Novel Analytical Methods for Improved Analysis of Biological Compounds

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Martin Joseph Beres
Graduate Program in Chemistry

The Ohio State University
2015

Dissertation Committee:

Dr. Susan V. Olesik – Advisor
Dr. Prabir K. Dutta
Dr. Vicki H. Wysocki
Dr. Pravin T.P. Kaumaya
Copyright by
Martin Joseph Beres
2015
Abstract

The work contained within this dissertation focuses on innovative technologies in the field of analytical chemistry, particularly within high-performance liquid chromatography (HPLC) and mass spectrometry (MS). Enhanced-fluidity liquids (EFL), which have low viscosity and high diffusivity, were studied as alternative mobile phases in mixed-mode hydrophilic interaction strong ion-exchange chromatography (HILIC/SCX). Additionally, these mobile phases were evaluated as environmentally friendly alternatives to traditional HILIC solvents in gradient separations. Finally, electrospun nanofibrous materials with high surface area to volume ratios were assessed as substrates in surface-assisted laser desorption/ionization mass spectrometry (SALDI-MS).

The potential of enhanced-fluidity liquid chromatography (EFLC) HILIC/SCX was explored, using amino acids as analytes. EFL mobile phases were prepared by adding liquefied CO₂ to methanol:water (MeOH:H₂O) mixtures, which increases the diffusivity and decreases the viscosity of the mixture. The optimized chromatographic performance of these MeOH:H₂O:CO₂ EFL mixtures was compared to traditional acetonitrile:water (ACN:H₂O) and MeOH:H₂O liquid chromatography (LC) mobile phases. MeOH:H₂O:CO₂ mixtures offered higher efficiencies and resolution of the ten amino acids relative to the MeOH:H₂O LC mobile phase, and decreased the required
isocratic separation time by a factor of two relative to the ACN:H₂O LC mobile phase. Large differences in selectivity were also observed between the EFLC and LC mobile phases. Retention mechanism studies revealed that the EFLC mobile phase separation was governed by a mixed-mode retention mechanism of HILIC/SCX. On the other hand, separations with ACN:H₂O and MeOH:H₂O LC mobile phases were strongly governed by only one retention mechanism, either HILIC or SCX, respectively.

EFLC was then evaluated for “green” HILIC separations. The impact of CO₂ addition to a MeOH:H₂O mobile phase was studied as an alternative to traditional ACN:H₂O HILIC mobile phases, while also optimizing buffer type, ionic strength, and pH. Using EFLC mixtures, a separation of 16 RNA nucleosides/nucleotides was achieved in 16 minutes with greater than 1.3 resolution for all analyte pairs. By using a reverse CO₂ gradient, analysis time was reduced by over 100% in comparison to isocratic conditions. The optimal separation using MeOH:H₂O:CO₂ mobile phases was also compared to that using MeOH:H₂O and ACN:H₂O mobile phases. Based on the chromatographic performance parameters (efficiency, resolution, and speed of analysis) and the overall environmental impact of the mobile phase mixtures, MeOH:H₂O:CO₂ mixtures were preferred to ACN:H₂O or MeOH:H₂O mobile phases for the separation of mixtures of these RNA nucleosides and nucleotides.

Finally, electrospun nanofibrous substrates were studied for the improvement of SALDI-MS analysis of large molecular weight proteins and polymers without the use of a chemical matrix. Various polymers (including polyacrylonitrile, polyvinyl alcohol, and SU-8 photoresist) and carbon substrates were examined. SALDI analysis using these
substrates eliminated “sweet spot” formation typically seen in matrix-assisted laser desorption/ionization (MALDI), which lead to greater shot-to-shot reproducibility. The fiber diameter of these substrates played a significant role in the quality of the mass spectra generated, with smaller fiber diameter yielding higher signal to noise ratio (S/N). Additionally, the degree of pyrolysis also impacted the degree of fragmentation and overall S/N for the prepared carbon substrates.
For Grant W. Gretta and Joseph C. Zwolinski
Acknowledgments

I could never have completed this Ph.D. without support and guidance from many individuals. First and foremost, I would like to thank my family. To my parents, Tom and Susan Beres, you have both shaped me into the man I am today, and I can never thank you enough for all you have done for me throughout the years. I hope you know how much I appreciated your emotional (and occasionally financial) support throughout these last five years. I also want to thank my sister, Justine, for her never-ending encouragement and reassurance. Your tough love was always just what I needed to give me some perspective, especially whenever I felt down and discouraged. To my grandmother, Josephine Zwolinski, I know a phone call to you can always brighten my day. I know you would literally do anything for me, and my debt to you (and St. Joe) can never be repaid. Finally, to my aunt Carol and cousin Autumn, both of you have been there for me in countless ways throughout the years, and I know I can continue to count on you whenever I need you.

Secondly, I would like to acknowledge Dr. Susan Olesik for her leadership and influence throughout my graduate school career. Your guidance has allowed me to grow as a scientist, and I am very appreciative that you were always willing to help me and critique my work. Even when I was struggling with a project, you showed me the importance of keeping a positive attitude and of believing in my abilities. For that, I
thank you. I have learned a lot from you over the last five years, and I know that I will be prepared for my career thanks to your education and leadership.

Next, I would like to thank the Olesik group members, past and present, for both creating a positive work environment where science and fun can coexist. I especially would like to thank Joseph Zewe, Cherie Pomeranz, Toni Newsome, Mike Beilke, Hui Wang, Xin Fang, June Kampaalanonwat, Jiayi “Seven” Liu, Yanhui “Celia” Wang, and Raffeal Bennett, all of whom I formed strong friendships with throughout the last 5 years. The memories we shared will always mean so much to me, and I wish the best of luck to all of you in your future endeavors.

Last but certainly not least, I need to thank my lovely fiancée, Courtenay Samsel. The moment I learned of our mutual love for The Office, mac and cheese, and Taco Bell, I knew you were the one for me. We have had our ups and our downs, especially when our relationship went long-distance, but I’ve never doubted for a moment that I want to spend the rest of my life with you. We have now both survived the trials and tribulations of graduate school, and I can’t wait to see what is in store for us next as we move to the next chapter of our lives in Boston. But most importantly, I can’t wait to marry you next fall. I could not have made this journey without you, and I hope that you will continue to be my “pickle” and allow me to be your “moose.”

To all of you, thank you so much. Each and every one of you is an important part of my life and I really could not have made it to this point without your love, friendship, and support.
Vita

2006.................................................................Rocky River High School

2009-2010 .......................................................Undergraduate Research Associate,
Department of Chemical and Biomolecular Engineering, University of Notre Dame

2010.................................................................B.S. Chemical Engineering, University of
Notre Dame

2010-2011 ..........................................................Graduate Teaching Associate, Department
of Chemistry and Biochemistry, The Ohio State University

2011-2014 ..........................................................Graduate Metro Fellow, College of Arts and
Sciences, The Ohio State University

2014-present.......................................................Graduate Research Associate, Department
of Chemistry and Biochemistry, The Ohio State University
Publications


Presentations


Martin J. Beres, William F. Schneider, Grand-Canonical Monte Carlo Simulations of Oxygen on Platinum(111)” – Notre Dame Undergraduate Research Conference 2010, Notre Dame, IN.

