represents the metal ion and L represents the ligand. The coordination number and net charge of the metal-ligand complex are different for different types of metal ions and ligands, though this will be ignored here and in the following sections to simplify the discussion. The metal-ligand complex is introduced postcolumn and will be called a “postcolumn interaction component” for convenience. Detection of the analyte is provided by monitoring the presence of either ML or L, either of which may provide a detectable signal indicating the presence of the analyte. The analyte, A, and product, MA, are not detectable under the experimental conditions. Depending on the spectral properties of ML and L, indirect detection of the analyte may be achieved by measuring either a decrease or an increase in the absorbance or fluorescence intensity, as shown in Figure 2.1. A summary of applications based on this approach to indirect detection is presented in Table 2.3. In the following sections, these applications are described in more detail.

2.4.1.1 Measuring a Decrease in Absorbance or Fluorescence Intensity

When utilizing this approach to indirect detection, the analyte is detected indirectly by monitoring a decrease in the absorbance or fluorescence intensity of the solution. For example, if the metal-ligand complex, ML, absorbs or fluoresces more strongly at a given wavelength than the ligand, L, then the presence of analyte A is indicated by a decrease in the absorbance or fluorescence intensity due to ML. Yoza et al [155] employed this approach when they used the colored, copper complex of methylthymol blue to detect the elution of aminopolycarboxylic acids. A solution
Figure 2.1. Indirect detection resulting from interaction: $A + ML \rightarrow MA + L$: (a) Measuring a decrease in absorbance or fluorescence intensity, (b) Measuring an increase in absorbance or fluorescence intensity. Solid circle for $L$ indicates the analyte ($L$) either absorbs UV-vis light or fluoresces. Open circle for $L$ indicates the analytes ($L$) neither absorbs UV-vis light nor fluoresces.
Table 2.3. Summary of the applications based on the indirect detection approach: A + ML \(\rightarrow\) MA + L

<table>
<thead>
<tr>
<th>Analyte (A)</th>
<th>ML</th>
<th>L</th>
<th>Signal monitored</th>
<th>Linear range</th>
<th>LOD (e)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminopolycarboxylic acids Clodronate and its esters</td>
<td>Copper-methymol blue</td>
<td>Methylmol blue</td>
<td>Decrease in absorbance of ML</td>
<td>—</td>
<td>0.1mM</td>
<td>155</td>
</tr>
<tr>
<td></td>
<td>Thorium-EDTA-xylenol orang</td>
<td>Xylenol orange</td>
<td>Decrease in absorbance of ML</td>
<td>0.3-16mg/l</td>
<td>0.3-1.4mg/l</td>
<td>156</td>
</tr>
<tr>
<td>Galactitol</td>
<td>Molybdate-chloranilate</td>
<td>Chloranilate</td>
<td>Decrease in absorbance of ML</td>
<td>0-100mg/l</td>
<td>10.5mg/l</td>
<td>157</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td></td>
<td></td>
<td></td>
<td>16mg/l</td>
<td>17.5mg/l</td>
<td></td>
</tr>
<tr>
<td>D-Glucitol</td>
<td></td>
<td></td>
<td></td>
<td>24mg/l</td>
<td>28mg/l</td>
<td></td>
</tr>
<tr>
<td>D-Arabinitol</td>
<td></td>
<td></td>
<td></td>
<td>36mg/l</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xylitol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Galactitol</td>
<td>Molybdate-chloranilate</td>
<td>Chloranilate</td>
<td>Increase in absorbance of L</td>
<td>0-100mg/l</td>
<td>17mg/l</td>
<td>157</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td></td>
<td></td>
<td></td>
<td>26mg/l</td>
<td>28mg/l</td>
<td></td>
</tr>
<tr>
<td>D-Glucitol</td>
<td></td>
<td></td>
<td></td>
<td>38mg/l</td>
<td>59mg/l</td>
<td></td>
</tr>
<tr>
<td>D-Arabinitol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xylitol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphorus oxo acids, fluoride, sulfate, bisphosphonates, N-(phosphonomethyl)-glycine, aminomethylphosphonic acid</td>
<td>Aluminum-morin</td>
<td>Morin</td>
<td>Decrease in fluorescence of ML</td>
<td>39-824ng</td>
<td>4-15ng</td>
<td>158</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>36mg/l</td>
<td>160</td>
</tr>
<tr>
<td>Cysteine</td>
<td>8-Hydroxyquinoline-5-sulfonic-Cd(II)</td>
<td>8-Hydroxyquinoline-5-sulfonic acid</td>
<td>Decrease in fluorescence of ML</td>
<td>0-430(\mu)M</td>
<td>0.1(\mu)M</td>
<td>161</td>
</tr>
<tr>
<td>Homocysteine</td>
<td></td>
<td></td>
<td></td>
<td>0-370(\mu)M</td>
<td>0.1(\mu)M</td>
<td></td>
</tr>
<tr>
<td>Glutathione</td>
<td></td>
<td></td>
<td></td>
<td>0-430(\mu)M</td>
<td>0.2(\mu)M</td>
<td>162</td>
</tr>
<tr>
<td>Cystine</td>
<td></td>
<td></td>
<td></td>
<td>0-100(\mu)M</td>
<td>0.6(\mu)M</td>
<td></td>
</tr>
<tr>
<td>Homocystine</td>
<td></td>
<td></td>
<td></td>
<td>0-150(\mu)M</td>
<td>0.3(\mu)M</td>
<td></td>
</tr>
<tr>
<td>Glutathione disulfide</td>
<td></td>
<td></td>
<td></td>
<td>0-200(\mu)M</td>
<td>0.3(\mu)M</td>
<td></td>
</tr>
<tr>
<td>Biotin</td>
<td>Avidin-2,6-ANS *</td>
<td>2,6-ANS *</td>
<td>Decrease in fluorescence of ML</td>
<td>1-200(\mu)M</td>
<td>0.5(\mu)M</td>
<td>163</td>
</tr>
<tr>
<td>Biocytin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biotin</td>
<td>Avidin-HABA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>HABA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Increase in absorbance of L</td>
<td>0.01-2mM</td>
<td>7.3µM</td>
<td>164</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------</td>
<td>-----------------</td>
<td>----------------------------</td>
<td>----------</td>
<td>-------</td>
<td>----</td>
</tr>
<tr>
<td>Biocytin</td>
<td>Palladium(II)-calcein</td>
<td>Calcein</td>
<td>Increase in fluorescence of L</td>
<td>Two- to three-order concentration range</td>
<td>0.5-1.0ng (in 100µl)</td>
<td>165</td>
</tr>
<tr>
<td>Organosulphur compounds</td>
<td>Fe(III)-methylcalcein blue</td>
<td>Methylcalcein blue</td>
<td>Increase in fluorescence of L</td>
<td>10-1000ng</td>
<td>10ng</td>
<td>3ng</td>
</tr>
<tr>
<td>D-Myo-1,2,6-inositol trisphosphate</td>
<td>Copper-tryptophan</td>
<td>Tryptophan</td>
<td>Increase in fluorescence of L</td>
<td>5-1000pmol</td>
<td>5-10pmol</td>
<td>3-5</td>
</tr>
<tr>
<td>Diamines, polyamines</td>
<td>Copper-APA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>APA&lt;sup&gt;c&lt;/sup&gt; and ADT&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Increase in fluorescence of L</td>
<td>2ng-20µg</td>
<td>8.7µg/l</td>
<td>167</td>
</tr>
<tr>
<td>Aminoglycoside antibiotics</td>
<td>Copper-ADT&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>4-400µg (in 200µl)</td>
<td>6.1µg/l</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> 2,6-ANS: 2-anilinonaphthalene-6-sulfonic acid
<sup>b</sup> HABA: 2-[4'-hydroxyphenylazo]benzoic acid
<sup>c</sup> APA: N-(9-anthrylmethyl)-2-pyridylmethylamine
<sup>d</sup> ADT: N-(9-anthrylmethyl)2-diethylenetriamine
<sup>e</sup> LOD: limit of detection
containing copper methylthymol blue (i.e., the ML component) was introduced postcolumn. The molar absorptivity of this complex differs from that of the free ligand at 595 nm. The presence of the aminopolycarboxylic acids in the eluent was detected by monitoring the decrease in absorbance at 595 nm. Another example of this mode of indirect detection is monitoring the elution of the compound clodronate and some of its esters using a mixed-ligand complex, thorium-EDTA-xylenol orange, as the postcolumn interaction component [156]. Clodronate and its esters, which were separated by anion-exchange chromatography, interact with this mixed-ligand complex to release the ligand, xylenol orange, resulting in a decrease in the absorbance at 550 nm. Dona and Verchère [157] detected the presence of alditols utilizing a molybdate-chloranilate complex as the postcolumn interaction component. The molybdate-chloranilate complex has a maximum absorbance at 347 nm while the free chloranilate ligand has a maximum absorbance at 318 nm. The presence of the alditols in the eluent is indicated by a decrease in the level of absorbance at 347 nm. It should be noted that the presence of the analyte can also be detected by monitoring an increase in absorbance at 318 nm, due to an increase in the concentration of the free chloranilate ligand in solution (additional examples of this mode of indirect detection will be covered in the next section). In this case, the limit of detection based on measuring the decrease in absorbance at 347 nm was found to be better than when measuring the increase in absorbance at 318 nm. Pietrzyk and coworkers [158-160] used an aluminum-morin complex as the postcolumn interaction component to detect the presence of phosphorus oxo acids, fluoride, sulfate, bisphosphonates, and N-(phosphonomethyl)glycine and its metabolite, aminomethylphosphonic acid. The aluminum-morin complex fluoresces more intensely...
at 505 nm, when excited at 420 nm, than does the free ligand, morin. Complexation of the analytes by the aluminum ion results in a decrease in the concentration of the aluminum-morin complex in the eluent. The resulting decrease in the fluorescence intensity of the solution in the detector flow cell indicates the presence of the analytes in the eluent. Pelletier and Lucy [161] monitored the elution of thiols by indirect fluorometric detection. They mixed a solution containing the fluorescent complex, 8-hydroxyquinoline-5-sulfonic-Cd(II), with the eluent from the column. The eluting thiols complex with the Cd(II) ion, resulting in a decrease in the fluorescence intensity at 510 nm. This approach was also employed for the detection of disulfides by first reducing the disulfides to the corresponding thiols following elution from the column but prior to mixing with a solution containing the 8-hydroxyquinoline-5-sulfonic-Cd(II) complex [162]. Reduction of the disulfides was accomplished by mixing the eluent with tris(2-carboxyethyl)phosphine.

An example of a complex which is not a metal-ligand complex but which has been used in a similar manner for indirect fluorometric detection is avidin-2,6-ANS (2-anilinonaphthalene-6-sulfonic acid) [163]. This complex has been utilized as the postcolumn interaction component for the indirect detection of biotin and biocytin. Biotin and biocytin form complexes with avidin, thereby releasing 2,6-ANS from the fluorescent complex. The resulting decrease in the fluorescence intensity of the solution at 438 nm indicates the presence of these analytes in the eluent.
2.4.1.2 Measuring an Increase in Absorbance or Fluorescence Intensity

In this section examples are described where the presence of the analyte is inferred by observing an increase in the measured signal. Here the ligand L absorbs more strongly or fluoresces more intensely at a given wavelength than the postcolumn interaction component, ML. Indirect detection of the analyte, A, under such circumstance can be achieved by measuring the increase in either the absorbance or fluorescence intensity due to the presence of component L.

This approach was employed by Przyjazny et al [164] to detect the elution of biotin and biocytin from an HPLC column. The detection of analytes were achieved by monitoring the increase in absorbance at 345 nm due to the presence of 2-[4'-hydroxyphenylazo]benzoic acid (HABA) in the eluent, which was released from an avidin-HABA complex introduced post-column. The molar absorptivity at 345 nm is higher for the free HABA in solution than for the avidin-HABA complex. HABA is released from the avidin-HABA complex as it interacts with biotin or biocytin as they elute from the column. Thus, the increase in absorbance at 345 nm indicates the presence of either biotin or biocytin. Werkhoven-Goewie et al [165] mixed a solution containing a palladium (II)-calcein complex with the eluent to analyze urine and serum samples containing organosulphur compounds. The fluorescence of calcein is quenched efficiently by palladium(II) ion. In the presence of the organosulphur compounds, which compete for the palladium(II) ion, calcein is released from the palladium(II)-calcein complex, resulting in an increase in the fluorescence intensity of the eluent. Similarly, a weakly fluorescent complex of Fe(III) ion with methylcalcein blue was the postcolumn interaction component used for indirect detection of D-myo-1,2,6-inositol trisphosphate.
In this example, the analytes form stronger complexes with Fe(III), which releases highly fluorescent methylcalcein blue into solution. Yang and Tomellini [3] introduced a copper(II)-tryptophan complex postcolumn to determine aliphatic biogenic polyamines and diamines. The fluorescence of tryptophan is quenched when complexed with copper(II) ion. These analytes bind with copper to release the fluorescent tryptophan from the copper(II)-tryptophan complex providing the fluorescence response. This approach to indirect detection was extended to the analysis of amino acids [4] and formulated aminoglycoside antibiotics [5]. Tanaka et al [167] employed a copper(II) complex of either N-(9-anthrylmethyl)-2-pyridylmethylamine (APA) or N-(9-anthrylmethyl)-diethylenetriamine (ADT) as the postcolumn interaction components for the detection of N-(phosphonomethyl)glycine and aminomethylphosphonic acid. The fluorescence of APA or ADT is quenched when complexed with copper(II). The fluorescence of APA or ADT recovers indicating the presence of the analytes after the analytes in the eluent mix with these postcolumn interaction components, another variation of this general approach to indirect detection, called postsuppressor ion replacement [168-170], employs equipment and columns similar to what is used in suppressed-ion chromatography. The primary difference between this approach and the ones described in the previous paragraph is that another column is used to introduce ML to replace eluting solute ions or co-ions, A, with a "replacement ion", L. Here M is not a metal ion but a co-ion of L. The concentration of the replacement ion in the eluent is monitored by measuring the UV-vis absorbance or fluorescence. Dasgupta included this approach as a postcolumn technique for detection in ion chromatography [171]. Common replacement ions include Ce(III), anthranilate [168], β-
naphthalenesulphonate [169], iodate and nitrate [170]. Using this approach, anions such as chloride, bromide, sulfate, phosphate, formate, acetate, and citrate [168,170], and cations such as lithium, potassium, sodium, and ammonium [169,170] have been detected in the eluent.

2.4.2 Interaction Mode: \( A + ML \rightarrow A' + M'L \)

Indirect detection based on a redox reaction occurring between the analyte, A, and the metal ion, M, of a complex, ML, which is mixed with the eluent has been demonstrated. Due to differences in the oxidation states of the metal ion, the reactant, ML, and the product, M'L, have different maximum absorption wavelengths or fluorescence emission wavelengths. Indirect detection of the analyte is accomplished by monitoring either an increase or decrease in absorbance or fluorescence due to the presence of M'L.

The use of a Cu(II)-2,2'-bicinchononate complex to detect sugars and uronic acids [172-177] is an example of this mode of indirect detection. The analytes reduce copper(II) ion in the complex to copper(I) ion. Copper(I) ion complexes with 2,2'-bicinchononate to form Cu(I)-2,2'-bicinochoninate, which has a maximum absorption at 562 nm. Thus, the reducing analytes are detected by monitoring the increase in absorbance at 562 nm. In another example, a peptide, dynorphin A, reduces tris(2,2'-bipyridyl)ruthenium(III) to tris(2,2'-bipyridyl)ruthenium(II). The tris(2,2'-bipyridyl)ruthenium(II) complex fluoresces when excited by visible light indicating the presence of dynorphin A in the eluent [178].
Similarly the presence of analytes that are capable of oxidizing the component which is added to the eluent can also be detected. For example, Mullertz et al. [179] detected phospholipid hydroperoxides by mixing a colorless Fe(II)-thiocyanate solution with the eluent. The hydroperoxides oxidize the Fe(II) to Fe(III) resulting in a violet-colored Fe(III)-thiocyanate complex which absorbs at 505 nm. An increase in the absorbance at this wavelength indicates the presence of the complex, and indirectly, the presence of the phospholipid hydroperoxides in the eluent.

2.4.3 Interaction Mode: \( A + B \rightarrow A' + B' \)

The methods of indirect detection described here are similar to those employing a metal ion in the redox reaction except the postcolumn interaction component, B, for these methods is not a metal-ligand complex.

Katz et al. [180-182] developed a method for monitoring the elution of reducing agents, including organic acids and carbohydrates, based on the production of fluorescent Ce(III) ion in aqueous solution. The analytes undergo a redox reaction with Ce(IV) ion as they elute from the column, to produce the fluorescent Ce(III) ion. Detection of these analytes is achieved by monitoring the fluorescence of Ce(III) produced at 350nm when excited at 260nm. Another approach is based on producing the UV-vis absorbing or fluorescent compound to be detected via a redox reaction which is catalyzed by enzymes immobilized postcolumn [183-190]. The enzymes are covalently bound to aminopropyl-controlled pore glass (CPG) [183] or tresylate-poly(vinyl alcohol) beads [189] which are packed into PTFE tubing [183], or a stainless-steel column [189]. For example, the fluorescence of reduced nicotinamide-adenine dinucleotide (NADH) at 470nm, when
excited at 365nm, is commonly monitored to indicate the presence of the reducing analytes in the eluent. NADH is produced when the redox reaction between the reducing analytes and nicotinamide-adenine dinucleotide (NAD) is catalyzed by an immobilized enzyme. Bile acids have been analyzed by micro-HPLC coupled with a fluorescence detector using this approach to indirect detection [183,184]. The presence of the bile acids in the eluent is indicated by the fluorescence signal resulting from reduced nicotinamide-adenine dinucleotide (NADH) which is produced by the redox reaction between NAD and the bile acids catalyzed by hydroxysteroid dehydrogenase. The same approach has been applied to the detection of hydroxysteroids [185], glycerol [186], and branched-chain amino acids [187-189] with the use of appropriate enzymes. NADH instead of NAD has been employed for detecting oxidizing agents such as sucrose and fructose [190]. To provide a detectable signal for these sugars, an enzyme, sorbitol dehydrogenase, was used to catalyze the reduction of the ketones of sucrose and fructose and the oxidization of the fluorescent NADH to produce the non-fluorescent NAD. The decreased fluorescence intensity of the solution indicates the presence of these analytes as they elute from the column. An interesting feature of this approach to indirect detection is that although the redox reaction takes place postcolumn, NAD or NADH may be added to either the mobile phase [183,184,186,189,190] or the eluent [185,187,188]. One advantage of pre-mixing the NAD or NADH with the mobile phase is the elimination of noise due to pulsation introduced by the pump adding NAD or NADH postcolumn, thereby providing increased sensitivity [183]. However, adding NAD or NADH into the mobile phase may affect the chromatographic separation. Introducing the postcolumn interaction component in the mobile phase may not be practical if the chromatographic
conditions and the conditions required for the postcolumn redox reaction are so different. The same approach for indirect detection has also been used to detect hydrophilic organic peroxides [191]. In this case, an enzyme, horseradish peroxidase, immobilized postcolumn, was used to catalyze the oxidation of (p-hydroxyphenyl)acetic acid by the peroxides to produce a fluorescent biphenyl derivative.

A more complicated strategy for indirect detection, requiring the use of two immobilized enzymes, has been employed to produce a UV-vis-absorbing or fluorescent species. The purpose of the first enzyme is to oxidize the analytes to produce hydrogen peroxide. The second enzyme catalyzes the oxidation of the postcolumn interaction component by the hydrogen peroxide to form a UV-vis-absorbing or fluorescent species. An example demonstrating this approach is the determination of hypoxanthine and xanthine by reversed-phase HPLC coupled with an immobilized enzyme reactor and a fluorescence detector [192,193]. The postcolumn interaction component is p-hydroxyphenylacetic acid. Hypoxanthine and xanthine are oxidized in the presence of immobilized xanthine oxidase as they elute from the column to produce hydrogen peroxide. The resulting hydrogen peroxide reacts with p-hydroxyphenylacetic acid in the presence of the immobilized peroxidase to produce the highly fluorescent product 6,6'-dihydroxy-3,3'-biphenyldiacetate. The postcolumn interaction component, p-hydroxyphenylacetic acid, is mixed with the eluent solution after the hydrogen peroxide is produced. This method was also employed to detect sugars such as stachyose, raffinose, melibiose, and galactose [194]. The same approach has been employed to analyze urine samples containing 1,5-anhydro-D-glucitol [195]. The postcolumn interaction component used was the sodium salt of N-(carboxymethylaminocarbonyl)-
4,4'-bis(dimethylamino)-diphenylamine (also called DA-64). In this case, the hydrogen peroxide produced by the oxidization of the 1,5-anhydro-D-glucitol reacts with DA-64 to produce Bindscheidler's Green, which has a maximum absorbance at 727 nm.

2.4.4 Interaction Mode: \( A + B \rightarrow A-B^* \)

This approach to indirect detection is based on measuring a decrease or increase in the absorbance or fluorescence intensity of the postcolumn interaction component, \( B \), due to the presence of analyte \( A \). In Section 3.2, examples were presented describing the detection of several cyclodextrins due to the enhanced absorbance of UV-vis-absorbing compounds or the fluorescence intensity of fluorescent compounds in the presence of cyclodextrins [150-154]. In those cases, the detectable components were introduced in the mobile phase. For this approach to indirect detection of cyclodextrins, the detectable components are introduced postcolumn. Phenolphthalein and 2-p-toluidinyl-6-naphthalenesulfonate are examples of detectable components which have been introduced postcolumn for detecting the elution of cyclodextrins [153,196]. Proteins have also been detected indirectly by postcolumn introduction of the fluorescent compounds, either 2-p-toluidinyl-6-naphthalene sulfonate or 1-anilino-8-naphthalene sulfonate, after separation by size-exclusion chromatography [197]. Indirect detection for these proteins is based on monitoring an increase in the fluorescence intensity of the detectable component resulting from hydrophobic interactions with the proteins. Stalikas et al [198] developed a method for monitoring the elution of nitrite and nitrate after ion chromatographic separation. A decrease in the fluorescence intensity of tryptophan, the postcolumn interaction.
component, due to dynamic queching of the fluorescence of tryptophan by nitrite and nitrate ions in the eluent, is indicative of the presence of these ions.

2.5 Conclusions

As demonstrated by the many examples presented in this review, indirect photometric and fluorometric HPLC detection provides a reasonable alternative to direct detection for analytes which do not possess native chromophores or fluorophores. Many different types of interactions between the analyte and the detectable component have been exploited to provide different approaches to indirect detection for a wide range of analytes. While general approaches to indirect detection have been presented, a detailed review of the literature shows the choice of detectable component, mode of interaction, and specific experimental conditions will often need to be developed and adapted for specific applications.
CHAPTER 3

INDIRECT FLUORESCENCE DETECTION OF AMINO SUGARS WITH THE USE OF COPPER(II) COMPLEXES OF TRYPTOPHAN AND ITS ANALOGUES FOLLOWING LIQUID CHROMATOGRAPHIC SEPARATION

3.1 Introduction:

Amino sugars are biologically important natural products. They are found in various animal species, plants, seeds, fungi, and bacteria [199]. The amino sugars are also important constituents of the soil nitrogen pool [200]. D-glucosamine (GlcN) and D-galactosamine (GalN) are the most prevalent amino sugars in nature and are considered to play important roles in various biological phenomena [200]. D-mannosamine (ManN) is a component of the sialic acids which are widely distributed in nature [201]. These three amino sugars differ only in the orientation of the substituents at the C-2 (NH₂) and/or the C-4 (OH) positions of the molecule (Figure 3.1).

Separation and analysis of amino sugars, especially these three amino sugars (D-glucosamine, D-galactosamine, D-mannosamine), have been of interest for many years [201]. Traditional methods of analysis include separation and detection by gas chromatography or liquid chromatography when coupled with suitable detectors. The low volatility of most carbohydrates requires derivatization of the hydroxyl, amino or carboxyl groups associated with this class of compounds prior to analysis by GC (gas chromatography). The derivatized amino sugars have been analyzed by GC coupled with
Figure 3.1. Molecular structures of the three amino sugars

D-Glucosamine    D-Galactosamine    D-Mannosamine
a nitrogen-specific flame thermionic detector (FTD) or a flame ionization detector (FID) [203-211].

Liquid chromatography (LC) has become the method of choice for most carbohydrate analyses. However, the lack of chromophores or fluorophores in these compounds makes their detection following LC separation challenging. The relatively insensitive and non-selective method of monitoring the refractive index (RI) of the eluent is commonly employed for HPLC detection [212,213]. Direct UV photometric detection of the amino sugars at 210 nm [214] is subject to interferences from the multitude of other compounds which absorb in this spectral region. Detection based on the UV absorption of amino sugar-copper complexes at 254 nm has been reported. This approach to detection has been accomplished by eluting the amino sugars from copper-loaded ion exchange resins with aqueous ammonia [215]. Indirect detection of glucosamine, galactosamine, and N-acetylgalactosamine, without derivatization, has been reported by adding phenol to the mobile phase [216]. Phenol was used not only to elute the analytes but also to monitor the presence of the analytes in the eluent. Photometric or fluorometric detection after pre-column or post-column derivatization of the amino sugars has been widely studied [217-228]. High-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD), which does not require derivation, has become the method of choice for the analysis of amino sugars [229-235]. Another direct detection method has been developed which uses electrospray ionization-ion mobility spectrometry to detect the amino sugars following microbore HPLC separation [236]. The differentiation and quantification of GlcN, GalN, and ManN have been reported using MS-MS after derivatizing the amino sugars with
[Co(diaminopropane)$_2$Cl$_2$]Cl [237]. Nagaveni et al. recently reported a mass spectrometric method for differentiating these three amino sugars without derivatization [238]. This method involves the decomposition of proton-bound heterodimers formed between the amino sugars and N-acetylated hexosamines or amino acids using electrospray ionization spectrometry.

As described previously in Chapter 1, a simple, indirect HPLC detection method, based on the recovery of L-tryptophan (L-Trp) fluorescence, has been applied to the detection of amino-containing compounds such as the aliphatic biogenic polyamines, amino acids, and aminoglycoside antibiotics [3-5]. Here, the application of this indirect fluorescence detection scheme using a copper-tryptophan complex was investigated for three amino sugars, GlcN, GluN, and ManN following their separation by HPLC. Studies were also undertaken to evaluate two tryptophan analogues, 5-hydroxy-L-tryptophan (5-HTP) and DL-5-methoxytryptophan (5-MTP) (Figure 3.2), as alternative fluorescent reagents.

3.2 Experimental

3.2.1 Reagents

D-Glucosamine hydrochloride (GlcN) (minimum purity, 99%), D-mannosamine hydrochloride (ManN) (minimum purity 99.4%), L-tryptophan (L-Trp) (minimum purity, 98%), and 5-hydroxy-L-tryptophan (5-HTP) (minimum purity, 99%) were purchased from Sigma (St. Louis, MO, USA). D-Galactosamine hydrochloride (GalN) (minimum purity, 98%) and DL-5-methoxytryptophan (5-MTP) (minimum purity, 95%) were purchased from Aldrich (Milwaukee, WI, USA). Reagent-grade copper sulfate was
Figure 3.2. Molecular structures of tryptophan and its analogue compounds: (a) L-
Tryptophan (L-Trp), (b) 5-Hydroxy-L-tryptophan (5-HTP), (c) DL-5-
Methoxytryptophan (5-MTP)
purchased from Fisher Scientific (Pittsburgh, PA, USA). Reagent-grade sodium borate
(Na₂B₄O₇·10H₂O) was purchased from J.T. Baker (Phillipsburg, NJ, USA). Certified
dihydroethylenediaminetetraacetic acid (EDTA) (99.8%) was purchased from Fisher Scientific
(Fair Lawn, NJ, USA). The copper complexes of tryptophan and its analogues were
made by adding copper sulfate and the corresponding ligand at a 1:2 (mole/mole) ratio.
The deionized water used in the preparation of the standard solutions and mobile phases
was obtained from a Milli-Q water system (Millipore, Bedford, MA, USA). All mobile
phases and reagent solutions were vacuum filtered through a 0.45 μm nylon filter
(Whatman, Hillboro, OR, USA) prior to use. The pHs of the mobile phases and other
solutions were adjusted using dilute solutions prepared from certified sodium hydroxide
solution (50% w/w) and reagent-grade hydrochloric acid (36.5% by weight) purchased
from Fisher Scientific (Fair Lawn, NJ, USA).

3.2.2 Apparatus

A Cary Eclipse fluorescence spectrophotometer (Varian, Palo Alto, CA, USA)
was used for the static fluorescence studies. The chromatographic separations were
performed using a Nicolet LC 9560 HPLC system (Madison, WI, USA) equipped with a
Rheodyne Model 7125 injector (Cotati, CA, USA) having a 10μl injection loop.
Separation of the amino sugars was achieved with the use of a Hamilton PRP-X100
column (250×4.1 mm, 10 μm, P/N 79433; Reno, NV, USA) containing a polymer-based
strong anion-exchanger. A Hitachi 655A-11 LC pump (Tokyo, Japan) was employed to
deliver the postcolumn reagent via a mixing tee. A Varian flow control dampener (Palo
Alto, CA, USA) was placed between the postcolumn reagent pump and the mixing tee to
improve flow stability. Chromatographic detection was provided by a Kratos Spectroflow 980 fluorescence detector (Ramsey, NJ, USA) fitted with a 10μl flow cell. The excitation wavelength of the detector was set at 280nm. The emission wavelengths detected were selected using longpass filters (320nm or 350nm).

3.3 Results and Discussions

3.3.1 Emission Spectra of Tryptophan and Its Analogues

The emission spectra obtained with an excitation wavelength of 280 nm for L-Trp, 5-HTP, and 5-MTP solutions having pHs of 9.0, 8.6 and 8.4, respectively, are given in Figure 3.3. The wavelengths of maximum emission for L-Trp, 5-HTP, and 5-MTP occur at 360 nm, 338 nm, and 337 nm, respectively. Based on our observations, the wavelength of maximum emission varies for these compounds by approximately ±3 nm for solutions ranging in pH from 2.0 to 12.0, which is small compared to the overall breadth of the emission bands for these compounds under the conditions studied. Static fluorescence measurements were made using an excitation wavelength set at 280 nm. The emission wavelength was set at 360 nm for L-Trp and 340 nm for 5-HTP and 5-MTP.

3.3.2 Evaluation of Fluorescence Quenching of Tryptophan and Its Analogues by Copper ion as a Function of Solution pH

The approach to indirect detection of analytes described here is based on the recovery of the fluorescence intensity of tryptophan, or one of its analogues, which is the difference between the fluorescence intensity of the compound while complexed to
Figure 3.3. Emission spectra of (a) 10 μM L-Trp in 32mM pH 9.0 sodium borate solution, (b) 8 μM 5-HTP in 32mM pH 8.6 sodium borate solution, (c) 10 μM 5-MTP in 32mM pH 8.4 sodium borate solution, excitation wavelength = 280 nm

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
copper ion and the fluorescence intensity of the compound which is unbound in solution. Experimentally, the pH of the solution is chosen such that the difference in the fluorescence intensity between the bound and unbound species will be as large as possible while minimizing any residual background fluorescence due to the copper complexes of tryptophan or its analogues.