Fields of Study

Major Field: Chemistry
Table of Contents

Abstract ...................................................................................................................................................... ii

Acknowledgments ...................................................................................................................................... vi

Vita ............................................................................................................................................................... viii

Publications ................................................................................................................................................. ix

Presentations ............................................................................................................................................... ix

Fields of Study .......................................................................................................................................... ix

Table of Contents ...................................................................................................................................... x

List of Tables ............................................................................................................................................... xv

List of Figures .............................................................................................................................................. xviii

List of Abbreviations ................................................................................................................................. xxvii

CHAPTER 1: INTRODUCTION ......................................................................................................................... 1

1.1 Concepts in chromatography .................................................................................................................. 1

1.1.1 Fundamental parameters in chromatography ................................................................................... 1

1.1.2 Separation mechanisms in liquid chromatography .............................................................................. 11

1.1.3 Isocratic vs. gradient elution ............................................................................................................. 13

1.2 Introduction to enhanced-fluidity liquid chromatography ..................................................................... 15
1.2.1 Properties of enhanced-fluidity liquids................................................................. 15
1.2.2 Enhanced-fluidity liquids as mobile phases for liquid chromatography ........ 16
1.3 Introduction to electrospinning .................................................................................. 19
1.4 Electrospun substrates for laser desorption/ionization mass spectrometry .......... 22
  1.4.1 Matrix-assisted laser/desorption ionization................................................................. 22
  1.4.2 Surface-assisted laser/desorption ionization................................................................. 23
  1.4.3 Matrix-enhanced surface-assisted laser/desorption ionization............................ 23
1.5 Research focus........................................................................................................... 25
1.6 References ................................................................................................................ 26

CHAPTER 2: ENHANCED-FLUIDITY LIQUID CHROMATOGRAPHY FOR
MIXED-MODE HYDROPHILIC INTERACTION/STRONG CATION-
EXCHANGE SEPARATION OF AMINO ACIDS ....................................................... 32
  2.1 Introduction .............................................................................................................. 32
  2.2 Materials and methods ............................................................................................ 37
    2.2.1 HPLC/EFLC setup................................................................................................. 37
    2.2.2 Mobile phase preparation ...................................................................................... 41
    2.2.3 Sample preparation .............................................................................................. 42
    2.2.4 Chromatography and data analysis ...................................................................... 43
  2.3 Results and discussion ............................................................................................. 43
CHAPTER 3: GRADIENT ENHANCED-FLUIDITY LIQUID HYDROPHILIC INTERACTION CHROMATOGRAPHY - A PATH TOWARDS “GREEN” SEPARATIONS

3.1 Introduction ....................................................................................... 89

3.2 Materials and methods .................................................................... 96

3.2.1 Instrumentation ............................................................................ 96

3.2.2 Chemicals ................................................................................... 96

3.2.3 Mobile phase preparation ............................................................ 98

3.2.4 Data analysis .............................................................................. 98

3.3 Results and discussion .................................................................... 99
CHAPTER 4: ELECTROSPUN NANOFIBERS AS SUBSTRATES FOR SURFACE-ENHANCED LASER DESORPTION/IONIZATION MASS SPECTROMETRY ANALYSIS OF LARGE MOLECULES

4.1 Introduction ................................................................................. 148

4.2 Materials and methods ................................................................. 152

4.2.1 Chemicals .............................................................................. 152

4.2.2 Instrumentation........................................................................ 152

4.2.3 Substrate preparation............................................................... 153

4.2.4 Sample preparation................................................................. 156

4.3 Results and discussion............................................................... 156
4.3.1 Preparation of electrospun polymeric SALDI substrates ................................ 156
4.3.2 SALDI of high molecular weight proteins ................................................. 157
4.3.3 ME-SALDI of high molecular weight proteins ........................................... 166
4.3.4 Fabrication of nanofibrous carbon SALDI substrates .................................. 170
4.3.5 Impact of fiber diameter on carbon substrate SALDI performance ............. 178
4.3.6 Impact of pyrolysis temperature on carbon substrate SALDI performance ... 182
4.4 Conclusions ...................................................................................................... 187
4.5 References ....................................................................................................... 188

CHAPTER 5: SUMMARY AND FUTURE WORK .................................................. 193

5.1 Research summary ............................................................................................ 193
5.2 Enhanced-fluidity liquid chromatography - Future work ................................. 194
5.3 Electrospun nanofibrous SALDI substrates – Future Work .............................. 196
5.4 References ....................................................................................................... 197

BIBLIOGRAPHY ................................................................................................. 199
List of Tables

Table 2.1. Slopes of $H$ vs $u$ curves above the optimum flow velocity for both LC and EFLC conditions, calculated using least-squares linear regressions. R-squared values are indicated in parenthesis. ................................................................. 56

Table 2.2. Enthalpy and entropy of transfer of amino acids from the mobile phase to the stationary phase for each mobile phase composition studied, as determined from van’t Hoff plots. The uncertainty in each value is reported as ± one standard deviation. .......... 74

Table 3.1. Nucleoside and nucleotide structures with corresponding logP values .......... 93

Table 3.2. Retention factors for nucleosides (A, U, C, G), monophosphate nucleotides (AMP, UMP, CMP, GMP), diphosphate nucleotides (ADP, UDP, CDP, GDP), and triphosphate nucleotides (ATP, UTP, CTP, GTP) under isocratic conditions using various ACN:100 mM sodium phosphate (pH=2.65) and MeOH:400 mM sodium phosphate (pH=2.65) mobile phases. ........................................................................................................... 111

Table 3.3. Retention factor ranges for nucleosides (A, U, C, G), monophosphate nucleotides (AMP, UMP, CMP, GMP), diphosphate nucleotides (ADP, UDP, CDP, GDP), and triphosphate nucleotides (ATP, UTP, CTP, GTP) under isocratic conditions using 80:20 (v:v) MeOH:H$_2$O (40 mM sodium phosphate, pH=2.65) mobile phases with various amounts of CO$_2$ added. ........................................................................................................... 116
Table 3.4. S value ranges for nucleosides (A, U, C, G), monophosphate nucleotides (AMP, UMP, CMP, GMP), diphosphate nucleotides (ADP, UDP, CDP, GDP), and triphosphate nucleotides (ATP, UTP, CTP, GTP) with varying mobile phases. 

Table 3.5. Method parameters and HPLC-EAT scores for optimized ACN:H₂O, MeOH:H₂O, and MeOH:H₂O:CO₂ separations. Optimized conditions are shown in Figure 3.13.

Table 4.1. Average fiber diameter of electrospun polymer nanofibers, reported with standard deviation.

Table 4.2. Average S/N for synthetic polymers [26] and proteins, with relative standard deviation (in parenthesis) indicating shot-to-shot reproducibility. SU-8 was used for PS (Mₗ = 5120) and PVA was used for TF.