The fluorescence intensities at 360nm of a solution containing L-Trp and a solution containing Cu(L-Trp)$_2$ as a function of pH, ranging from 2.0 to 12.0, are presented in Figure 3.4. Previous studies [3-5] established the working pH for detection when using tryptophan is between 8.5 and 9.0 by comparing the chromatographic peak intensities obtained for solutions having different pH values. The working pH range used for detection was also determined by choosing the pH which provided the maximum difference between the fluorescence intensities for the free and complexed L-Trp while providing the minimum fluorescence background due to Cu(L-Trp)$_2$. It was determined that the minimum fluorescence background occurs at a pH of approximately 8.5, while the difference between the fluorescence intensities of the L-Trp and Cu(L-Trp)$_2$ increases from pH 8.5 to 11.0. While the fluorescence signal may increase from pH 8.5 to 11.0, assuming the same amount of L-Trp is released from a Cu(L-Trp)$_2$ complex, using a higher pH solution may not result in improved analyte detection. One problem may be an increased fluorescence background. The fluorescence background may be so large that it is out of the range of the detector and an increase in the level of the background may also affect the noise level. Taking these factors into account, the working pH for the detection of amino sugars with the use of Cu(L-Trp)$_2$ as the postcolumn reagent was chosen to be approximately 9.0.
Figure 3.4. Fluorescence intensity of 4 μM L-Trp and 2 μM Cu(L-Trp)$_2$ as a function of pH, excitation wavelength = 280nm, emission wavelength = 360nm.
Plots of the fluorescence intensity at 340nm for solutions containing 5-HTP and Cu(5-HTP)$_2$ as a function of pH ranging from 2.0 to 12.0 are presented in Figure 3.5. The plots show the fluorescence of 5-HTP is quenched efficiently by copper ion at a pH of approximately 9.0. At this pH, a minimum in the fluorescence intensity of the Cu(5-HTP)$_2$ complex is observed. However, the optimal pH for analyte detection is approximately 8.5 which is where the difference in the fluorescence intensity of 5-HTP and that of Cu(5-HTP)$_2$ is greatest. Compared to the fluorescence profiles of L-Trp and Cu(L-Trp)$_2$, shown in Figure 3.4, the difference in the fluorescence intensities between 5-HTP and Cu(5-HTP)$_2$ at pH 8.5 is larger than the difference between L-Trp and Cu(L-Trp)$_2$ at pH 9.0. These data indicate 5-HTP might be a suitable alternative to L-Trp for use in this indirect fluorescence detection method, possibly providing enhanced detection sensitivity.

The data plotted in Figure 3.6 show the fluorescence intensities at 340nm of 5-MTP and Cu(5-MTP)$_2$ as a function of solution pH ranging from 2.0 to 12.0. Efficient fluorescence quenching of 5-MTP by copper ion occurred at a pH of approximately 8.4, where the difference in the fluorescence intensity of 5-MTP and that of Cu(5-MTP)$_2$ is greatest. This difference is also larger than was observed between L-Trp and Cu(L-Trp)$_2$ at pH 9.0 (Figure 3.4). These data suggest that a solution containing Cu(5-MTP)$_2$ at a pH of 8.4 might also be suitable for this approach to indirect detection of the amino sugars and has potential for providing a stronger chromatographic signal than would be obtained using L-Trp.
Figure 3.5. Fluorescence intensity of 4 μM 5-HTP and 2 μM Cu(5-HTP)$_2$ as a function of pH, excitation wavelength = 280nm, emission wavelength = 340nm
Figure 3.6. Fluorescence intensity of 4 μM 5-MTP and 2 μM Cu(5-MTP)$_2$ as a function of pH, excitation wavelength = 280nm, emission wavelength = 340nm
3.3.3 Evaluation of the Stability of Potential Postcolumn Reagents in a Basic Sodium Borate Buffer Solution

3.3.3.1 Evaluation of the Stability of Cu(5-HTP)₂ in a 32 mM, pH 8.6, Sodium Borate Solution

The autoxidation of 5-HTP in strongly basic solution has been reported to result in the formation of a free-radical semiquinone imine [239]. Studies of the electrochemical and enzymatic oxidation of 5-HTP indicate the compound is relatively easily oxidized at physiological pH [240]. The presence of Cu²⁺ ion in the Cu(5-HTP)₂ postcolumn reagent may also act either as an oxidizing agent or a catalytic agent for the oxidation of this tryptophan analog. It is reasonable, therefore, to be concerned with the stability of 5-HTP under the solution conditions utilized here.

The stability of Cu(5-HTP)₂ in sodium borate buffer solution at pH 8.6 was evaluated by taking aliquots of a Cu(5-HTP)₂ stock solution over time. EDTA at a concentration 1.5 time higher than that of Cu²⁺ was added to each aliquot to release the 5-HTP from the Cu(5-HTP)₂ complex. The concentration of the free 5-HTP in solution was monitored by measuring the fluorescence intensity of the solution. A sample of 5-HTP, initially mixed with Cu²⁺ and EDTA, was also prepared for comparison.

It was observed that the fluorescence intensity due to 5-HTP decreased over time for the aliquots taken from the Cu(5-HTP)₂ stock solution and treated with EDTA to release the 5-HTP, as shown in Figure 3.7. Assuming the EDTA is capable of releasing whatever species are complexed with the copper ion, these data suggest that 5-HTP in the presence of Cu²⁺ in basic sodium borate buffer solution decomposes to form either a different species which does not fluorescence or which has a lower fluorescence intensity.
Figure 3.7. Stability study: fluorescence intensity of 8μM Cu(5-HTP)$_2$ in a pH of 8.6 sodium borate buffer solution as a function of time, excitation wavelength = 280 nm, emission wavelength = 360 nm.
than 5-HT P. The decrease in the fluorescence intensity was not due to the shift of the maximum emission wavelength. This was proven by looking at the emission spectra of the stock solution of Cu(5-HTP)₂ treated with EDTA 75 hours after preparation, which are presented in Figure 3.8. Since the fluorescence intensity for 5-HTP in the comparative sample, in which the Cu²⁺ was chelated by EDTA, proved to be stable over the same time frame, it is reasonable to suggest that free Cu²⁺ induced the decomposition of 5-HTP in the basic sodium borate solution. It is also noted, a sodium borate solution with a pH of 8.6 containing Cu(5-HTP)₂, which was originally clear, turned brown overnight when held at room temperature, proving the occurrence of decomposition under the conditions studied. Due to the observed instability of Cu(5-HTP)₂ in sodium borate buffer solution at the pH under consideration, it was eliminated from consideration as a new postcolumn reagent.

### 3.3.3.2 Evaluation of the Stability of Cu(5-MTP)₂ in a 32 mM, pH 8.4 Sodium Borate Solution

The presence of a methoxy group at the C-5 position of 5-MTP, instead of a hydroxyl group as in 5-HTP, is expected to improve the stability of this compound in basic solution. To test this hypothesis, a study similar to the one described previously was also performed to evaluate the stability of Cu(5-MTP)₂ in a pH of 8.4 sodium borate solution. The fluorescence intensity of the 5-MTP released from the Cu(5-MTP)₂ complex by adding EDTA at different times was measured. No significant trend was observed in the fluorescence intensity vs. time profile, indicating that Cu(5-MTP)₂ is
Figure 3.8. Emission spectra of 5-HTP released from 8μM Cu(5-HTP)$_2$ in a pH of 8.6 sodium borate solution by adding EDTA after (a) 3 hours, (b) 75 hours, excitation wavelength = 280 nm.
stable in a room temperature, pH 8.4, sodium borate solution for at least 80 hours. Based on the results of these studies, Cu(5-MTP)₂ was evaluated as a potential postcolumn reagent for the detection of the amino sugars.

3.3.4. Chromatographic Detection of the Amino Sugars Using two Different Postcolumn Reagents

3.3.4.1. Chromatographic Detection of the Amino Sugars via Postcolumn Addition of Cu(L-Trp)₂ in Sodium Borate Solution, pH=9.0

An example of the detection of the three amino sugars after chromatographic separation using Cu(L-Trp)₂ in 40 mM sodium borate at pH 9.0 as the postcolumn reagent is presented Figure 3.9. As had been used in previous studies [3-5], a 350 nm longpass filter was utilized for selecting the L-Trp fluorescence signal. The linear dynamic range for GlcN, GalN and ManN was determined by injecting six standard solutions in triplicate with amounts from 0.5 nmol to 10 nmol under the chromatographic conditions given in Figure 3.9(1). The results are presented in Table 3.1. The detection limits for these three amino sugars were determined to be at the nmol injected level. Lower detection limits in the pmol range for diamines, polyamines and amino acids have been achieved using this indirect fluorescence detection method [3,5]. Since indirect detection of these analytes is based on the fluorescence of L-Trp released from Cu(L-Trp)₂ postcolumn reagent by the analytes, the affinities of the analytes for Cu²⁺ is one of the important factors affecting the amount of L-Trp released by the analytes. Previous studies [241] have shown that GlcN, GalN and ManN act as bidentate ligands with the amino group as the main donor towards Cu²⁺, and one of the hydroxyl groups as the
Figure 3.9. Fluorescence detection of amino sugars with two different longpass glass filters. Separation was achieved with the use of Hamilton anion exchange PRP-X100. Mobile phase: 1.6mM sodium borate, pH=9.0, 1ml/min. Postcolumn reagent: 2x10^{-5} M Cu(L-Trp)_2 in 40mM sodium borate at pH=9.0, 1ml/min. Detector sensitivity: 0.005μAFS. Chromatogram 1: longpass glass filter λ_{cutoff} = 350nm. Chromatogram 2: longpass glass filter λ_{cutoff} = 320nm. Peak (a) D-Glucosamine (2.5nmol); (b) D-Galactosamine (2.5nmol); (c) D-Mannosamine (2.5nmol). Excitation wavelength = 280 nm.
Table 3.1. Statistical summary for detection of three amino sugars following chromatographic separation, as represented in Figure 3.9(1)

<table>
<thead>
<tr>
<th>Amino sugar</th>
<th>Linear range (nmol)</th>
<th>Regression equation(^a)</th>
<th>(r^b)</th>
<th>DL (^c) (nmol)</th>
<th>RSD (^d) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcN</td>
<td>0.5 - 10</td>
<td>(H = 2.6817C + 0.2583)</td>
<td>0.9993</td>
<td>0.15</td>
<td>1.4</td>
</tr>
<tr>
<td>GalN</td>
<td>0.5 - 10</td>
<td>(H = 2.7439C + 0.2685)</td>
<td>0.9992</td>
<td>0.15</td>
<td>2.1</td>
</tr>
<tr>
<td>ManN</td>
<td>0.5 - 10</td>
<td>(H = 1.3704C - 0.2074)</td>
<td>0.9999</td>
<td>0.30</td>
<td>3.1</td>
</tr>
</tbody>
</table>

\(^a\) \(H\), peak height (mV), \(C\), analyte injected (nmol)
\(^b\) Correlation coefficient
\(^c\) Detection limit (S/N=3)
\(^d\) Relative standard deviation (n=3, for 2.5nmol GlcN, 2.5nmol GalN, and 2.5nmol ManN injected respectively)
second donor center. The overall stability constants \((\log \beta_2)\) of these amino sugars for 
\(\text{Cu}^{2+}\) range from 8.76 to 9.68, which are several orders lower than the overall stability 
constants of copper complexes of polyamines \((\log \beta_2 = 16.0-18.9)\) [242] and amino acids 
\((\log \beta_2 = 13.7-15.2)\) [243]. Additionally, polyol compounds, including carbohydrates, 
have been known to complex with borate ion, via the hydroxyl groups, to form negatively 
charged borate complexes which allow the separation of these compounds with anion 
exchange chromatography [244]. The possible complexation of the hydroxyl groups in the 
amino sugars with borate ion may decrease the affinity of one of the hydroxyl groups 
for \(\text{Cu}^{2+}\) and block the complexation of the amino groups with \(\text{Cu}^{2+}\). This would result in 
a smaller stability constants for these amino sugars to \(\text{Cu}^{2+}\) and a higher detection limit. 
The complicate equilibria for the postcolumn interaction component in the basic buffer 
solution and the kinetics of the competition reactions between the analytes and the 
detectable compound, L-Trp or 5-MTP, with copper ion may also contribute to the 
different detection limits obtained using this indirect fluorescence detection method for 
different analytes. Future studies may provide more information allowing a better 
understanding of the processes involved in this approach to indirect fluorescence 
detection. The detection limits based on the indirect detection method developed in this 
study are within the range of detection limits reported for these compounds with many 
other detection methods using either precolumn or postcolumn derivatization 
[217,220,221], which is in the range of 0.14-3.4 nmol injected, though derivatization 
approaches may choose a labeling reagent providing lower detection limits in the pmol 
range [218,224-226]. Detection limits as low as pmol can be also achieved when using
pulsed amperometric detection [230,231,233-235] or electrospray-ion mobility
spectrometry [235].

3.3.4.2. Chromatographic Detection Using Cu(5-MTP)₂ in Sodium Borate Solution
at pH 8.4

As indicated previously, the maximum emission wavelengths of L-Trp and 5-
MTP are 360 nm and 340 nm, respectively. While earlier studies [3-5] have employed a
350 nm longpass filter for selecting the fluorescence signal of L-Trp, using the same filter
for measuring the fluorescence signal for 5-MTP would result in filtering out the
maximum emission wavelength for this compound. For this reason, a 320 nm longpass
filter was chosen to select the emission intensity for 5-MTP. As expected, since a lower
wavelength cutoff filter will allow more light to reach the detector, the chromatographic
signal measured with the 320 nm longpass filter was greater than observed when using
the 350 nm filter. This is true with either L-Trp or 5-MTP as the fluorescent reagent.
An example of the detection of the three amino sugars using Cu(5-MTP)₂ in 40 mM
sodium borate at pH 8.4 as the postcolumn reagent after chromatographic separation is
presented in Figure 3.10. The intensity of the chromatographic signal was found to be
nearly equivalent to that obtained when using Cu(L-Trp)₂ in a pH of 9.0 sodium borate
solution and utilizing the 320 nm longpass glass filter (Figure 3.9(2)). As indicated by
equation (1.1), a higher fluorescence efficiency of the fluorescent agent may result in a
larger chromatographic signal for the analytes. Based on the results obtained for the
quenching of 5-MTP (Figure 3.6), which showed the difference in the fluorescence
intensities at pH 8.4 between 5-MTP and Cu(5-MTP)₂ was larger than the difference in
Figure 3.10. Detections of amino sugars by new postcolumn reagent. Separation was achieved with the use of Hamilton anion exchange PRP-X100. Mobile phase: 1.6mM sodium borate, pH=9.0, 1ml/min. Postcolumn reagents: $2 \times 10^{-5}$ M Cu(5-MTP)$_2$ in 40mM sodium borate at pH=8.4, 1ml/min. Detector sensitivity: 0.005μAFS. Longpass glass filter $\lambda_{\text{cutoff}} = 320$nm. Peak (a) D-Glucosamine (2.5nmol); (b) D-Galactosamine (2.5nmol); (c) D-Mannosamine (2.5nmol). Excitation wavelength = 280 nm.
the fluorescence intensities at pH 9.0 between L-Trp and Cu(L-Trp)₂ (Figure 3.4), it is reasonable to expect that using Cu(5-MTP)₂ postcolumn reagent should result in a more intense signal than would be observed using Cu(L-Trp)₂ as the postcolumn reagent. The reason why the chromatographic detection results are not consistent with what was predicted by the static fluorescence studies could be in part that the optical arrangement employed in the fluorescence detector is different from that used for the static fluorescence study. In the static fluorescence studies, the fluorescence intensity of different tryptophan analogues was measured at their maximum emission wavelengths, while the HPLC detector employed in this study responded to the total intensity of all the lights that pass through the longpass filter.

3.4 Conclusions

Indirect fluorescence detection based on fluorescence recovery of L-tryptophan was successfully applied to the detection of amino sugars following chromatographic separation. Two tryptophan analogue compounds, 5-hydroxy-L-tryptophan (5-HTP) and DL-5-methoxytryptophan (5-MTP) were evaluated as potential alternatives to L-tryptophan. The successful chromatographic detection of amino sugars employing the new postcolumn reagent Cu(5-MTP)₂ in pH 8.4 buffer showed that 5-MTP could be an alternative to L-Trp.