Table 4.3. Average fiber diameters for electrospun SU-8 under different electrospinning conditions.

Table 4.4. Fiber diameter shrinkage as a result of pyrolysis for SU-8 fibers with an as-spun average fiber diameter of 270 nm.

Table 4.5. S/N of SALDI spectra for PEG (Mₗ = 3400) on carbon substrates with different average fiber diameters. The pyrolysis temperature was 600 °C.

Table 4.6. S/N of SALDI spectra for PS (Mₗ = 5120) on carbon substrates with different average fiber diameters. The pyrolysis temperature was 600 °C.

Table 4.7. S/N of SALDI spectra for PEG (Mₗ = 3400) on carbon substrates processed to different pyrolysis temperatures.
Table 4.8. S/N of SALDI spectra for PS ($M_w = 5120$) on carbon substrates processed to different pyrolysis temperatures. ................................................................. 186
List of Figures

Figure 1.1. Typical chromatogram obtained from a chromatographic experiment. ........ 3

Figure 1.2. Example van Deemter plot. ................................................................. 7

Figure 1.3. Diagram illustrating how (A) efficiency, (B) asymmetry, and (C) resolution information can be obtained from a chromatogram.................................................. 10

Figure 1.4. Typical van Deemter plots under HPLC and EFLC mobile phase conditions. ............................................................................................................................ 18

Figure 1.5. Illustration of a typical electrospinning apparatus.................................... 21

Figure 1.6. Illustration of MALDI mechanism.......................................................... 24

Figure 2.1. Molecular structure of ten amino acids. ............................................... 36

Figure 2.2. Chemical functionality of PolySulfoethyl A\textsuperscript{TM} column. .................. 38

Figure 2.3. Diagram of instrumentation used for EFLC experiments. ....................... 40

Figure 2.4. Effect of methanol content on retention of amino acids in HPLC. Mobile phase conditions: methanol/water with 15 mM ammonium formate buffer (pH = 3.0).
Analytes: L-trp (•), L-phe (■), L-leu (▲), L-tyr (X), L-val (∗), L-pro (●), L-thr (—), L-ala (+), L-gln (═), L-asn (♦). ................................................................................................................................. 45

Figure 2.5. Effect of buffer concentration on retention of amino acids in methanol/water mobile phases. Mobile phase conditions: 80:20 methanol:water buffered with ammonium
formate to pH=3.0. Error bars are contained within the size of the data points. Analytes:
L-trp (♦), L-phe (■), L-leu (▲), L-tyr (X), L-val (*), L-pro (●), L-thr (—), L-ala (+), L-gln (—), L-asn (♦).

**Figure 2.6.** Effect of acetonitrile content on retention of amino acids in HPLC. Mobile phase conditions: acetonitrile/water with 15 mM ammonium formate buffer (pH = 3.0).

Analytes: L-trp (♦), L-phe (■), L-leu (▲), L-tyr (X), L-val (*), L-pro (●), L-thr (—), L-ala (+), L-gln (—), L-asn (♦).

**Figure 2.7.** Effect of buffer concentration on retention of amino acids in acetonitrile/water mobile phases. Mobile phase conditions: 80:20 acetonitrile/water buffered with ammonium formate to pH=3.0. Error bars are contained within the size of the data points. Analytes: L-trp (♦), L-phe (■), L-leu (▲), L-tyr (X), L-val (*), L-pro (●), L-thr (—), L-ala (+), L-gln (—), L-asn (♦).

**Figure 2.8.** Variation of amino acid retention as a function of CO₂ added to mobile phase. Mobile phase conditions: 75:25 methanol:water (pH = 3.0). Analytes: L-trp (♦), L-phe (■), L-leu (▲), L-tyr (X), L-val (*), L-pro (●), L-thr (—), L-ala (+), L-gln (—), L-asn (♦).

**Figure 2.9.** Effect of CO₂ addition on chromatographic efficiency. Mobile phase conditions: 75:25 methanol:water (pH = 3.0) with 0% (■), 15% (■), and 30% (■) CO₂ by volume and 80:20 acetonitrile:water (pH=3.0) (■). Flow rate = 1.0 mL/min. Error bars correspond to ± one standard deviation, n = 3.

**Figure 2.10.** van Deemter plots for L-proline (orange circles) and L-asparagine (black diamonds) under LC-methanol/water (hollow markers), LC-acetonitrile/water (patterned markers).
markers), and EFLC (shaded markers) conditions. LC conditions: 75:25 methanol:water (15mM NH$_4^+$ formate, pH=3.0) and 80:20 acetonitrile:water (15mM NH$_4^+$ formate, pH=3.0). EFLC conditions: 75:25 methanol:water (15mM NH$_4^+$ formate, pH=3.0) with 30% by vol. CO$_2$.  

**Figure 2.11.** Efficiency to retention time ratio (N/t) for optimized mobile phases. Mobile phase conditions: 75:25 methanol:water (15mM NH$_4^+$ formate, pH = 3.0) with 0% (■) and 30% (■) CO$_2$ by volume, and 80:20 acetonitrile:water (15mM NH$_4^+$ formate, pH = 3.0) (■). Flow rate = 1.0 mL/min. Error bars correspond to ± one standard deviation, n = 3.  

**Figure 2.12.** Separation of 10 amino acids with varying CO$_2$ content in methanol/water mobile phases. Mobile phase conditions: 75:25 methanol:water (pH = 3.0), flow rate = 1.0 mL/min. CO$_2$ concentrations were (A) LC-0% (B) EFLC-15% and (C) EFLC-30% by volume. Analytes: L-pro (1), L-phe (2), L-tyr (3), L-leu (4), L-val (5), L-thr (6), L-trp (7), L-ala (8), L-gln (9), L-asn (10).  

**Figure 2.13.** Effect of CO$_2$ addition on resolution of amino acid pairs. Mobile phase conditions: 75:25 methanol:water (pH = 3.0) with 0% (■), 15% (■), and 30% (■) CO$_2$ by volume. Flow rate = 1.0 mL/min. Error bars correspond to ± one standard deviation, n = 3.  

**Figure 2.14.** Separation of 10 amino acids with varying mobile phase compositions. (A) 80:20 acetonitrile:water (pH=3.0), (B) 75:25 methanol:water (pH = 3.0), and (C) 75:25 methanol:water (pH = 3.0) with 30% vol. CO$_2$. Buffer concentration was 15mM in each
mobile phase, and flow rate was 1.0 mL/min. Analytes: L-pro (1), L-phe (2), L-tyr (3), L-leu (4), L-val (5), L-thr (6), L-trp (7), L-ala (8), L-gln (9), L-asn (10).

**Figure 2.15.** van’t Hoff plot for LC – 80:20 acetonitrile:water (15mM NH$_4^+$ formate, pH = 3.0). The temperature range studied was 30-55ºC. Solid lines indicate linear regressions. Analytes: L-trp (♦), L-phe (■), L-leu (▲), L-tyr (X), L-val (*), L-pro (●), L-thr (—), L-ala (+), L-gln (──), L-asn (♦).