Detection limits obtained for the three amino sugars evaluated are in the range of 0.15-0.30 nmol injected. Smaller affinities of these three amino sugars for copper ion and the possible complexation of borate ion with the amino sugars may contribute to the
higher detection limits obtained using this indirect fluorescence detection, compared to
the detection limits previously obtained for diamines, polyamines, and amino acids.
Chapter 4

LIQUID CHROMATOGRAPHIC DETERMINATION OF GLUCOSAMINE IN COMMERCIAL DIETARY SUPPLEMENTS BY INDIRECT FLUORESCENCE DETECTION

4.1 Introduction

Glucosamine-containing products are used widely as dietary supplements to relieve the symptoms of osteoarthritis [245,246]. Glucosamine has been reported to decrease joint pain, improve joint strength, enhance joint function, and to rebuild and maintain connective tissue [245,246]. Several liquid chromatographic methods have been studied for the determination of glucosamine in raw materials and dietary supplements [247-251]. The lack of a suitable chromophore or fluorophore in the glucosamine molecule makes optical detection challenging. Glucosamine can be detected after elution by measuring changes in the refractive index [247] or UV absorbance at 195 nm [248,249]. Often, however, glucosamine is derivatized to incorporate a suitable chromophore or fluorophore before performing the liquid chromatographic separation to improve analyte detectability [225,249-251]. Possible derivatization reagents include o-phthalaldehyde (OPA)/3-mercaptopropionic acid (MPA) [225,249], phenylisothiocyanate (PITC) [250,251], and N-(9-fluorenyl-methoxycarbonyloxy) succinimide (FMOC-Su) [252].

As described in Chapter 3, an indirect detection method for amino sugars including glucosamine based on measuring the fluorescence signal of either L-tryptophan
(L-Trp) or DL-5-Methoxytryptophan (5-MTP) has been shown to be effective. Here, the use of this method for measuring the amount of glucosamine in several formulations of commercially available dietary supplements is explored. The use of L-Trp and 5-MTP, which are added postcolumn as their copper complexes, is compared for the indirect fluorescence detection of glucosamine following liquid chromatographic separation. We choose to compare the results obtained using this indirect fluorescence detection method to those obtained for pre-column derivatization of the glucosamine with PITC due to questions of the stability of glucosamine-OPA/MAP derivative which becomes evident as part of this study.

4.2 Experimental

4.2.1 Materials and Reagents

The glucosamine hydrochloride (GlcN·HCl) (USP reference standard) used as a standard was purchased from U.S. Pharmacopeia (Rockville, MD, USA). L-tryptophan (L-Trp) (minimum purity, 98%), phenylisothiocyanate (PITC) (minimum purity, 99%), o-phthalaldehyde (OPA) (minimum purity 99%), and 3-mercaptopropionic acid (MPA) (minimum purity, 99%) were purchased from Sigma (St. Louis, MO, USA). DL-5-methoxytryptophan (5-MTP) (minimum purity, 95%) was purchased from Aldrich (Milwaukee, WI, USA). Reagent-grade copper sulfate and HPLC grade methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Reagent-grade sodium borate (Na₂B₄O₇·10H₂O) and potassium phosphate monobasic were purchased from J.T. Baker (Phillipsburg, NJ, USA). Reagent-grade glacial acetic acid was purchased from VWR Scientific (San Francisco, CA). The copper complexes of L-Trp and 5-MTP were
prepared by adding aqueous solutions of copper sulfate and the corresponding ligand at a 1:2 (mole/mole) ratio. The deionized water used to prepare the solutions was obtained from a Milli-Q water system (Millipore, Bedford, MA, USA). All mobile phases were vacuum filtered through a 0.45 μm nylon filter (Whatman, Hillboro, OR, USA) prior to use. The pH of the mobile phases and other solutions was adjusted using dilute solutions prepared from certified sodium hydroxide solution (50%, w/w) and reagent-grade hydrochloric acid (36.5% by weight) purchased from Fisher Scientific (Fair Lawn, NJ, USA).

4.2.2 Dietary Supplement Samples

Fourteen commercial glucosamine-containing samples (A-N) were analyzed. Two commercial chondroitin-containing samples (P and Q), which do not contain glucosamine, were used to evaluate potential interference by chondroitin or other compounds since many commercially available products are mixtures of glucosamine and chondroitin. The data presented are the average of triplicate injections of solutions prepared from each sample.

4.2.3 Apparatus

The liquid chromatography system consisted of a Varian 9010 solvent delivery system (Palo Alto, CA, USA) and a Rheodyne Model 7125 injector (Cotati, CA, USA) with a 10μL injection loop. A Kratos Spectroflow 980 fluorescence detector was employed for the indirect fluorescence detection method. A Waters 484 tunable absorbance detector (Milford, MA, USA) was employed for the pre-column.
derivatization method. A Hamilton PRP-X100 column (250mm × 4.1mm, 10μm, Reno, NV, USA) containing a polymer-based strong anion-exchanger was used for the indirect fluorescence detection method, and a Waters Xterra MS C18 column (50mm × 4.6mm, 3.5μm) (Milford, MA, USA) was used for the pre-column derivatization method. A Hitachi 655A-11 liquid chromatography pump (Tokyo, Japan) delivered the postcolumn interaction components via a mixing tee for the indirect fluorescence detection method. A Varian flow control dampener (Palo Alto, CA, USA) was placed between the postcolumn solution pump and the mixing tee to improve flow stability. The stability of the reaction product of glucosamine with the derivatization reagent, o-phthalaldehyde (OPA)/3-mercaptopyrrolidione (MPA) was investigated with the use of a Varian Cary 50 Bio UV-Visible Spectrophotometer (Palo Alto, CA, USA).

4.2.4. Preparation of Glucosamine Standard Solutions

USP glucosamine hydrochloride was accurately weighed to prepare standard stock solutions in water at concentrations of 0.3065mg/ml and 1.063mg/ml. Glucosamine hydrochloride standard solutions were prepared from the stock solutions by serial dilution. The concentrations of the standards used for comparing the content of glucosamine in four commercial samples (A-D) with the use of L-Trp and 5-MTP were: 30.65μg/ml, 61.30μg/ml, 91.95μg/ml, 122.6μg/ml, and 153.2μg/ml. The concentrations of the standards used for comparing the content of glucosamine in the other ten commercial samples (E-N) were: 53.15μg/ml, 106.3μg/ml, 159.4μg/ml, 212.6μg/ml, and 265.8μg/ml.
4.2.5 Preparation of Glucosamine-Containing Sample Solutions

4.2.5.1 Solid Samples (Tablet, Capsule, and Powder)

For samples in the form of tablet or capsule, between three and six tablets or capsules were transferred, as is, to a beaker filled with 300ml of deionized water and dissolved with stirring. For powder samples, an appropriate amount of the powder was transferred, as is, to a beaker filled with 300ml of deionized water and dissolved with stirring. The solution was then sonicated for approximately 30 minutes. The solution was transferred to a 500ml volumetric flask and filled to the mark with deionized water resulting in a stock solution containing approximately 3mg/ml glucosamine. An aliquot of this stock solution was filtered through a 0.45μm syringe filter and diluted to make a solution for analysis containing approximately 0.15mg/ml glucosamine.

4.2.5.2 Liquid Sample

An appropriate liquid sample was transferred to a 25ml volumetric flask resulting in a solution containing approximately 0.15mg/ml glucosamine. This solution was filtered through a 0.45μm syringe filter.

4.2.6 Procedure for Reaction of Glucosamine with OPA/MPA

The procedure followed to prepare the glucosamine-OPA/MPA derivative was adapted from Refs.5 and 6. 320μl of a solution of OPA in methanol (10.798mg/ml), 100μl of MPA, 800μl of the aqueous solution of glucosamine hydrochloride (1.023mg/ml), and 9580μl of borate buffer (80mM, pH=9.5) was mixed at ambient temperature. To monitor the stability of the glucosamine-OPA/MPA derivative, 600μl of
this solution was mixed with 2400μl of borate buffer (80mM, pH=9.5) and transferred to a UV-vis spectrophotometer cell.

4.2.7 Procedure for Pre-column Derivatization Reaction of Glucosamine by PITC

The procedure followed was adapted from the procedures of Liang et al [250] and Ji et al [251]. 1ml of the solution containing approximately 0.15mg/ml glucosamine, 1ml of 0.3M phosphate buffer with a pH of 8.3, and 1ml of 5% (v/v) phenylisothiocyanate in methanol were transferred to a 20ml glass vial and mixed. The glass vial was capped tightly and placed in a water bath held at 60°C. During the derivatization reaction, the glass vial was removed from the water bath and vortexed for 1 minute every 20 minutes. After 120 minutes, the glass vial was removed from the water bath, put into an ice bath for 10 minutes and then restored to room temperature. The solution was filtered through a 0.45μm syringe filter. A blank for the pre-column derivation reaction was prepared by following the procedure given as above except transferring 1ml of water instead of 1ml of the glucosamine solution.

4.2.8 Chromatographic Conditions

4.2.8.1 Indirect Fluorescence Detection Method

Mobile phase: 1.6mM sodium borate, pH 9.0; flow rate: 1ml/min

Postcolumn interaction component: 2x10⁻⁵ M Cu(L-Trp)₂ in 40mM sodium borate at pH 9.0, or 2x10⁻⁵ M Cu(5-MTP)₂ in 40mM sodium borate at pH 8.4; flow rate: 1ml/min

Column: strong anion-exchange column, PRP-X100 (250mm × 4.1mm, 10μm)
Detector: Spectroflow 980 fluorescence detector, excitation wavelength = 280nm; emission wavelength selection was provided by a longpass glass filter (λcutoff = 320nm or 340nm).

4.2.8.2 Precolumn Derivatization Method
Mobile phase: methanol/water /acetic acid = 10.00/89.96/0.04 (v/v/v) [250]; flow rate: 1ml/min
Column: Reversed phase, Waters Xterra MS C18 (50mm × 4.6mm, 3.5μm)
Detector: Waters 484 tunable absorbance detector, λ=254nm

4.2.9 Calculations
The content of glucosamine in the commercial samples in the form of glucosamine hydrochloride is calculated as follows:

\[
GlcN \cdot HCl = \frac{h - b}{a} \times D \tag{4.1}
\]

where \(GlcN \cdot HCl\), in mg, is the content of glucosamine in the commercial samples in the form of glucosamine hydrochloride, \(h\) is the peak height in the sample chromatogram, \(b\) is the y-intercept of the calibration curve, \(a\) is the slope of the calibration curve, and \(D\) is the dilution factor.

For commercial samples, such as samples A, F and N, which state the content of glucosamine is in the form of glucosamine base, the content of glucosamine is calculated as follows:

\[
GlcN = GlcN \cdot HCl \times \frac{179.17}{215.63} \tag{4.2}
\]
where \( \text{GlcN} \), in mg, is the content of glucosamine in the form of glucosamine base, \( \text{GlcN-HCl} \) is the content of glucosamine in the form of glucosamine hydrochloride, 179.17 is the formula mass (in amu) of glucosamine base, and 215.63 is the formula mass (in amu) of glucosamine hydrochloride.

For commercial samples, such as samples C, D, G, H, I, and L, which state the content of glucosamine is in the form of glucosamine sulfate, the content of glucosamine is calculated as follows:

\[
(GlcN)_2 \cdot H_2SO_4 = \text{GlcN} \cdot HCl \times \frac{456.42}{431.26} \tag{4.3}
\]

where \((GlcN)_2 \cdot H_2SO_4\), in mg, is the content of glucosamine in the form of glucosamine sulfate, \( \text{GlcN-HCl} \) is the content of glucosamine in the form of glucosamine hydrochloride, 456.42 is the formula mass (in amu) of glucosamine sulfate, and 431.26 is twice of the formula mass (in amu) of glucosamine hydrochloride.

For commercial sample E, which states the content of glucosamine is in the form of glucosamine sulfate potassium chloride, the content of glucosamine in the form of glucosamine sulfate potassium chloride salt is calculated as follows:

\[
(GlcN)_2 \cdot H_2SO_4 \cdot 2KCl = \text{GlcN} \cdot HCl \times \frac{605.52}{431.26} \tag{4.4}
\]

where \((GlcN)_2 \cdot H_2SO_4 \cdot 2KCl\), in mg, is the content of glucosamine in the form of glucosamine sulfate potassium chloride salt, \( \text{GlcN-HCl} \) is the content of glucosamine in the form of glucosamine hydrochloride, 605.52 is the formula mass (in amu) of glucosamine sulfate potassium chloride, and 431.26 is twice the formula mass (in amu) of glucosamine hydrochloride.
4.3 Results and Discussion

4.3.1 Separation of Glucosamine from Other Compounds

Several ingredients are found in the formulation of glucosamine-containing samples. Chondroitin is one of the ingredients commonly found in these formulations. Potential interferences in the quantitative analysis of glucosamine due to the presence of these ingredients must be considered. Therefore, chondroitin-containing samples were mixed with the glucosamine samples before performing the chromatographic separation. Figure 4.1 shows the separation of glucosamine from the unknown compounds in the chondroitin-containing samples under the chromatographic conditions employed here with the use of indirect fluorescence detection. A higher than usual concentration of chondroitin-containing sample solution was used to enhance the detection signal of the unknown compounds which may interfere with glucosamine.

4.3.2 Comparison of Using Either of Two Postcolumn Interaction Compounds, Cu(L-Trp)₂ and Cu(5-MTP)₂ for Indirect Fluorescence Detection of Glucosamine

The study in Chapter 3 demonstrated the successful application of both Cu(L-Trp)₂ and Cu(5-MTP)₂ for indirect fluorescence detection of glucosamine, galactosamine, and mannosamine following liquid chromatographic separation. Here, a study was conducted to compare the results obtained by using these two postcolumn interaction compounds for the determination of glucosamine in the commercial samples. The purpose of this study was to evaluate the use of different postcolumn interaction compounds for quantitative analysis of glucosamine. The calibration curves produced

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Figure 4.1. Separation of glucosamine from other compounds
Mobile phase: 1.6 mM sodium borate, pH=9.0, 1ml/min; Postcolumn interaction component: 2x10^{-5} M Cu(L-Trp)_{2} in 40 mM sodium borate at pH=8.4, flow rate: 1ml/min; Column: Hamilton PRP-X100, anion exchange column; Excitation wavelength = 280 nm. Longpass filter \lambda_{cutoff} = 340 nm.

a: mixture of glucosamine standard (GlcN-HCl: 0.0766mg/ml) and chondroitin-containing sample P (sample P: 2.04mg/ml)
b: glucosamine-containing sample B (sample B: 0.21mg/ml)
c: chondroitin-containing sample P (sample P: 4.08mg/ml)
d: chondroitin-containing sample Q (sample Q: 4.46mg/ml)

Peaks 1 and 2: unknown compounds in chondroitin-containing sample P

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
using Cu(L-Trp)$_2$ and Cu(5-MTP)$_2$ in the postcolumn solution are presented in Figure 4.2. The results obtained with the use of these calibration curves for evaluating the content of glucosamine in four samples, A-D, are presented in Table 4.1. It is important to realize that using different ratios of Cu$^{2+}$ to L-Trp (or 5-MTP) to prepare the postcolumn interaction component solutions will affect the resulting calibration curves. However, the ratio of Cu$^{2+}$ to L-Trp (or 5-MTP) will not affect quantitation of the unknown if the postcolumn same interaction component solution is used for analyzing standard solutions and the solutions containing the unknown. The confidence limits (CL) for the content of glucosamine in the samples were calculated using an equation described in the literature [253]. This value expresses the uncertainty of estimating the content of glucosamine in the samples by the calibration curve at a chosen confidence level. A paired $t$-test was performed to compare the results obtained by using these two different postcolumn reagents resulting in $t_{calculated} = 2.71$, which is less than the value of $t_{critical}$, 3.18, for 95% confidence and 3 degrees of freedom [254]. This $t_{calculated}$ value indicates that the results obtained using the two methods which employed two different postcolumn interaction compounds, Cu(L-Trp)$_2$ and Cu(5-MTP)$_2$, are not significantly different at the 95% confidence level. In the following study, Cu(L-Trp)$_2$ was used for the indirect detection of glucosamine for the commercial samples E-N.