**Figure 2.16.** van’t Hoff plot for LC – 75:25 methanol:water (15mM NH$_4^+$ formate, pH = 3.0). The temperature range studied was 30-55ºC. Solid lines indicate linear regressions. Analytes: L-trp (♦), L-phe (■), L-leu (▲), L-tyr (X), L-val (*), L-pro (●), L-thr (—), L-ala (+), L-gln (──), L-asn (♦).

**Figure 2.17.** van’t Hoff plot for EFLC – 75:25 methanol:water (15mM NH$_4^+$ formate, pH = 3.0) with 30% CO$_2$ by volume. The temperature range studied was 30-55ºC. Solid lines indicate linear regressions. Analytes: L-trp (♦), L-phe (■), L-leu (▲), L-tyr (X), L-val (*), L-pro (●), L-thr (—), L-ala (+), L-gln (──), L-asn (♦).

**Figure 2.18.** Enthalpy-entropy compensation plot at 25ºC for LC – 80:20 acetonitrile:water (15 mM NH$_4^+$ formate, pH = 3.0).

**Figure 2.19.** Enthalpy-entropy compensation plot at 25ºC for LC – 75:25 methanol:water (15 mM NH$_4^+$ formate, pH = 3.0).

**Figure 2.20.** Enthalpy-entropy compensation plot at 25ºC for EFLC – 75:25 methanol:water (15mM NH$_4^+$ formate, pH = 3.0) with 30% CO$_2$ by volume.

**Figure 2.21.** Enthalpy-entropy compensation plots at 40ºC for EFLC – 75:25 methanol:water (15mM NH$_4^+$ formate, pH = 3.0) with 30% CO$_2$ by volume.
Figure 3.1. Chromatogram of 16 nucleoside/nucleotide analyte mixture (1.25 x 10^{-4} M) with MeOH:H_{2}O:CO_{2} mobile phase - Component A: CO_{2}, Component B: 80:20 (v:v) MeOH:H_{2}O. Gradient program is (0-1.50 min) 70% B, (1.50-10.00 min), 70-90% B with 1.00 mL/min flow rate. Analyte key: (1) A, (2) U, (3) G, (4) C, (5) AMP, (6) UMP, (7) CMP, (8) GMP, (9) ADP, (10) UDP, (11) CDP, (12) GDP, (13) ATP, (14) UTP, (15) CTP, (16) GTP.

Figure 3.2. Isocratic separation of A, AMP, ADP, and ATP analyte mixture (1.25 x 10^{-4} M) with 80:20 (v:v) MeOH:H_{2}O mobile phase containing 10 mM ammonium phosphate buffer (pH=2.00).

Figure 3.3. Variation of average retention factor (k*) as a function of mobile phase pH. Mobile phase component A: CO_{2}, mobile phase component B: 80:20 (v:v) MeOH:H_{2}O containing 25 mM sodium phosphate buffer at pH=2.00 (■), pH=2.65 (■), and pH=3.15 (■). Gradient program: (0-1.00 min) 70% B, (1.00-6.67 min) 70-90% B with 1.50 mL/min flow rate.

Figure 3.4. Peak widths for 16 nucleoside/nucleotide analytes (100 ppm) as a function of pH. Mobile phase component A: CO_{2}, component B: 80:20 (v:v) MeOH:H_{2}O containing 25 mM sodium phosphate buffer at pH=2.00 (■), pH=2.65 (■), and pH=3.15 (■). Gradient program used is the same as in Figure 3.3.

Figure 3.5. Peak widths for 16 nucleoside/nucleotide analyte mixture (100 ppm) as a function of buffer concentration. Mobile phase component A: CO_{2}, component B: 80:20 (v:v) MeOH:aqueous phosphate buffer at pH=2.65 with an ionic strength of 10 mM (■).
25 mM (■), and 40 mM (▲) in the mobile phase. Gradient program used is same as in Figure 3.3. .............................................................. 107

**Figure 3.6.** Effect of ACN content on retention factor of A (♦), AMP (■), ADP (▲), and ATP (+). Remaining mobile phase content was 100 mM sodium phosphate (pH=2.65).

Note: Some retention factors of ADP and ATP are off scale (>25) in (B) – see Table 3.2. .................................................................................................................................. 109

**Figure 3.7.** Effect of MeOH on retention factor of A (♦), AMP (■), ADP (▲), and ATP (+). Remaining mobile phase content was 100 mM sodium phosphate (pH=2.65). .... 110

**Figure 3.8.** Effect of CO₂ content on retention factor of A (♦), AMP (■), ADP (▲), and ATP (+). Mobile phase component A: CO₂, mobile phase component B: 80:20 (v:v) MeOH:H₂O with 40 mM sodium phosphate buffer, pH=2.65. .............................................. 115

**Figure 3.9.** Log k vs. φ plots for 100 ppm of A (♦), AMP (■), ADP (▲), and ATP (+) for ACN:H₂O mobile phases. Mobile phase component A: ACN, mobile phase component B: 100 mM sodium phosphate (pH=2.65). Lines represent linear regressions.......... 117

**Figure 3.10.** Log k vs. φ plots for 100 ppm of A (♦), AMP (■), ADP (▲), and ATP (+) for MeOH:H₂O mobile phases. Mobile phase component A: MeOH, mobile phase component B: 400 mM sodium phosphate (pH=2.65). Lines represent linear regressions. ........................................................................................................................................ 118

**Figure 3.11.** Log k vs. φ plots for 100 ppm of A (♦), AMP (■), ADP (▲), and ATP (+) for MeOH:H₂O:CO₂ mobile phases. Mobile phase component A: CO₂, mobile phase component B: 80:20 (v:v) MeOH:H₂O (40 mM sodium phosphate, pH=2.65). Lines represent linear regressions................................................................. 119
Figure 3.12. Isoelutropic nomogram comparing percentage of weak eluent needed to obtain identical solvent strength for (A) nucleosides, (B) monophosphate nucleotides (C) diphosphate nucleotides, and (D) triphosphate nucleotides. The strong eluent is H$_2$O for ACN and MeOH and 80:20 (v:v) MeOH:H$_2$O for CO$_2$......................................................... 121

Figure 3.13. Optimized separation of the 16 nucleoside/nucleotide analyte mixture (1.25 x 10$^{-4}$ M) with (A) ACN:100 mM sodium phosphate (pH=2.65) (0-7.5 min, 30% B, 7.5-15 min, 30-40% B, hold 40% B), (B) MeOH:400 mM sodium phosphate (pH=2.65) (0-4 min, 10% B, 4-6 min, 10-20% B, hold 20% B), and (C) MeOH:H$_2$O:CO$_2$ with gradient and sodium phosphate as described in Section 3.3.4. Analyte key is the same as Figure 3.1. Mobile phase gradients indicated with dashed lines......................................................... 127

Figure 3.14. Resolution data for (■) ACN:H$_2$O, (■) MeOH:H$_2$O and (■) MeOH:H$_2$O:CO$_2$ optimized separations (mobile phase conditions listed in Figure 3.13). Dashed line represents R=1.0. ................................................................. 128

Figure 3.15. Variation in elution order (from top to bottom) for nucleosides/nucleotides under different mobile phase conditions................................................................. 129

Figure 3.16. Efficiency data for early-eluting peaks (those which elute prior to the gradient reaching the column) for (■) MeOH:H$_2$O:CO$_2$, (■) MeOH:H$_2$O and (■) ACN:H$_2$O optimized separations (mobile phase conditions listed in Figure 3.13). ....... 132

Figure 3.17. Pseudo van Deemter plots for 100 ppm of (A) A, (B) AMP, (C) ADP, and (D) ATP for the MeOH:H$_2$O:CO$_2$ (♦), MeOH:H$_2$O (■), and ACN:H$_2$O (▲) optimized mobile phases. Peak volumes were measured at various flow rates under optimized mobile phase conditions in Figure 3.13, adjusting gradient parameters accordingly. .... 133
Figure 3.18. Resolution of neighboring analyte pairs under optimized EFLC gradient conditions (Section 3.3.4) using 0.50 mL/min (■), 1.00 mL/min (■), 1.50 mL/min (■), and 2.00 mL/min (■) flow rates, adjusting gradient parameters accordingly. The dashed line marks a resolution of 1.0................................................................. 135

Figure 3.19. Separation of the 16 nucleoside/nucleotide analyte mixture \((1.25 \times 10^{-4} \text{ M})\) with (A) 0.50 mL/min, (B) 1.00 mL/min, (C) 1.50 mL/min, and (D) 2.00 mL/min flow rates, using optimized EFLC gradient conditions (Section 3.3.4). Analyte key is the same as Figure 3.1...................................................................................... 136

Figure 4.1. Electrospinning setup modified for SALDI substrate preparation on a commercial MALDI target plate............................................................................................................. 155

Figure 4.2. Chemical structures of (A) PAN, (B) PVA, and (C) SU-8 photoresist...... 159

Figure 4.3. SEM images of electrospun (A) PAN, (B) PVA, and (C) SU-8 photoresist. .............................................................................................................................................. 160

Figure 4.4. SALDI mass spectrum of 2 mg/mL BSA on PAN substrate.................... 163

Figure 4.5. SALDI mass spectrum of 1 mg/mL TF on PVA substrate...................... 164

Figure 4.6. ME-SALDI spectra of (A) 2 mg/mL BSA on PAN substrate and (B) 1 mg/mL TF on PVA substrate. Matrix concentration = 20 mg/mL sinapinic acid. ........ 167

Figure 4.7. S/N for varying concentrations of (A) BSA and (B) TF on stainless steel (■), PVA (■), and PAN (■) substrates. Matrix concentration = 20 mg/mL sinapinic acid.. 168

Figure 4.8. ME-SALDI spectrum of 0.05 mg/mL BSA on PVA substrate. Matrix concentration = 40 mg/mL sinapinic acid................................................................. 169
Figure 4.9. Digital photographs of SU-8 nanofibers (A) as-spun, (B) pyrolyzed to 450°C, (C) pyrolyzed to 600°C, and (D) pyrolyzed to 750°C on a stainless target plate........... 174

Figure 4.10. SEM images of SU-8 nanofibers (A) as-spun, (B) pyrolyzed to 450°C, (C) pyrolyzed to 600°C, and (D) pyrolyzed to 750°C. .............................................................. 176

Figure 4.11. Mass spectra of 40 mg/mL PEG (M_w = 3400) using carbon nanofibrous substrates with average fiber diameters of (A) 250 nm, (B) 180 nm, and (C) 160 nm. The final pyrolysis temperature was 600°C. ................................................................. 179

Figure 4.12. Mass spectra obtained from 40 mg/mL PEG (M_w = 3400) on carbon substrates processed to different final temperatures during pyrolysis. ......................... 184
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_s$</td>
<td>peak asymmetry factor</td>
</tr>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>DHB</td>
<td>2,5-dihydroxybenzoic acid</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>EFL</td>
<td>enhanced-fluidity liquid</td>
</tr>
<tr>
<td>EFLC</td>
<td>enhanced-fluidity liquid chromatography</td>
</tr>
<tr>
<td>GA</td>
<td>glutaraldehyde</td>
</tr>
<tr>
<td>$H$</td>
<td>theoretical plate height</td>
</tr>
<tr>
<td>HILIC</td>
<td>hydrophilic interaction liquid chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>HPLC-EAT</td>
<td>high-performance liquid chromatography environmental assessment tool</td>
</tr>
<tr>
<td>IEC</td>
<td>ion-exchange chromatography</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>$K$</td>
<td>distribution constant</td>
</tr>
<tr>
<td>$k$</td>
<td>retention factor</td>
</tr>
<tr>
<td>$k^*$</td>
<td>average retention factor</td>
</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
<tr>
<td>MALDI</td>
<td>matrix-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>ME-SALDI</td>
<td>matrix-enhanced surface-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>$m/z$</td>
<td>mass to charge ratio</td>
</tr>
<tr>
<td>$N$</td>
<td>theoretical plate number (chromographic efficiency)</td>
</tr>
<tr>
<td>$N/t$</td>
<td>efficiency to time ratio</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>PAN</td>
<td>polyacrylonitrile</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PS</td>
<td>polystyrene</td>
</tr>
<tr>
<td>PVA</td>
<td>polyvinyl alcohol</td>
</tr>
<tr>
<td>R</td>
<td>resolution</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SALDI</td>
<td>surface-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>SCX</td>
<td>strong cation-exchange</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>SFC</td>
<td>supercritical fluid chromatography</td>
</tr>
<tr>
<td>S/N</td>
<td>signal to noise ratio</td>
</tr>
<tr>
<td>SubFC</td>
<td>subcritical fluid chromatography</td>
</tr>
<tr>
<td>$t_R$</td>
<td>retention time</td>
</tr>
<tr>
<td>TF</td>
<td>transferrin</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TOF</td>
<td>time-of-flight</td>
</tr>
<tr>
<td>$u$</td>
<td>linear velocity</td>
</tr>
<tr>
<td>UV/Vis</td>
<td>ultraviolet/visible</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>separation factor</td>
</tr>
<tr>
<td>$\Delta H$</td>
<td>enthalpy of transfer</td>
</tr>
<tr>
<td>$\Delta S$</td>
<td>entropy of transfer</td>
</tr>
<tr>
<td>$\phi$</td>
<td>phase ratio</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION

1.1 Concepts in chromatography

1.1.1 Fundamental parameters in chromatography

While chromatography has evolved into numerous differing techniques throughout the years, all chromatography is essentially a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (mobile phase) moves in a definitive direction [1]. This separation is driven by differences in the relative distribution of the sample components between the two phases, which is described by the distribution constant, $K$, for a single component (Equation 1). $K$ is given by Equation 1.1:

$$K = \frac{C_S}{C_M}$$ (1.1)

where $C_S$ and $C_M$ represent the concentration of an analyte in the stationary phase and the concentration of an analyte in the mobile phase, respectively.