4.3.3 Stability of the Glucosamine-OPA/MPA Derivative

A precolumn derivatization method was employed to compare the results obtained with the indirect fluorescence detection method. The most commonly used precolumn
Figure 4.2. Calibration curves for the methods using (a) $2 \times 10^{-5}$ M Cu(L-Trp)$_2$ in 40mM sodium borate at pH 9.0 (1ml/min), (b) $2 \times 10^{-7}$ M Cu(5-MTP)$_2$ in 40mM sodium borate at pH 8.4 (1ml/min) as the postcolumn interaction component. Mobile phase: 1.6 mM sodium borate, pH=9.0, 1ml/min; Column: Hamilton PRP-X100, anion exchange column; Excitation wavelength = 280 nm. Longpass filter $\lambda_{\text{cutoff}} = 320$ nm.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Table 4.1. Comparison of results obtained using \( \text{Cu(L-Trp)}_2 \) and \( \text{Cu(5-MTP)}_2 \)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Stated ingredient\textsuperscript{a}</th>
<th>Assay\textsuperscript{b}</th>
<th>RSD\textsuperscript{c}</th>
<th>95% CL\textsuperscript{d}</th>
<th>Assay\textsuperscript{b}</th>
<th>RSD\textsuperscript{c}</th>
<th>95% CL\textsuperscript{d}</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>GlcN</td>
<td>628mg/tablet</td>
<td>1.5%</td>
<td>608-648mg/tablet</td>
<td>625mg/tablet</td>
<td>1.2%</td>
<td>616-633mg/tablet</td>
</tr>
<tr>
<td>B</td>
<td>GlcN-HCl</td>
<td>466mg/tablet</td>
<td>1.3%</td>
<td>442-491mg/tablet</td>
<td>441mg/tablet</td>
<td>1.2%</td>
<td>431-451mg/tablet</td>
</tr>
<tr>
<td>C</td>
<td>(GlcN\textsubscript{2})\textsubscript{H_2}SO\textsubscript{4}</td>
<td>517mg/capsule</td>
<td>1.2%</td>
<td>491-542mg/capsule</td>
<td>500mg/capsule</td>
<td>1.5%</td>
<td>489-511mg/capsule</td>
</tr>
<tr>
<td>D</td>
<td>(GlcN\textsubscript{2})\textsubscript{H_2}SO\textsubscript{4}</td>
<td>294mg/capsule</td>
<td>2.3%</td>
<td>268-319mg/capsule</td>
<td>287mg/capsule</td>
<td>2.1%</td>
<td>276-298mg/capsule</td>
</tr>
</tbody>
</table>

\textsuperscript{a} GlcN: glucosamine; GlcN-HCl: glucosamine hydrochloride; (GlcN\textsubscript{2})\textsubscript{H_2}SO\textsubscript{4}: glucosamine sulfate

\textsuperscript{b} Average of triplicate injections

\textsuperscript{c} RSD: relative standard deviation of triplicate injections

\textsuperscript{d} Confidence limit at 95% confidence level
derivatization reagents for glucosamines are o-phthalaldehyde (OPA) combined with thiols including MPA [225,249,255] and phenylisothiocyanate (PITC) [250,251,256], though another derivatization reagent, N-(9-fluorenyl-methoxycarbonyloxy) succinimide (FMOC-Su), has been developed recently [252,257]. An experiment was conducted to investigate the stability of the glucosamine-OPA/MPA derivative under the conditions utilized here since a question of the stability of the amine-OPA/MPA derivatives was raised in literature [258]. To evaluate the stability of the glucosamine-OPA/MPA derivative, the UV absorption of the glucosamine-OPA/MPA derivatization reaction was monitored over time. According to the reports in the literature, the use of a derivatization reagent with an OPA/MPA molar ratio of 1:50 results in increased derivative stability for selected mono-, di- and polyamines, and amino acids [258]. For this reason, the stability of the glucosamine-OPA/MPA derivative was investigated under similar conditions. A derivatization reaction of glucosamine with an OPA/MPA molar ratio of 1:50 was performed according to a method described in the literature [225,249]. The maximum absorption of the glucosamine-OPA/MPA derivative in borate buffer (80mM, pH=9.5) was found to occur at 335nm. The absorbance for the glucosamine-OPA/MPA derivative solution at 335nm was monitored for over four and half hours after mixing the reagents with the results presented in Figure 4.3. The results show that the absorbance of the glucosamine and OPA/MPA reaction solution reached a maximum after about 60 minutes and then declined. The absorbance of the glucosamine-OPA/MPA derivative decreased to almost half of the maximum absorbance after five hours. These results are consistent with the glucosamine-OPA/MPA derivative being unstable over the time frame studied.
Figure 4.3. Absorbance for glucosamine-OPA/MPA derivative solution vs. reaction time at room temperature.
under the conditions of this reaction. The instability in the absorbance due to the glucosamine-OPA/MPA derivative resulted in a RSD (relative standard deviation) for the chromatographic analysis much higher than 2% for peak heights measured for triplicate injections of the glucosamine-OPA/MPA derivative. This observation led us to compare the results obtained using the indirect fluorescence detection with the results obtained using a PITC pre-column derivatization method where the RSD was less than 2%.

4.3.4 Comparison of the Indirect Fluorescence Detection Method and the PITC Pre-column Derivatization Method

A representative chromatogram showing the isocratic elution of the glucosamine-PITC derivative is given in Figure 4.4. The column was washed with methanol:water (80:20, v/v) at 1ml/min between injections of solutions containing glucosamine derivatized with PITC. Ten glucosamine-containing samples, E-N, were analyzed by the method based on indirect fluorescence detection described here and by the method based on pre-column derivatization of glucosamine with PITC. The calibration curves for these two methods are presented in Figure 4.5. The results for the content of glucosamine in the samples obtained based on these calibration curves are summarized in Table 4.2 and presented graphically in Figure 4.6. Though the goal of this study was to compare the results obtained for the two methods utilized and not to analyze the particular samples, the content of glucosamine in the samples analyzed ranged from 81% to 128% of the nominal value provided by the manufacturers. The range of these values is similar to the range observed for the analysis of commercially available glucosamine samples [256].
Figure 4.4. Representative chromatogram of glucosamine-PITC derivative
Mobile phase: CH$_3$OH:H$_2$O:CH$_3$COOH = 10.00:89.96:0.04 (v/v/v), 1ml/min; Column: X Terra C18 (50×4.6mm, 3µm); Detector: Waters 484 UV-Vis detector, λ=254nm
Figure 4.5. Calibration curves for the determination of glucosamine in ten samples based on (a) indirect fluorescence detection method, (b) PITC pre-column derivatization method. Chromatographic conditions: (a) Mobile phase: 1.6 mM sodium borate, pH=9.0, 1ml/min; Column: Hamilton PRP-X100, anion exchange column; Postcolumn interaction component: 2x10^{-5} M Cu(L-Trp)_2 in 40mM sodium borate at pH 9.0 (1ml/min); Excitation wavelength = 280 nm; Longpass filter λ_{cutoff} = 340 nm. (b) Mobile phase: CH$_3$OH:HO$_2$:CH$_2$COOH = 10.00:89.96:0.04 (v/v/v), 1ml/min; Column: XTerra C18 (50x4.6mm, 3μm); Detector: Waters 484 UV-Vis detector, λ=254nm

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
Table 4.2. Content of glucosamine determined by indirect fluorescence detection method and PITC pre-column derivatization method

<table>
<thead>
<tr>
<th>Sample</th>
<th>Stated ingredient</th>
<th>Indirect fluorescence detection method</th>
<th>PITC pre-column derivatization method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Assay b</td>
<td>RSD c</td>
</tr>
<tr>
<td>E</td>
<td>(GlcN)$_2$H$_2$SO$_4$-2KCl</td>
<td>523mg/tablet</td>
<td>0.8%</td>
</tr>
<tr>
<td>F</td>
<td>GlcN</td>
<td>310mg/tablet</td>
<td>1.5%</td>
</tr>
<tr>
<td>G</td>
<td>(GlcN)$_2$H$_2$SO$_4$</td>
<td>409mg/capsule</td>
<td>0.4%</td>
</tr>
<tr>
<td>H</td>
<td>(GlcN)$_2$H$_2$SO$_4$</td>
<td>879mg/3tablets</td>
<td>1.1%</td>
</tr>
<tr>
<td>I</td>
<td>(GlcN)$_2$H$_2$SO$_4$</td>
<td>823mg/tablet</td>
<td>0.8%</td>
</tr>
<tr>
<td>J</td>
<td>GlcN-HCl</td>
<td>521mg/capsule</td>
<td>1.8%</td>
</tr>
<tr>
<td>K</td>
<td>GlcN-HCl</td>
<td>503mg/tablet</td>
<td>0.2%</td>
</tr>
<tr>
<td>L</td>
<td>(GlcN)$_2$H$_2$SO$_4$</td>
<td>549mg/tablet</td>
<td>0.2%</td>
</tr>
<tr>
<td>M</td>
<td>GlcN-HCl</td>
<td>1505mg/11.2g</td>
<td>0.8%</td>
</tr>
<tr>
<td>N</td>
<td>GlcN</td>
<td>947mg/29.57ml</td>
<td>0.4%</td>
</tr>
</tbody>
</table>

* GlcN: glucosamine; GlcN-HCl: glucosamine hydrochloride; (GlcN)$_2$H$_2$SO$_4$: glucosamine sulfate; (GlcN)$_2$H$_2$SO$_4$-2KCl: glucosamine sulfate potassium chloride salt

b Average of triplicate injections

c RSD: relative standard deviation of triplicate injections

d Confidence limit at 95% confidence level
Figure 4.6. Comparison of indirect fluorescence detection method and PITC pre-column derivatization method for the determination of glucosamine in ten samples.
For all of the samples, the results obtained using the method based on indirect fluorescence detection are close to those obtained using PITC pre-column derivatization. A paired $t$-test was performed to compare the results obtained for two methods with a result of $t_{\text{calculated}} = 0.665$, which is less than the value of $t_{\text{critical}} = 2.262$, for 95% confidence and 9 degrees of freedom. This indicates that the results obtained by the method based on indirect fluorescence detection and those obtained by the method based on pre-column derivatization are not significantly different at the 95% confidence level.

4.4 Conclusions

It has been demonstrated that indirect fluorescence detection based on the approach using either Cu(L-Trp)$_2$ or Cu(5-MTP)$_2$ as the postcolumn interaction compound is applicable for analyzing glucosamine in the commercial dietary supplement samples. The results for ten samples analyzed using the indirect fluorescence detection method were not significantly different from the results obtained using a PITC pre-column derivatization method at the 95% confidence level. The detection limit of glucosamine for the method based on indirect fluorescence detection reported in Chapter 3 is 0.15nmol in 10µL of solution injected, which corresponds to a concentration of 3.2µg/ml. This detection limit is higher than the detection limit reported for the method based on pre-column derivatization of glucosamine by PITC, 0.075µg/ml [251]. However, advantages of using indirect fluorescence detection, such as avoiding a time-consuming pre-column derivatization step and possible stability problems of derivatization products, allow it to be a suitable alternative to methods based on pre-column derivatization for determining glucosamine concentrations for analyses where the
detection limit is not a significant consideration. For example, when analyzing commercial dietary supplements, the concentration of glucosamine in the analyzed samples may easily be maintained well above the detection limit for the method. The results presented here also indicate caution should be exercised when using OPA/MPA for derivatization of glucosamine if the waiting time between initiating the reaction and injection into the column varies.
PART II. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION
OF COPPER(II)-AZAMACROCYCLIC COMPLEXES
CHAPTER 5

REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC
SEPARATION OF COPPER(II)-AZAMACROCYCLIC COMPLEXES

5.1 Introduction

Copper radionuclides offer a wide range of half-lives and several decay profiles, providing a useful selection of both diagnostic and therapeutic isotopes [259-261]. Among the available copper radionuclides, $^{64}\text{Cu}$ has favorable properties for use in both positron emission tomography (PET) imaging and targeted radiotherapy. Among these properties are its half-life, decay scheme, and the potential for large-scale production with high specific activity using a biomedical cyclotron [259-263]. The increasing use of $^{64}\text{Cu}$ and other copper radioisotopes in nuclear medicine has led to a need for the development of bifunctional chelators (BFCs). A BFC consists of a chelator which complexes the radiometal and a functional group for attaching the chelated metal to a biomolecule. The BFC-metal complex must possess high \textit{in vivo} stability against the loss of copper radioisotope during \textit{in vivo} delivery [10-12,264]. Synthesis of several novel cross-bridged tetraamine ligands having non-adjacent nitrogens connected by an ethylene (CH$_2$CH$_2$) bridge and their Cu(II) complexes have been reported [6-9]. The \textit{in vivo} stabilities of some $^{64}\text{Cu}$-labeled conventional and cross-bridged tetraazamacroyclic complexes have been studied and compared [10-15]. It has been shown that the charge and the size of the macrocyclic backbone of the complexes have significant effects on the \textit{in vivo} stability of the copper complexes. The development of a routine method for

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
separating and characterizing these complexes is critical for assessing their properties, evaluating rediolabeling purity, and monitoring their in vivo behavior.

Reversed-phase HPLC (RP-HPLC) has emerged as the most popular liquid chromatographic separation technique due to its selectivity and versatility. It can be applied to a great variety of samples, such as polar compounds, including many pharmaceuticals. Ionic compounds can be separated by means of ion-pair liquid chromatography [265] and ligand exchange liquid chromatography [266]. RP-HPLC has also been employed for the assessment of physico-chemical properties of solutes [267,268], including the acid dissociation constant (pKₐ) [269-271], complex formation constant [272,273], and the lipophilicity or hydrophobicity parameter [274-278]. A major advantage of measuring such properties using RP-HPLC methods is that only small amounts of an impure sample are needed.

Separation of copper complexes from other metal complexes with the same type of ligand by RP-HPLC has been reported [279-281]. RP-HPLC separations of copper complexes having different types of ligands have also been reported, such as the separation of copper(II)-chelated bleomycin congeners [282] and copper complexes of chlorophylls and their derivatives [283,284]. The copper-64-labeled azamacrocyclic complexes discussed herein have been previously analyzed by LC-MS [285]. The separation of Cu-azamacrocyclic complexes was achieved with the use of an octyl-bonded phase (C8) and a mobile phase containing 0.1% aqueous formic acid at pH 2.5. Problematic peak tailing and variable retention times of one of the most important Cu-azamacrocyclic complexes were observed [285].
A study was undertaken to investigate conditions which provide improved RP-HPLC separations of three Cu-azamacrocyclic complexes. Two of these complexes are uncharged in aqueous solution, Cu(II)-4,11-bis(carboxymethyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane (Cu-CB-TE2A) and Cu(II)-4,10-bis(carboxymethyl)-1,4,7,10-tetraazabicyclo[5.5.2]tetradecane (Cu-CB-DO2A), while the third, Cu(II)-1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (Cu-TETA), is anionic at neutral pH. The structures of these complexes and their corresponding ligands are presented in Figure 5.1. Absorbance at 280 nm was used to monitor the complexes as they eluted from the reversed-phase column. Separation was achieved with the use of two different mobile phases, a citric acid-buffered mobile phase and a formic acid mobile phase, and two different stationary phases, an octadecyl bonded phase (C18) and an octyl bonded phase (C8). The effects of the pH of the buffered mobile phase, the concentration of the buffer, and the concentration of the organic modifier, methanol, on the separation were studied. Separation of these complexes based on ion-pair liquid chromatography was also investigated with the use of a MS-compatible ion-pair reagent, triethylammonium acetate (TEAA). Estimations of the pKₐ’s of Cu-TETA and the lipophilicity of the complexes based on RP-HPLC data are also discussed.

5.2 Experimental

5.2.1 Reagents

Certified anhydrous citric acid was purchased from Fisher (Fairlawn, NJ, USA). Reagent-grade formic acid solution (88% w/w) was purchased from J.T. Baker (Phillipsburg, NJ, USA). Glacial acetic acid (99.7%) was purchased from VWR.
Figure 5.1. Structures of (a) Cu(II)-azamacrocyclic complexes: Cu-CB-TE2A, Cu-CB-DO2A, and Cu-TETA, and (b) the corresponding ligands: H₂CB-TE2A, H₂CB-DO2A, and H₄TETA.
Scientific (San Francisco, CA, USA). Triethylamine (99%) was purchased from Lancaster Synthesis (Windham, NH, USA). HPLC-grade methanol was purchased from Pharmco (Brookfield, CT, USA). A 1.0 M stock solution of the ion-pair reagent, triethylammonium acetate (TEAA), was prepared by titrating aqueous triethylamine with acetic acid to a pH of 6.3 [286]. Certified sodium hydroxide solution (50% w/w) and reagent-grade hydrochloric acid (36.5% w/w) were purchased from Fisher (Fairlawn, NJ, USA). Dilute solutions of these reagents were used to adjust the pH of the mobile phase. The deionized water used in the preparation of the standard solutions and eluents was obtained from a Milli-Q water system (Millipore, Bedford, MA, USA). All mobile phases were filtered through a 0.45 μm nylon filter (Whatman, Hillboro, OR, USA) prior to use.

5.2.2 Apparatus

Chromatographic separations were performed at ambient temperature using a Nicolet LC 9560 HPLC system (Madison, WI, USA) fitted with a Reodyne 7125 injector (Cotati, CA, USA) having a 10μl injection loop. A Kratos Spectroflow 783 UV/Vis absorbance detector with the wavelength set at 280 nm was used for chromatographic detection in combination with a Kipp & Zonen BD41 chart recorder. Two reversed-phase HPLC analytical columns were used for the chromatographic separation: (1) Betabasic C18 column (150×4.6 mm, 5 μm; Keystone Scientific, Bellefonte, PA, USA); (2) Zorbax SB-C8 column (75×4.6 mm, 3.5 μm; Agilent, Wilmington, DE, USA).
5.2.3 Chromatographic Conditions

The reversed-phase HPLC column was washed for at least 30 minutes using 60:40 (v/v) methanol:water at 1.0 ml/min after daily use. The chromatographic column was equilibrated with the desired mobile phase for at least 30 minutes before use. For ion-pair HPLC separations, the column was equilibrated with the mobile phase containing the ion-pair reagent for at least 60 minutes. A mobile phase flow rate of 1.0 ml/min was used throughout the study. The pH of 0.1% formic acid solution (approximately equivalent to 22 mM formic acid) was 2.5.

The dead time ($t_0$) used for the calculation of the capacity factor, $k' = (t_R - t_0)/t_0$, where $t_R$ is the retention time of analyte, was measured as the time of the first distortion of the baseline after the injection of water [287].

5.2.4 Sample Preparation

Cu(II)-TETA, Cu(II)-CB-TE2A, and Cu(II)-CB-D02A were provided by Prof. Wong’s group. Another Cu(II)-CB-TE2A sample (called crude Cu(II)-CB-TE2A) was also provided by Prof. Wong’s group. This crude Cu(II)-CB-TE2A was recovered from Cu(II)-CB-TE2A samples which had been used to study acid decomplexation in aqueous hydrochloric acid solution. These Cu(II)-CB-TE2A samples were evaporated to dryness, washed with ether, and re-dissolved in 95% ethanol. After centrifugation to remove the small amount of precipitate, the clear blue solutions were placed in ether chambers to precipitate the crude Cu(II)-CB-TE2A. All samples of the complexes for analysis by HPLC were dissolved in deionized water.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.
5.2.5 Data Analysis

The statistical software JMP (SAS Institute Inc., Cary, NC, USA) was employed to perform non-linear fitting to estimate the pKₐ’s of Cu(II)-TETA.

5.3 Results and Discussion

5.3.1 Effect of the Concentration of the Citric Acid Buffer on the Capacity Factors

The separation of four Cu(II)-azamacrocyclic complexes on a C₈ reversed-phase column was previously studied with a mobile phase of 22 mM aqueous formic acid solution at pH 2.5 [285]. However, severe peak tailing and inconsistent retention times of one of the most important Cu-azamacrocyclic complexes, Cu-CB-TE2A, were encountered. A buffer may be added to the mobile phase to control pH, affect selectivity, and with these chromatographic conditions, achieve reproducible separations with acceptable peak shape. Citric acid has three pKₐ values, 3.128, 4.761, and 6.396 [288], thereby providing a wide range of useful buffering regions (pKₐ ± 1 pH unit) including the region of pH 2.5. For these reasons, citrate was chosen as a mobile phase buffer for the separation of these Cu-azamacrocyclic complexes. The effect of the concentration of citric acid buffer at pH 2.5 from 12 mM to 150 mM on the capacity factors of the Cu-azamacrocyclic complexes was evaluated with the use of a C18 column. As the concentration of the citric acid buffer increased, the capacity factors of the Cu-azamacrocyclic complexes decreased slightly. The decreased capacity factors of the complexes at citric acid buffer concentrations over 60 mM led to a reduction in the resolution of Cu-TETA and Cu-CB-DO2A and the co-elution of an impurity with Cu-CB-DO2A. Figure 5.2 shows...
Figure 5.2. Separations of Cu-CB-TE2A, Cu-TETA, and Cu-CB-DO2A with the use of citric acid buffer at (a) 30 mM, (b) 60 mM, and (c) 150 mM. Mobile phase: citric acid at pH 2.5; Stationary phase: Betabasic C18. Peaks: 1 = Cu-CB-DO2A (0.10 mg/ml); 2 = Cu-TETA (0.13 mg/ml); 3 = Cu-CB-TE2A (0.10 mg/ml); 4 = impurity.
representative chromatograms at three concentrations of the citric acid in the mobile phase, 30 mM, 60 mM, and 150 mM.

5.3.2 Effect of the pH of the Mobile Phase on the Capacity Factors

Of the three Cu-azamacrocyclic complexes, two complexes, Cu-CB-TE2A and Cu-CB-DO2A, are expected to be uncharged in aqueous solution. The third complex, Cu-TETA, is ionizable in aqueous solution over a wide pH range due to its having two extra carboxylic acid functionalities (Figure 5.1). The retention of the ionizable Cu-TETA is expected, therefore, to be highly dependent on the pH of the mobile phase. The effect of the pH of the mobile phase on the retention of these three complexes on a C18 column is presented in Figure 5.3. As the pH of the mobile phase increased from 2.2 to 4.5, the capacity factor of Cu-TETA decreased, while the capacity factors of Cu-CB-TE2A and Cu-CB-DO2A increased only slightly. Increasing the pH promotes the ionization of Cu-TETA. This results in a decrease in the retention of Cu-TETA because the ionic form of Cu-TETA, a negatively charged species, is more hydrophilic than the neutral form. When the pH is over 2.5 and below 3.2, the lowest resolution of Cu-TETA and Cu-CB-DO2A is observed. At a mobile phase pH over 3.2, the resolution of Cu-TETA and Cu-CB-DO2A becomes better but the co-elution of an impurity and the Cu-TETA occurs. Taking these results into consideration, the separation of these Cu-azamacrocyclic complexes is better carried out at pH below 2.5.
Figure 5.3. Effect of the pH of the mobile phase on the capacity factor of Cu-CB-TE2A, Cu-TETA, and Cu-CB-DO2A. Mobile phase: 30 mM citric acid. Stationary phase: Betabasic C18. Fitted curve of Cu-TETA is plotted according to Eq. (1), see the text for details.
5.3.3 Effect of the Concentration of the Methanol in the Mobile Phase on the Capacity Factors

It has been reported that a mobile phase containing less than 10% methanol may not sufficiently wet an octadecylsilated silica-based stationary phase [289]. For this reason, adding varying mounts of methanol in the citric acid buffered mobile phase was evaluated. The effect of the concentration of the methanol in the mobile phase on the capacity factors of the three Cu-azamacrocyclic complexes with the use of a C18 column is presented in Figure 5.4. It is apparent that the concentration of methanol in the mobile phase greatly affects the capacity factor of these copper complexes. Adding only 2% methanol to the mobile phase results in a reduction in the capacity factor by half. When a mobile phase containing 10% methanol is used, these copper complexes are hardly retained on the C18 column. When the logarithm of the capacity factor is plotted vs. the fraction of the methanol in the mobile phase (Figure 5.5), the slightly non-linear trend was similar to that was observed by Hsieh and Dorsey [290] and attributed to a structural change of the stationary phase within highly aqueous mobile phases.

5.3.4 Comparison of the Separations with the Use of Two Different Mobile Phases on C8 and C18 Columns

The separation of the three complexes with the use of 30 mM citric buffer at pH 2.5 on a C18 column was achieved with a better symmetry of the Cu-CB-TE2A peak as shown in Figure 5.2, compared to the severe tailing of the corresponding peak eluted from the C8 column using 22 mM formic acid as the mobile phase, possibly due to the residual silanols of the C8 stationary phase [285]. To investigate the effects of different
Figure 5.4. Effect of the concentration of methanol in the mobile phase on the capacity factor of Cu-CB-TE2A, Cu-TETA, and Cu-CB-D02A. Mobile phase: 30 mM citric acid at pH 2.5; Stationary phase: Betabasic C18.
Figure 5.5. Plots of the log($k'$) values of Cu-CB-TE2A, Cu-TETA, and Cu-CB-D02A vs. the concentration of methanol and their trends. Mobile phase: 30 mM citric acid at pH 2.5; Stationary phase: Betabasic C18.
mobile phases and different stationary phases on peak tailing for Cu-CB-TE2A, C8 and C18 columns were used to compare the profiles of the peak tailing obtained with the use of two different mobile phases. The mobile phases used were 22 mM formic acid (pH 2.5) and 30 mM citric acid buffer at pH 2.5. In addition to the mixture of the three Cu-azamacrocyclic complex standards, an impure Cu-CB-TE2A sample called the "crude Cu-CB-TE2A", recovered from acid decomplexation studies, was also used to evaluate the effects of mobile phase and column on the separations obtained.

The chromatograms showing the separation of the three complexes on the C18 column with the use of 22 mM formic acid (pH 2.5) and on the C8 column with the use of 22 mM formic acid (pH 2.5) or 30 mM citric acid buffer are presented in Figure 5.6. Compared to the separation on a C18 column with the use of 30 mM citric acid buffer at pH 2.5 (Figure 5.2a), the separation on a C18 column using 22 mM formic acid (pH 2.5) resulted in a longer retention time and slight band-broadening for the Cu-TETA peak (Figure 5.6a), while the band profiles for Cu-CB-TE2A did not show a significant difference between these mobile phases. When the C8 column was used, separation of the three complexes with the two different mobile phases, 22 mM formic acid (pH 2.5) and 30 mM citric buffer at pH 2.5 both result in severe tailing of Cu-CB-TE2A peak, as well as the co-elution of Cu-CB-DO2A with the impurity peak from Cu-TETA sample (Figure 5.6b and 5.6c). Thus, it is possible that the severe tailing of the Cu-CB-TE2A peak is not due to the use of 22 mM formic acid (pH 2.5) but the column employed. The C18 column used here provided better peak symmetry for the Cu-CB-TE2A peak observed than when using C8 column.
Figure 5.6. Separations of Cu-CB-TE2A, Cu-TETA, and Cu-CB-D02A with the use of different mobile phases and different columns. (a) Mobile phase: 22 mM formic acid (pH 2.5); Column: Betabasic C18; (b) Mobile phase: 22 mM formic acid (pH 2.5); Column: Zorbax SB-C8; (c) Mobile phase: 30 mM citric acid buffer at pH 2.5; Column: Zorbax SB-C8. Peaks: 1 = Cu-CB-D02A (0.10 mg/ml); 2 = Cu-TETA (0.13 mg/ml); 3 = Cu-CB-TE2A (0.10 mg/ml); 4 = impurity.
While the use of citric acid buffer at pH 2.5 did not improve the peak symmetry, there are advantages to using citric acid buffer in the mobile phase, such as the capability of maintaining pH over a wider range than for formic acid, potentially resulting in improved reproducibility, and the ability to optimize the separation by adjusting the pH over a wide range. The latter advantage can be observed in Figure 5.7 by comparing the separation obtained for the crude Cu-CB-TE2A sample on the C18 column with the use of a mobile phase containing 6 mM citric acid at pH 2.5 and 6.2 as well as 22 mM formic acid (pH 2.5). Impurities were better separated with the use of 6 mM citric acid buffer at pH 6.2 (Figure 5.7a) than with the use of 6mM citric acid buffer at pH 2.5 (Figure 5.7b) or 22 mM formic acid (pH 2.5) (Figure 5.7c).

5.3.5 Separation of Cu-Azamacrocyclic Complexes by Ion-Pair HPLC Using a C8 Column

Though citric acid buffer has some advantages over formic acid as the mobile phase in the separation of Cu-azamacrocyclic complexes by reversed-phase liquid chromatography, two obvious challenges remained: (1) The pH of the mobile phase must be lower than the pKₐ of acidic solutes to force the association of acid, thereby increasing the retention of the solutes on the reversed-phase column. If the mobile phase pH required is too low, damage to silica-based alkyl-bonded phases may occur due to the hydrolysis of the siloxane bond; (2) An additional requirement may be that the mobile phase buffer be volatile so that it will be compatible with LC-MS, a technique which has been widely used in drug discovery and development. Ion-pair reversed-phase liquid chromatography [265] has the advantage of simultaneously separating ionic and neutral...
Figure 5.7. Separations of the crude Cu-CB-TE2A (1.0 mg/ml) with the use of different mobile phases. (a) Mobile phase: 6 mM citric acid buffer at pH 6.2; (b) Mobile phase: 6 mM citric acid buffer at pH 2.5; (c) 22 mM formic acid (pH 2.5). Stationary phase: Betabasic C18.
solutions with the use of a reverse-phase column and a volatile ion-pair reagent in the mobile phase at a moderate pH. Therefore this method was employed as an alternative for separating the Cu-azamacrocyclic complexes. Triethylammonium acetate (TEAA) has been used as a volatile ion-pair reagent for the analysis of nucleic acids by LC-MS [286,291]. The separation of the Cu-azamacrocyclic complexes by ion-pair HPLC on the C8 column with the use of 40 mM TEAA in the mobile phase at pH 6.3 is presented in Figure 5.8a. The complexes are well separated from several small peaks which were not previously observed under reversed-phase conditions (Figure 5.6). Compared to the separation obtained using reversed-phase conditions on a C8 column, shorter retention times were observed for the three Cu-complexes when using ion-pair conditions and the same C8 column. This is attributed to the competition between the solutes and the hydrophobic triethylammonium for the stationary phase, hence the reduced hydrophobic interaction between the reversed-phase and the solutes. The crude Cu-CB-TE2A sample was also used to evaluate the separations produced by the ion-pair reversed-phase HPLC method. The separation of the crude Cu-CB-TE2A with the use of 40 mM TEAA in the mobile phase at pH 6.3, presented in Figure 5.8b, shows a similar resolution of the impurity peaks as was obtained using 6 mM citric acid buffer in the mobile phase at pH 6.2 (Figure 5.7a).
Figure 5.8. Ion-pair HPLC separations of (a) Cu-CB-TE2A, Cu-TETA, and Cu-CB-DO2A; (b) the crude Cu-CB-TE2A (1.0 mg/ml). Mobile phase: 40 mM triethylammonium acetate (TEAA) at pH 6.3. Stationary phase: Zorbax SB-C8. Peaks: 1 = Cu-CB-DO2A (0.10 mg/ml); 2 = Cu-TETA (0.13 mg/ml); 3 = Cu-CB-TE2A (0.10 mg/ml).
5.3.6 Estimation of the pKₐ's of Cu-TETA Using Reversed-Phase Chromatographic Data