The chromatography work described in this dissertation is a form of column chromatography, where the stationary phase is packed within a column and the mobile phase is a liquid. This is known as high-performance liquid chromatography (HPLC). Accordingly, the rest of the definitions and parameters that follow will be described as they are defined in HPLC.
The information obtained from a chromatographic experiment is contained within a chromatogram, which is typically a plot of detector response (y-axis) versus time or versus the volume of mobile phase that has passed through the column (x-axis). An example is shown in Figure 1.1. This signal normally contains a number of peaks of various sizes, rising from a baseline signal. This chromatogram can then be analyzed to determine information about the completed separation.

The position of a peak in a chromatogram is characterized by its retention time \(t_R\) or its retention volume \(V_R\). Both \(t_R\) and \(V_R\) are different ways of expressing the same information, however \(t_R\) is directly observable and more commonly used [2]. \(t_R\) is made of two components, the time the analyte spends in the mobile phase, or dead time \(t_M\), and the time it spends in stationary phase, or adjusted retention time \(t_R'\). It is given by Equation 1.2.

\[
t_R = t_M + t_R' \tag{1.2}
\]

All analytes spend the same amount of time in the mobile phase, which can be observed from an unretained solvent peak in a chromatogram. However, \(t_R'\) varies depending on a sample’s interaction with the stationary phase. It is this variation that results in the physical separation of the sample components. This difference is characterized by the individual retention factor \((k)\) of each sample component, which is the ratio of the time a substance spends in the stationary phase to the time it spends in the mobile phase, and is given by Equation 1.3.

\[
k = \frac{t_R'}{t_M} = \frac{t_R - t_M}{t_M} \tag{1.3}
\]
Figure 1.1. Typical chromatogram obtained from a chromatographic experiment.
As it turns out, these retention factors are directly proportional to the distribution coefficient (K), according to equation 1.4:

\[ K = k\beta \]  

(1.4)

where \( \beta \) is the phase ratio, which is defined as the ratio of the volume of mobile phase to the volume of accessible stationary phase.

Since \( t_{R'} \), \( k \), and \( K \) are all related by some constant, they can all be used to describe the separation (or selectivity) factor (\( \alpha \)) of two peaks within a chromatogram, which is a measure of the ability of a chromatographic system to separate two solutes, \( a \) and \( b \). (Equation 1.5).

\[ \alpha = \frac{t_{R_b'}}{t_{R_a'}} = \frac{k_b}{k_a} = \frac{K_b}{K_a} \]

(1.5)

By convention, the earlier eluting compound is defined as \( a \), so that \( \alpha \) is always greater or equal to 1, with higher values indicating a higher degree of separation.

Retention time, retention factor, and separation factor are all important chromatographic parameter determined from the positions of peaks within a chromatogram. However, the width and shape of these peaks are also indicators of chromatographic performance. The width of a chromatographic peak is a measure of the how much an analyte spreads out from the time it is injected onto the column to the time it reaches the detector. This phenomenon is called band broadening and it is what determines the chromatographic efficiency of a separation, expressed as either plate number \( (N) \) or plate height \( (H) \). In terms of plate height, the extent of band broadening is
predicted by the van Deemter equation [3] (Equation 1.6), where smaller values of $H$ indicate higher efficiencies.

$$H = A + \frac{B}{u} + (C_S + C_m)u$$  \hspace{1cm} (1.6)

A, B, C_s, and C_m are constants and $u$ is the linear velocity of the mobile phase through the column. The $A$-term represents the contribution from multipath flow through a packed column, and is proportional to particle packing diameter according to Equation 1.7,

$$A \propto 2\lambda d_p$$  \hspace{1cm} (1.7)

where $\lambda$ is the packing factor of the stationary phase and $d_p$ is the diameter of the particles of the stationary phase. The $B$-term signifies band broadening due to longitudinal diffusion, and is given by Equation 1.8,

$$B \propto \frac{2\gamma D_m}{u}$$  \hspace{1cm} (1.8)

where $D_m$ is the diffusion coefficient in the mobile phase and $\gamma$ is a tortuosity factor.

Finally, the $C$-terms characterize the contributions from resistance to mass transfer within the stationary ($C_s$) and mobile ($C_m$) phases, according to equations 1.9 and 1.10,

$$C_s \propto \frac{R d_f^2 u}{D_s}$$  \hspace{1cm} (1.9)

$$C_m \propto \frac{\omega d_p^2 u}{D_m}$$  \hspace{1cm} (1.10)

where $d_f$ is film thickness of the stationary phase, and $R$ and $\omega$ are also tortuosity factors.

From these definitions, it is evident that $H$ is largely dependent on characteristics of the column stationary phase packing ($d_f, d_p, D_s, \gamma, \omega$, and $R$), characteristics of the mobile phase ($D_m$), and the mobile phase velocity ($u$). Additionally, the various contributions
from individual terms are largely a function of $u$. Therefore, for a given stationary phase and mobile phase, there exists an optimum mobile phase velocity, $u_{opt}$, at which a minimum plate height, $H_{min}$, will be achieved. $u_{opt}$ and its corresponding $H_{min}$ can be determined from a van Deemter plot (Figure 1.2). This is the mobile phase flow rate at which maximum efficiency will be achieved for a specific chromatographic system.
Figure 1.2. Example van Deemter plot.
One can also express chromatographic efficiency in terms of plate number, $N$, which is related to plate height according to equation 1.11,

$$N = \frac{L}{H}$$

(1.11)

where $L$ is the length of the column. Since $N$ is inversely related to $H$, larger plate numbers indicate increased efficiency. $N$ can also be directly measured from a chromatogram, by measuring the peak width. For a Gaussian peak, $N$ is given by:

$$N = \left(\frac{t_R}{\sigma_t}\right)^2$$

(1.12)

where $t_R$ is retention time and $\sigma_t$ is the band variance in time units. The peak width can be measured at different peak heights, for which $N$ can be calculated according to equation 1.13,

$$N = a\left(\frac{t_R}{w}\right)^2$$

(1.13)

where $a = 5.54$ if $w = \text{width of the peak at half height ($w_{1/2}$)}$, and $a = 16$ if $w = \text{width of the peak at its base ($w_b$)}$. This is illustrated in Figure 1.3A.

While the efficiency of peaks within a chromatographic separation is very important, the shape of the peak is important as well. While simple theory assumes that all peaks should be Gaussian, the reality is often quite different for many separations. This can be due to many sources, including extra column effects, incomplete resolution of sample components, slow mass transfer processes, and column packing voids [4,5]. Since significant errors can result from the calculation of chromatographic parameters based on this false assumption, today’s software provides integration tools or curve
fitting routines to correctly assess these values. A common example of deviation from Gaussian peak shape is peak asymmetry. This is described by a peak asymmetry factor ($A_s$) for each peak within a chromatogram, according to equation 1.14:

$$A_s = \frac{BC}{AB}$$  \hspace{1cm} (1.14)

where $AB$ is the horizontal distance between the left side of the peak and its center and $BC$ is the horizontal distance between the center of the peak and its right side, generally measured at 10% of the peak height (Figure 1.3B). When $A_s$ is greater than 1, the peak is said to have positive skew (called “tailing”), When $A_s$ is less than 1, the peak is said to have negative skew (called “fronting”).