Since two of four carboxylic acid arms of TETA complex with the Cu²⁺ ion, Cu-TETA is considered to be a diprotic acid (Figure 5.1). As shown in Figure 5.3, the capacity factor of Cu-TETA is dependent on pH. This relationship can be described using an equation for the capacity factor of diprotic acids as a function of the concentration of proton (H⁺) in the mobile phase [269]:

\[
k' = \frac{k'_0 + k'_{-1} \frac{K_{a1}}{[H^+]} + k'_{-2} \frac{K_{a1}K_{a2}}{[H^+]^2}}{1 + \frac{K_{a1}}{[H^+]} + \frac{K_{a1}K_{a2}}{[H^+]^2}}
\]  

(5.1)

where \(k'_0\), \(k'_{-1}\), \(k'_{-2}\) are the capacity factors of the undissociated, half dissociated, and fully dissociated diprotic acid and \(K_{a1}\) and \(K_{a2}\) are the corresponding acid dissociation constants in the mobile phase, respectively. These five parameters, \(k'_0\), \(k'_{-1}\), \(k'_{-2}\), \(K_{a1}\) and \(K_{a2}\), can be determined by fitting the appropriate model, i.e. Eq. (5.1), by nonlinear regression [292], to a series of capacity factor values measured at different mobile phase pH for the solute. The statistical software JMP was used to perform the nonlinear fitting of the data. In order to reduce the uncertainties in the estimation of five parameters by nine data points when JMP performs nonlinear fitting, some parameters were locked. The parameters being locked were \(k'_0\), \(k'_{-1}\), and \(k'_{-2}\), because \(k'_0\) and \(k'_{-2}\) can be easily estimated as \(k'_0 \approx 2.4\) by extrapolating the curve to pH=0 where Cu-TETA is undissociated, and \(k'_{-2} \approx 0\) by extrapolating the curve at pH above 4.5 where Cu-TETA is almost fully dissociated, while \(k_{-1}\) is estimated as having a value between \(k_0\) and \(k_2\). Thus, fitting of the data was performed by varying the values of \(k_0\), \(k_{-1}\), and \(k_2\) to
calculate different values of the sum of square error (SSE), which is an indicator of how well the line fits. When $k'_0$, $k'_{-1}$, and $k'_{-2}$ are locked at 2.6, 1.1, and 0.01, respectively, a minimum value of SSE is achieved and the estimated values of $K_{a1}$ and $K_{a2}$ are obtained: $pK_{a1} = 2.32 \pm 0.09/-0.08$ and $pK_{a2} = 3.19 \pm 0.06/-0.05$. These values of the five parameters are employed to plot the corresponding fitted curve shown in Figure 5.3 with the use of Eq. (5.1). Clarke and Martell [293] reported a $pK_{a1}$ of 2.50 ± 0.01 and a $pK_{a2}$ of 3.91 ± 0.01 for Cu-TETA. These $pK_a$ values were determined by potentiometric titration in aqueous KCl solution (0.1 M, 25.0 °C). They also reported the values for $pK_{a1} = 2.90 \pm 0.03$ and $pK_{a2} = 3.68 \pm 0.03$ for Cu-TETA based on Delgado and Frausto Da Silva's data [294], which were determined by potentiometric titration in aqueous KNO$_3$ solution (0.1 M, 25.0 °C) (Delgado and Frausto Da Silva did not report directly the $pK_{a1}$ and $pK_{a2}$ of Cu-TETA, but these two values can be derived mathematically from their data). Our $pK_{a1}$ value is close to the result reported by Clarke and Martell (0.2 logarithm unit difference), while $pK_{a2}$ is close to Delgado and Frausto Da Silva's result (0.5 logarithm unit difference). The ionic strength of the 30 mM citric acid-buffered mobile phase we used is different from the ionic strength of the solutions for the potentiometric titration reported, which may result in the $pK_a$ values of the Cu-TETA based on HPLC data differing from the values obtained by potentiometric titration. It is noted that the other parameters such as $k'_0$, $k'_{-1}$, and $k'_{-2}$ in Eq. (5.1) are constants which will be determined by performing non-linear fitting. However, previous discussion has shown that the capacity factors of the two neutral complexes, Cu-CB-TE2A and Cu-CB-DO2A vary slightly as the pH increases (Figure 5.3). The same effect may also be applied to the undissociated, half dissociated, and fully dissociated Cu-TETA, which means $k'_0$, $k'_{-1}$,
and \( k'-2 \) in Eq.(5.1) may not be constant when performing nonlinear fitting to obtain the values of \( pK_{a1} \) and \( pK_{a2} \), and may introduce some uncertainty into the results of \( pK_{a1} \) and \( pK_{a2} \). There are also outliers in our experimental data that may reduce the accuracy of the nonlinear fitting.

### 5.3.7 Estimation of the Lipophilicity of the Complexes by Reversed-Phase HPLC

Although the terms “lipophilicity” and “hydrophobicity” are frequently considered to be synonymous and both are used in the literature, their scientific meanings are quite different. The following definitions have been recommended by IUPAC [295].

- “Lipophilicity represents the affinity of a molecule or a moiety for a lipophilic environment. It is commonly measured by its distribution behaviour in a biphasic system, either liquid-liquid (e.g., partition coefficient in octan-1-ol/water) or solid/liquid (retention on reversed-phase high performance liquid chromatography (RP-HPLC) or thin-layer chromatography (TLC) system).”

- “Hydrophobicity is the association of non-polar groups or molecules in an aqueous environment which arises from the tendency of water to exclude non-polar molecules.”

Discussions of the difference between these two terms can be found in the literature [296,297]. It is well known that lipophilicity plays an important role in the physico-chemical properties and pharmacokinetic behavior of pharmaceuticals and it is usually expressed as the octanol-water partition coefficient [296], which has traditionally been measured by the so-called shake-flask method. Estimating the lipophilicity of a
solute by reversed-phase liquid chromatography is based on developing a relationship between the logarithm of the partition coefficient and the logarithm of the capacity factor of the solute described by the following equation [274]:

\[
\log P = a \log(k') + b \tag{5.2}
\]

The values of \(a\), \(\log(k')\), and \(b\) are highly dependent on the concentration of the organic modifier in the mobile phase. For this reason, the capacity factor obtained using a 100% aqueous mobile phase, \(\log(k'_w)\), is often used to replace \(\log(k')\) in Eq. (5.2). In most cases, the use of a 100% aqueous mobile phase is experimentally unrealistic since it is too weak to elute the solutes in a reasonable period of time. For this reason, \(\log(k'_w)\) values are often obtained by extrapolating from the plot of \(\log(k')\) versus the fraction of organic modifier to 0% organic modifier by fitting the experimental data to a suitable model. Octadecyl stationary phases are most often used to measure the \(\log(k'_w)\) values. However, other stationary phases have also been used for this purpose. Several reviews [276,277,297] have discussed the effect of the stationary phase on the determination of \(\log(k'_w)\). It is generally agreed that with the use of methanol-water mobile phases, the capacity factors obtained using a C18 stationary phase correlate well with literature values of octanol-water partition coefficients. Though the regression coefficients for the correlation between \(\log P\) and \(\log(k'_w)\) vary for different solutes and under different chromatographic conditions, Braumann [26] concluded that the regression coefficients approach 1.0 and 0.0 for the slope and intercept, respectively, resulting in \(\log P = \log(k'_w)\). Though views differ over whether \(\log(k'_w)\) and \(\log P\) are identical,
log($k''_w$) has become the most widely used chromatographic parameter of lipophilicity [298-300].

The log($k''_w$) values of the neutral complexes, Cu-CB-DO2A and Cu-CB-TE2A, were obtained directly with the use of a totally aqueous mobile phase using an octadecyl stationary phase, as shown in Figure 5.5. The log($k''_w$) values for Cu-CB-DO2A and Cu-CB-TE2A were found to be 0.11 and 0.77, respectively. For an ionic compound, the log($k''_w$) value of the undissociated form is generally considered [277]. Therefore, the log($k''_w$) value of the ionic complex, Cu-TETA, is given by the logarithm of the capacity factor of undissociated Cu-TETA, $\log k_0' \approx \log 2.6 = 0.41$, where $k_0'$ is estimated by non-linear regression as previously discussed. This study provides a simple and reasonable method for estimating the relative lipophilicity of the three complexes. Additional studies would be required to explore the relationship between log($k''_w$) and the structural properties of the Cu-azamacrocyclic complexes in terms of quantitative structure-retention relationships (QSRR) or to correlate log($k''_w$) with their relevant bio-partitioning processes.

5.4 Conclusion

The separation of three Cu-azamacrocyclic complexes, Cu-CB-TE2A, Cu-TETA, and Cu-CB-DO2A, was achieved by reversed-phase HPLC with the use of 30 mM citric acid buffer in the mobile phase at a pH below 2.5. Addition of methanol in the mobile phase greatly affected the retention of these polar copper complexes. A better separation of the crude Cu-CB-TE2A sample was achieved with the use of 6 mM citric acid buffer,
pH 6.2, than when using 22 mM formic acid (pH 2.5). Differences in tailing profiles observed for Cu-CB-TE2A appeared to be the result of differences in the interactions of Cu-CB-TE2A with the C8 and C18 stationary phases. Ion-pair HPLC was shown to be an effective alternative for the separation of these complexes with the use of 40 mM triethylammonium acetate (TEAA) as the ion-pair reagent in the mobile phase at pH 6.3. Finally, the pKₐ's of the acidic solute, Cu-TETA, and the lipophilicity parameter, log($k'_w$), of three Cu-azamacroyclic complexes were estimated with the use of reversed-phase HPLC.
CHAPTER 6
SEPARATION OF COPPER(II)-AZAMACROCYCLIC COMPLEXES WITH A
GRAPHITIC CARBON STATIONARY PHASE

6.1. Introduction

As discussed in Chapter 5, an improved separation of several copper(II)-
azamacrocyclic complexes was achieved based on reversed-phase liquid
chromatography. Separation was produced with the use of two different mobile phases, a
citric acid-buffered mobile phase and a formic acid mobile phase, and two different
stationary phases, octyl- (C8) and octadecyl- (C18) bonded stationary phases. Separation
of these complexes based on ion-pair liquid chromatography was also investigated with
the use of a mass spectrometry-compatible ion-pair reagent, triethylammonium acetate, in
the mobile phase. However, it was observed that the complexes are weakly retained on
C8 and C18 bonded stationary phases, which led us to consider other stationary phases
which provide increased retention thereby improve the separation of these highly polar
complexes.

Porous graphitic carbon stationary phase has been utilized in HPLC to provide
unique retention and separation of very polar compounds [301-305]. For example, the
separations of oxalic acid, creatine, and creatinine, which could not be obtained using
bonded silica stationary phases, were easily achieved with a graphitic carbon stationary
phase [306]. This stationary phase is a very hydrophobic reversed-phase stationary phase
which retains strongly polar molecules. It is also stable under extreme conditions of pH,
salt concentration, and temperature. The retention mechanisms on graphitic carbon stationary phase are different from those observed on silica-based bonded phases. The overall retention on graphitic carbon stationary phase is a combination of two mechanisms [302-304]: 1) the dispersive interactions between analyte-mobile phase and analyte-graphite surface, by which retention increases as the hydrophobicity of the molecule increases; 2) charge induced interaction of a polar analyte with the polarizable surface of graphite. Thus, the molecular area in contact with the graphite surface as well as the type and positioning of the functional groups in relation to the graphite surface highly affect the strength of analyte interactions with the graphitic carbon stationary phase [302-304].

A study was conducted to evaluate the retention behavior and experimental conditions required to produce a separation of four copper(II)-azamacrocyclic complexes using a graphitic carbon stationary phase. The structures of these complexes and their corresponding ligands are presented in Figure 6.1. Separation of these complexes under both isocratic elution and gradient elution was investigated. The effects of the organic mobile phase modifier (methanol or acetonitrile), the column temperature, and the sample concentration, on the retention of these complexes with the use of graphitic carbon stationary phase are also investigated.
Figure 6.1. Structures of (a) copper complexes: Cu-CB-TE2A, Cu-CB-D02A, Cu-CB-TE2LA, Cu-CB-D02A, and (b) the corresponding ligands: H$_2$CB-TE2A, H$_2$CB-D02A, H$_2$CB-TE2LA, H$_2$CB-D02LA.
6.2. Experimental

6.2.1. Reagents

HPLC-grade methanol was purchased from EMD Chemicals Inc (Gibbstown, NJ, USA). HPLC-grade acetonitrile was purchased from Fisher (Fairlawn, NJ, USA). The deionized water used to prepare the mobile phases was obtained from a Milli-Q water system (Millipore, Bedford, MA, USA). All mobile phases were filtered through a 0.45μm nylon filter (Whatman, Hillboro, OR, USA) prior to use. Reagent-grade uracil (minimum purity, 99%) was purchased from Sigma (St. Louis, MO, USA).

6.2.2. Apparatus

The liquid chromatography system consisted of a Varian 9010 solvent delivery system (Palo Alto, CA, USA) and a Rheodyne Model 7125 injector (Cotati, CA, USA) with a 10μL injection loop. A Waters 484 tunable absorbance detector (Milford, MA, USA) set at 280nm was used for chromatographic detection in combination with a Kipp & Zonen BD41 (Holland) chart recorder. The column temperature was controlled with the use of a VWR Scientific 1160 circulating water bath (West Chester, PA, USA) coupled with a column water jacket. The mobile phase was preheated by placing the inlet tubing through the same water bath. Preheating of mobile phase prevented the band broadening from the temperature gradient from the column walls to the interior of the column [307,308]. The column used for the chromatographic separation was Hypercarb (100×3mm, 5μm; Thermo Electron, Waltham, MA).
6.2.3. Chromatographic Conditions

The Hypercarb column was washed for at least 20 minutes using 95:5 (v/v) acetonitrile:water at 0.4ml/min after daily use. The column was equilibrated with the desired mobile phase for at least 30 minutes before each use. A mobile phase flow rate of 0.4ml/min was used throughout the study.

The dead time used for the calculation of the capacity factor was assigned as the time of the first distortion of the baseline after injection of water [287]. The gradient delay time was obtained by the following procedure [309]: 1) replace the HPLC column with a short piece of PEEK tubing; 2) run a gradient profile from 0% to 100% of 0.2% acetone in 10 minutes; 3) extrapolate the gradient straight line to the baseline. The peak asymmetry ($T$) is determined by the following equation [310]:

$$T = \frac{b_{0.1}}{a_{0.1}}$$  \hspace{1cm} (6.1)

where $a_{0.1}$ is the distance between the peak front and the peak maximum, measured at 10% of the total peak height, and $b_{0.1}$ is the distance between the peak maximum and peak end, measured at 10% total peak height.