Finally, arguably the most important parameter in a chromatographic separation is that of resolution, $R$, which is a measurement of the degree of separation between two compounds/peaks. This depends on both the width and shape of the peak, and is given by equation 1.15:

$$R = \frac{2\Delta t}{(w_{b1} + w_{b2})}$$  \hspace{1cm} (1.15)

where $\Delta t$ is the separation of the two peak maxima, and $w_{b1}$ and $w_{b2}$ are the average widths at the base of the two peaks (Figure 1.3C). An $R$ value of 1.0 corresponds to a valley separation of about 94% and is generally an adequate goal for an optimized separation, but an $R$ of 1.5 is typically required for two peaks to be fully separated at the baseline.
Figure 1.3. Diagram illustrating how (A) efficiency, (B) asymmetry, and (C) resolution information can be obtained from a chromatogram.
1.1.2 Separation mechanisms in liquid chromatography

The term “liquid chromatography” technically includes any chromatographic technique that uses a liquid mobile phase. However, given the large number of possible chemical interactions between solutes and a stationary phase, there are easily over a dozen separation techniques used today. All of these techniques utilize one or more of five mechanisms of separation: adsorption, partitioning, ion-exchange, affinity, or size-exclusion interactions [2]. The main separation techniques discussed within this dissertation are summarized here.

Normal-phase liquid chromatography (NPLC) is an adsorption-based technique which uses a polar stationary phase and a nonpolar (typically organic) mobile phase. Analytes are separated based on their ability to participate in polar interactions with the stationary phase. As a result, highly polar compounds are retained longer and elute later than nonpolar compounds. Increasing the polarity of the mobile phase leads to decreases in retention. Although it was one of the first modes of chromatography to be developed, NPLC is relatively limited by the solubility of polar analytes in the required nonpolar, organic solvents. Additionally, retention reproducibility is often highly sensitive to even low concentrations of polar contaminants (like H$_2$O) [6,7]. As a result, NPLC has since mostly been replaced by a counterpart technique, called reversed-phase liquid chromatography (RPLC).

Reversed-phase chromatography is essentially the opposite of NPLC, and has become the most popular liquid chromatographic method due to its versatility and simplicity of operation. It has an exceptional range of applications, including both neutral
and ionic solutes. In RPLC, the stationary phase is a nonpolar material and the mobile phase is relatively polar. Generally, the mobile phase is an aqueous solution with some portion of organic modifier. Increasing the amount of organic solvent decreases the relative polarity of the mobile phase, which leads to decreases in retention, which is a reversal of the trend seen in NPLC. RPLC is generally considered to be governed by analyte partitioning, although adsorption processes cannot be ruled out entirely [8,9].

Hydrophilic interaction liquid chromatography (HILIC) is a chromatographic technique that has gained significant traction for separations of highly polar or charged analytes. First proposed in 1990 [10], HILIC is a method that uses polar stationary phases (like NPLC) with mobile phases similar to those used in RPLC. Retention of analytes is very similar to NPLC, with compounds of low polarity eluting first. Increasing the amount of organic content decreases the relative polarity of the mobile phase, which leads to increased retention, which is opposite of the behavior seen in RPLC. The exact retention mechanisms present in HILIC are still poorly understood, but present theories propose a partitioning mechanism with varying degrees of adsorption and electrostatic interactions also playing a role [11]. The partitioning mechanism is based on the differential distribution of the analyte solute molecules between an organic-rich mobile phase and a water-enriched layer that forms on the surface of a hydrophilic stationary phase [10,12]. Analytes are separated according to their differences in partitioning between the bulk of the mobile phase and the water-enriched layer immobilized at the surface of the stationary phase. Regardless HILIC has numerous advantages over either NPLC or RPLC methods for highly polar analytes. For example, solubility of highly
polar analytes is much greater in HILIC solvents than in NPLC solvents and retention of these compounds is much greater than the negligible retention often seen in RPLC.

Lastly, ion-exchange chromatography (IEC) is a mode of chromatographic separation used to separate ions or easily ionizable substances. Separation occurs entirely due to electrostatic interactions between sample ions dissolved in a mobile phase and immobilized centers of opposite charge in the stationary phase. Cation-exchange occurs when the stationary phase carries a negative charge and sample ions are positively charged, and anion-exchange occurs when the stationary phase carries a positive charge and sample ions are negatively charged. Samples with greater charge density are retained longer, and analyte retention is strongly correlated to the presence of other ions in the mobile phase. Presence of competing ions decreases analyte-stationary phase interactions and thus can be increased or decreased to achieve a desired level of retention. These separations can also be further classified into strong ion-exchange or weak ion-exchange, depending on whether the stationary phase maintains its charge across the entire pH range (strong) or only a small range (weak).

1.1.3 Isocratic vs. gradient elution

The simplest chromatography experiments are conducted using a mobile phase of fixed composition throughout the entire separation. These methods are referred to as “isocratic” methods. These methods work well for many samples and are the most convenient form of liquid chromatography. For some samples, however, no set of mobile phase conditions exists to provide a satisfactory separation within a reasonable amount of
time. This is known as the “general elution problem,” and generally occurs when a sample has components with a wide retention range [13].

One of the solutions to the general elution problem is the use of mobile phase gradients in chromatographic experiments, which is referred to as gradient elution. This practice involves starting with a mobile phase composition with weak eluent strength, and gradually adjusting the composition so that the eluent strength is also gradually increased. This provides better separation for weakly retained components at the beginning of the separation, while still eluting strongly retained components within a reasonable time.

The chromatographic parameters discussed in Section 1.1.1 are inherently defined for isocratic separations, but much work has been completed to define nearly equivalent parameters and equations for linear gradients using a linear solvent strength model [13]. For example, the retention factor, $k$, used in gradient separations is referred to as the average retention factor, $k^*$, which is the median value of $k$ (which is equal the instantaneous value of k when a sample component has migrated halfway through the column). Near exact equations for retention times, peak widths, and resolution have been derived as well, making gradient separations less intimidating to even novice chromatographers.

Gradient and isocratic separations each have their advantages/disadvantages. Isocratic separations are generally easier to develop, easier to transfer between instruments, and require no re-equilibration time between sample injections. As a result, they are often preferred for simple separations and high-throughput methods. Gradient
separations can be more difficult to develop, but they can offer greater separation capability, reduced analysis time, and reduced peak tailing. Accordingly, experienced chromatographers prefer gradient elution for the separation of many samples.

1.2 Introduction to enhanced-fluidity liquid chromatography

1.2.1 Properties of enhanced-fluidity liquids

Enhanced-fluidity liquids (EFL), also called gas expanded liquids (GXL) [14], are liquid mixtures to which high proportions of a liquefied gas have been added [15]. These mixtures are subcritical fluids that have characteristic properties in between those of traditional liquids and those of supercritical fluids [16].