6.2.4. Sample Preparation

Cu(II)-CB-TE2A, Cu(II)-CB-DO2A, Cu(II)-CB-TE2LA, and Cu(II)-CB-DO2LA were provided by Prof. Wong’s group. All samples of the complexes and uracil were dissolved in deionized water for analysis by HPLC.
6.3. Results and Discussion

6.3.1. Effect of the Concentration of Methanol in the Mobile Phase on Solute Retention

The effect of the concentration of methanol in the mobile phase on the retention of these four copper(II)-azamacrocyclic complexes when using the graphitic carbon stationary phase was investigated. A plot of the logarithm of the capacity factor vs. the fraction of the methanol in the mobile phase is presented in Figure 6.2. As discussed in chapter 5, the log \(k'\) values of Cu(II)-CB-DO2A and Cu(II)-CB-TE2A obtained using a C18 stationary phase column and a mobile phase with no organic modifier are 0.11 and 0.77, respectively. To obtain similar log \(k'\) values of these two complexes with a graphitic carbon stationary phase, the concentration of the methanol in the mobile phase must be 21% (v/v) for Cu(II)-CB-DO2A, and 33% (v/v) for Cu(II)-CB-TE2A. These values demonstrate the enhanced retention of the complexes on the graphitic carbon stationary phase. A representative chromatogram of these four complexes under isocratic elution with the use of methanol as the organic modifier in the mobile phase is presented in Figure 6.3. Under these elution conditions, three of the complexes eluted early while the last complex eluted much later. A mobile phase gradient was applied to obtain a better separation for the first three peaks and reduce the retention time of the last peak thereby reducing the overall separation time. A representative chromatogram of the gradient elution is presented in Figure 6.4.
Figure 6.2. Plots of log(k') vs. methanol concentration for Cu-CB-DO2A (0.11mg/ml), Cu-CB-DO2LA (0.11mg/ml), Cu-CB-TE2A (0.13mg/ml), and Cu-CB-TE2LA (0.14mg/ml)
Figure 6.3. Separation of Cu-CB-TE2A, Cu-CB-DO2A, Cu-CB-TE2LA, and Cu-CB-DO2LA under isocratic elution conditions. Mobile phase: 30% (v/v) methanol/water; Column: Hypercarb (100×3mm, 5μm), column temperature: 35 °C; Detection: UV absorbance λ=280nm; Peaks: 1= Cu-CB-DO2A (0.11mg/ml); 2= Cu-CB-DO2LA (0.11mg/ml); 3= Cu-CB-TE2A (0.13mg/ml); 4= Cu-CB-TE2LA (0.14mg/ml).
Figure 6.4. Separation of Cu-CB-TE2A, Cu-CB-DO2A, Cu-CB-TE2LA, and Cu-CB-DO2LA under gradient elution conditions. Mobile phase: A: 60% (v/v) methanol/water, B: water; Gradient profile: 0-3.5min, 30%A-30%A; 3.5-18.5min, 30%A-100%A; Column: Hypercarb (100×3mm, 5μm), column temperature: 25 °C; Detection: UV absorbance λ=280nm; Peaks: 1= Cu-CB-DO2A (0.11mg/ml); 2= Cu-CB-DO2LA (0.11mg/ml); 3= Cu-CB-TE2A (0.13mg/ml); 4= Cu-CB-TE2LA (0.14mg/ml).
6.3.2. Effect of the Concentration of Acetonitrile in the Mobile Phase on Solute Retention

The effect of the concentration of the acetonitrile in the mobile phase on the retention of these four copper(II)-azamacrocyclic complexes when using a graphitic carbon stationary phase was studied. The results are presented in Figure 6.5 as the plots of the logarithm of the capacity factor vs. the fraction of the acetonitrile in the mobile phase for each of the complexes. The elution order of four complexes by using either acetonitrile or methanol as the mobile phase is the same. As expected, the elution strength of acetonitrile is much greater than that of methanol. A representative chromatogram for these four complexes under isocratic elution with the use of acetonitrile as the mobile phase modifier is presented in Figure 6.6. To obtain a better separation of the first two eluted peaks, and reduce the entire separation time, gradient elution was applied with the resulting chromatogram being presented in Figure 6.7.

6.3.3. Effect of the Column Temperature on the Retention

The relationship between the capacity factor and the column temperature can be described by the following van’t Hoff equation [311]:

\[
\log k' = -\frac{\Delta H^0}{2.3RT} + \frac{\Delta S^0}{2.3R} + \log \Phi \tag{6.2}
\]

where \( R \) is the universal gas constant, \( T \) is the temperature, \( \Delta H^0 \) and \( \Delta S^0 \) are the enthalpy and entropy of a solute transfer from the mobile phase to the stationary phase, respectively, and \( \Phi \) is the phase ratio of the column (the stationary to mobile phase volume ratio). This linear relationship between \( \log k' \) and \( 1/T \) was found in most studies with reversed-phase liquid chromatography. It has also been observed that a linear
Figure 6.5. Plots of log(k') vs. acetonitrile concentration for Cu-CB-DO2A (0.11mg/ml), Cu-CB-DO2LA (0.11mg/ml), Cu-CB-TE2A (0.13mg/ml), and Cu-CB-TE2LA (0.14mg/ml).
Figure 6.6. Separation of Cu-CB-TE2A, Cu-CB-DO2A, Cu-CB-TE2LA, and Cu-CB-DO2LA under isocratic elution conditions. Mobile phase: 10% (v/v) acetonitrile/water; Column: Hypercarb (100×3mm, 5µm), column temperature: 25 °C; Detection: UV absorbance λ=280nm; Peaks: 1= Cu-CB-DO2A (0.11mg/ml); 2= Cu-CB-DO2LA (0.11mg/ml); 3= Cu-CB-TE2A (0.13mg/ml); 4= Cu-CB-TE2LA (0.14mg/ml).
Figure 6.7. Separation of Cu-CB-TE2A, Cu-CB-DO2A, Cu-CB-TE2LA, and Cu-CB-DO2LA under gradient elution conditions. Mobile phase: A: 50% (v/v) acetonitrile/water, B: water; Gradient profile: 0-3.5min, 15%A-15%A; 3.5-18.5min, 15%A-40%A; Column: Hypercarb (100x3mm, 5μm), column temperature: 25°C; Detection: UV absorbance λ=280nm; Peaks: 1= Cu-CB-DO2A (0.11mg/ml); 2= Cu-CB-DO2LA (0.11mg/ml); 3= Cu-CB-TE2A (0.13mg/ml); 4= Cu-CB-TE2LA (0.14mg/ml).
relationship between log $k'$ and $1/T$ for the solutes, fenuron ($N,N$-dimethyl-$N'$-phenylurea) and benzene, using a graphitic carbon stationary phase column (Hypercarb) and a mobile phase of methanol/water or acetonitrile/water over a range of temperature, 20-180 °C [312].

Van’t Hoff plots for the four complexes studied here over the temperature range from 25 °C to 65 °C, with the use of a graphitic carbon stationary phase and a mobile phase containing either methanol/water or acetonitrile/water are presented in Figure 6.8. The correlation coefficients for the linear fits range from 0.9193 to 0.9964. A representative chromatogram of an improved separation for these four complexes provided by increasing the column temperature to 65 °C is presented in Figure 6.9. Increasing the temperature resulted in improved peak shape and a shorter elution time.

### 6.3.4. Effect of the Sample Concentration on the Observed Peak Profile

During the course of this study, it was observed that the peak profile for the last complex which eluted, Cu-CB-TE2LA (Figure 6.3, peak #4) was asymmetric. Further investigation showed that the asymmetry of the elution peaks increased for the other three complexes, as the retention time was increased by reducing the concentration of the mobile phase organic modifier. An example of these results is presented in Figure 6.10 for complex, Cu-CB-DO2LA. One of the potential sources of peak asymmetry is the HPLC system employed. Uracil was used as a reference to evaluate the HPLC system. The reason why uracil was chosen includes the following: it is a polar compound that is hardly retained on C8 and C18 stationary phases, and is widely used as a dead volume...
Figure 6.8. Plot of log $k'$ vs. $1/T$ for the complexes: Cu-CB-D02A (0.11mg/ml), Cu-CB-D02LA (0.11mg/ml), Cu-CB-TE2A (0.13mg/ml), and Cu-CB-TE2LA (0.14mg/ml), with the use of a graphitic carbon stationary phase for mobile phase: (a) methanol/water or (b) acetonitrile/water.
Figure 6.9. Separation of Cu-CB-TE2A, Cu-CB-DO2A, Cu-CB-TE2LA, and Cu-CB-DO2LA with a column temperature of 65 °C. Mobile phase: 30% (v/v) methanol/water; Column: Hypercarb (100×3mm, 5μm); Detection: UV absorbance λ=280nm; Peaks: 1= Cu-CB-DO2A (0.11mg/ml); 2= Cu-CB-DO2LA (0.11mg/ml); 3= Cu-CB-TE2A (0.13mg/ml); 4= Cu-CB-TE2LA (0.14mg/ml).
Figure 6.10. Peak profiles for Cu-CB-DO2LA (0.11mg/ml) obtained by using different concentrations of methanol in the mobile phase. Column: Hypercarb (100×3mm, 5µm); column temperature: 25 °C; Detection: UV absorbance λ=280nm.
marker in HPLC. The peak profiles for uracil obtained using mobile phases with
different concentrations of methanol are presented in Figure 6.11. The peak asymmetry
is 1.2 for a mobile phase of 15% (v/v) methanol. This result indicates that the HPLC
system is not the source of the asymmetry observed for the four complexes.

Sample overloading is another potential contribution to the peak asymmetry
observed for these complexes. A representative chromatogram obtained using a mobile
phase containing acetonitrile and a sample mixture at one-tenth the concentration used to
obtain the chromatogram in Figure 6.6 is shown in Figure 6.12. The peak shapes
obtained for Cu-CB-TE2LA using the diluted sample (Figure 6.12) is clearly more
symmetric than for the more concentrated sample. Improved peak shape was also
observed for the complexes in the diluted sample mixture when using a mobile phase
containing methanol. This result suggests that sample overloading is likely one of the
sources of peak tailing for these compounds when using a graphitic carbon stationary
phase.

6.4. Conclusions

The separation of four Cu(II)-azamacrocyclic complexes was achieved with the
use of a graphitic carbon stationary phase and aqueous mobile phases containing either
methanol or acetonitrile as the organic modifier. The retention of these polar copper
complexes on the graphitic carbon stationary was enhanced significantly relative to what
was observed using C8 or C18 stationary phases. Gradient elution and increasing the
column temperature provided more symmetrical peak shapes, better separations and
Figure 6.11. The peak profiles for uracil (0.067mg/ml) obtained by using mobile phases having different concentration of methanol. Column: Hypercarb (100×3mm, 5μm), column temperature: 25 °C; Detection: UV absorbance λ=254nm.
Figure 6.12. Peak profiles for a one-tenth dilution of the sample concentration for the complexes with the use of a mobile phase of 10% (v/v) acetonitrile/water. Column: Hypercarb (100×3mm, 5μm), column temperature: 25 °C; Detection: UV absorbance λ=280nm; Peaks: 1= Cu-CB-DO2A (0.011mg/ml); 2= Cu-CB-DO2LA (0.011mg/ml); 3= Cu-CB-TE2A (0.013mg/ml); 4= Cu-CB-TE2LA (0.014mg/ml).
shorter separation times. Sample overloading was one of the factors that resulted in the peak tailing for these complexes.
CHAPTER 7
SUMMARY AND FUTURE STUDIES

Different approaches to indirect photometric and fluorometric HPLC detection have been reviewed based on the different types of interactions occurring between the analyte and the detectable component. This review provides guidelines for the choice of detectable component, mode of interaction, and specific experimental conditions to be developed and adapted for specific applications. One of the approaches to indirect detection, which is based on the fluorescence recovery of L-tryptophan and its analogue, DL-5-methoxytryptophan, has been successfully applied to the detection of amino sugars including glucosamine in dietary supplements following chromatographic separation. This indirect fluorescence detection method is a suitable alternative to methods based on pre-column derivatization for determining glucosamine concentrations for analyses where the detection limit is not a significant consideration. Advantages of using this approach to indirect fluorescence detection include avoiding a time-consuming pre-column derivatization step and possible stability problems of the derivatization products. In a separate study, improved separations of copper(II)-azamacroyclic complexes have been successfully achieved by reversed-phase HPLC and ion-pair HPLC. Enhanced retention and the separation of the complexes with a graphitic carbon stationary phase have also been demonstrated.

Considering that the ligands for these copper(II)-azamacroyclic complexes are capable of binding copper(II) ion, studies could be conducted to investigate the
application of this indirect fluorescence detection method for detecting those ligands that do not have a chromophore or a fluorophore. Several issues should be addressed for this application: 1) how the kinetics of the reaction between the copper(II) and the ligands to be detected indirectly affects the amount of detectable component, L-tryptophan or DL-5-methoxytryptophan, released from its copper(II) complex, therefore the detection limit of the ligands to be detected indirectly; 2) establishing chromatographic conditions which separate the ligands to be detected indirectly.

Efforts could also be extended to the study of estimating the lipophilicity of metal complexes by reversed-phase high-performance liquid chromatography (RP-HPLC) and capillary electrophoresis (CE). Metal complexes have been used increasingly as diagnostic agents and anti-tumor drugs. The most widely known is cisplatin (cis-diamminedichloroplatinum(II)), which has been employed to treat certain cancers for over thirty years [313,314]. Luminescent lanthanide(III) complexes have been used as probes for medical analysis and diagnosis [315,316]. Complexes of paramagnetic metal ions, such as Gd(III), Mn(II), and Fe(III), have been used as magnetic resonance imaging contrast agents [262,317]. The application of radiometal-labeled compounds as imaging and therapeutic agents has been studied [261,262]. Several reviews have discussed the properties and applications of such metal complexes [318]. The biological behavior of these metal complexes is influenced by the ligands that are employed to chelate the metal ions. The increasing use of metal complexes in medicine has led to the design of ligands that possess desirable chelating ability and result in suitable biological behavior.

A prime physico-chemical descriptor that is an important indicator of the biological behavior of compounds is lipophilicity. Lipophilicity, which is expressed as
log \( P \), has been estimated by measuring the 1-octanol-water partition coefficient \( (P) \), a measure of the distribution ratio of the compound between 1-octanol and water [298]. The so-called shake-flask method has been the traditional technique for determining the partition coefficient. Due to the limitations of this traditional method, such as the high-purity of samples required and the fact that the measurements are time-consuming, chromatographic and electrophoretic methods have been investigated as more efficient ways for estimating lipophobicity. Several reviews discussing the estimation of lipophobicity by reversed-phase high-performance liquid chromatography (RP-HPLC) and capillary electrophoresis (CE) have been published recently [279,299,301,319,320].

There are very few reports of the estimation of lipophobicity of metal complexes by RP-HPLC or CE [319,321,322]. Mostly the traditional shake-flask method is still utilized to measure the lipophobicity of metal complexes. The reliability of the log \( P \) data obtained by this method may be questionable since the presence of impurities in the metal complexes can significantly affect the magnitude of the partition coefficient measured. The log \( P \) data obtained using the shake-flask method have been applied to the construction of quantitative structure-activity relationships (QSAR) studies of metal complexes [323].

The following research projects could be conducted to advance the investigation of the lipophobicity estimation for metal complexes using RP-HPLC and CE methods.

- The role of the RP-HPLC stationary phases in the estimation of log \( P \) for metal complexes needs to be investigated. It is expected that differences in the log \( P \) values obtained using different stationary phases will provide information on the interactions which occur between the metal complexes and the stationary phases.
• The effect of the organic modifier in the RP-HPLC mobile phase used to estimate log $P$ values and to extrapolate to $\log(k_{\infty})$ should be also studied.

• The log $P$ values of the ligands should also be measured and correlated to the log $P$ values of the corresponding metal complexes. The lipophilicity of the metal complexes is dependent on the lipophilicity of the ligands and the coordination structure of the complexes defined by the metal ions and the ligands.

Future research projects should initially investigate copper(II) complexes since the results presented in Chapter 5 show the successful application of RP-HPLC for estimating the lipophilicity of three copper(II) azamacrocyclic complexes. Other metal complexes could be the subject of additional studies. These complexes could include complexes of gadolinium(III), gallium(III), iron(III), lanthanide(II), and ruthenium(II), which have promising medical applications.

As the impact of metals, which usually complex with inorganic, organic, or biological ligands, on the environment becomes the subject of public concern [324], studies on such metal complexes also become more important. Thus, an additional benefit of the research proposed here is that it will provide separation methods for supporting research in the future concerning the analysis of metal complexes in environmental as well as biological samples.
REFERENCES


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


[34] G. Vigh and A. Leitold, J. Chromatogr. 312 (1984) 345


[71] P. R. Haddad and M. Y. Croft, Chromatographia 21 (1986) 648


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.