The term “enhanced-fluidity” originated from the decrease in viscosity (inverse of fluidity) seen when liquefied gas is added to traditional liquids [15]. For example, both theoretical [17] and experimental [15,18] results indicate that addition of CO$_2$ to methanol and methanol/water mixtures substantially decreases the viscosity of the mixture in a way that is proportional to the amount of liquefied gas added. Typical EFL mixtures have a viscosity on the order of $10^{-3}$ g/cm·s, whereas the viscosity of liquids is on the order of $10^{-2}$ g/cm·s and that of supercritical fluids is on the order of $10^{-4}$ g/cm·s.

Another important property of enhanced-fluidity liquids is the increased diffusivity of solutes through the mixtures relative to traditional liquids. Typical values for diffusion coefficients are on the order of $10^{-5}$ cm$^2$/s, which is intermediate between those coefficients seen in liquids ($10^{-6}$ cm$^2$/s) and supercritical fluids ($10^{-3}$-$10^{-4}$ cm$^2$/s). Again, the increase in diffusivity is proportional to the amount of liquefied gas added. For
example, the diffusion coefficient of benzene in methanol/water mixtures increases by an order of magnitude as \( \text{CO}_2 \) is added to the mixture up to 50 mol\% [15].

It should be noted that there is often a limit to the amount of liquefied gas that can be added to a liquid mixture before miscibility issues arise. Phase diagrams can be generated for mixtures of different liquefied gases with different liquid solvents to indicate conditions where one phase is maintained and conditions where phase separations occur. A number of different organic solvents including tetrahydrofuran, dichloromethane, acetonitrile, and alcohols are highly miscible with \( \text{CO}_2 \) [19]. Our group has also shown that \( \text{CO}_2 \) is highly miscible with alcohol/water binary mixtures and to a small degree with acetonitrile/water mixtures [20].

1.2.2 Enhanced-fluidity liquids as mobile phases for liquid chromatography

The properties of enhanced-fluidity liquids (discussed in the previous section) make them interesting candidates for mobile phases in liquid chromatography. The increased diffusivity and decreased viscosity of EFL mobile phases have significant implications in the development of HPLC methods. Looking back to the van Deemter equation (equation 1.6), it is evident that the diffusivity of the mobile phase has a significant impact on both the B-term and C-term contribution to overall band broadening. By increasing the diffusivity of the mobile phase at constant linear velocity, the contribution from the B-term is increased while the C-term contribution is decreased. This effectively increases the flowrate at which optimum chromatographic efficiency is attained (Figure 1.4). Combined with the lower viscosity (and correspondingly lower
column back pressures), this makes EFL mobile phases ideal for fast, highly efficient separations. These methods which use EFL mobile phases are referred to by our group as enhanced-fluidity liquid chromatography (EFLC) methods.

EFLs as mobile phases in chromatography have been studied in depth by our group, mostly using CO₂ as the liquefied gas. To date, EFLC has offered improved separations in reversed-phase [21], normal-phase [22], size-exclusion [23], chiral [24], and HILIC [25]. However, EFL mobile phases have not been studied in separations governed by an ion-exchange mechanism nor has it been studied in gradient separations. This is the focus of Chapters 2 and 3 of this dissertation, respectively.
Figure 1.4. Typical van Deemter plots under HPLC and EFLC mobile phase conditions.
1.3 Introduction to electrospinning

Electrospinning is a method by which polymer nanofibers are generated by applying an electric potential difference between a syringe containing a conductive polymer solution and a grounded collector plate. Using a syringe pump, the polymer solution is slowly expelled from the tip of a syringe at a constant rate. A droplet of the polymer solution is held at the end of the syringe tip as a consequence of the polymer solution’s surface tension. At a certain critical applied voltage (usually > 6 kV), the surface tension of the polymeric solution is overcome by electrostatic forces, and the solution is pulled into a charged jet, forming a Taylor cone [26]. The charged jet begins to travel through space in an unstable whipping motion in the direction of a grounded, conductive collector, positioned some distance away from the end of the syringe tip [27]. As the jet travels towards the collector plate the solvent evaporates, leaving deposited nanofibers on the collector plate oriented in a random configuration [28]. The entanglement of the high molecular weight polymer chains prevents the electrically driven jet from breaking up, maintaining a continuous solution jet. This differentiates electrospinning from electrospraying, in which beads rather than fibers are formed [29,30,31].

A diagram of a typical electrospinning apparatus is shown in Figure 1.5. Hundreds of different polymers have been electrospun to produce nanofibers with varying fiber morphologies [28]. Numerous method parameters can be changed in order to alter physical characteristics of the produced nanofibers, including solution viscosity, solution conductivity, applied voltage, flow rate, ambient temperature/humidity, and
distance from syringe tip to collector [27]. Chapter 4 of this work highlights some optimization of these parameters in order to produce nanofibrous substrates for MALDI and SALDI analyses using polyacrylonitrile (PAN), polyvinyl alcohol (PVA), and SU-8 photoresist polymers.
Figure 1.5. Illustration of a typical electrospinning apparatus.
1.4 Electrospun substrates for laser desorption/ionization mass spectrometry

1.4.1 Matrix-assisted laser/desorption ionization

Matrix-assisted laser/desorption ionization (MALDI) is one of the most widely-used techniques in laser desorption ionization mass spectrometry. In MALDI, an organic matrix is used to absorb laser radiation and transfer the absorbed energy to analyte molecules (Figure 1.6). This matrix can either be mixed directly with the analyte sample or it can be spotted on the target plate separately [32,33]. Regardless, after the sample and matrix are spotted on the target plate, the solvent evaporates and analyte-matrix cocrystals form. Ideally a homogenous distribution results, free of “sweet spots” where the analyte is disproportionately concentrated. This is especially important for quantitative analysis [34]. A laser then irradiates the cocrystals, triggering ablation and desorption of the sample and matrix molecules. Finally, ionization of the sample molecules occurs and ions are accelerated into the mass spectrometer.

MALDI methods are relatively simple and fast, and work for thermally labile analytes over a large molecular weight range [35,36,37]. Typically these methods are used with a time-of-flight (TOF) mass spectrometer, due to its large mass range. Matrix selection is very important, although the selection of the matrix is usually based on empirical observations rather than theoretical predictions. Additionally, organic matrices can generate signals themselves, causing spectral interferences in the low mass region [38]. These interferences, along with “sweet spot” formation and reproducibility issues, are the main limitations of MALDI-MS.
1.4.2 Surface-assisted laser/desorption ionization

Surface-assisted laser/desorption ionization (SALDI) is a technique that was developed as an alternative to MALDI-MS methods [38]. In SALDI, a nanostructured inorganic material is used in place of an organic matrix. Like organic MALDI matrices, these inorganic materials typically exhibit strong UV absorption. Examples include carbon [38], silicon [39], and metal [40] nanoparticles. These materials absorb laser energy and transfer energy to analyte molecules, just as the organic matrices do in MALDI. The exact mechanism of SALDI, however, is a little less clear. What is known is that the nanostructure of the inorganic material is very important, as bulk materials do not generate signals that their nanostructured materials do [41].

1.4.3 Matrix-enhanced surface-assisted laser/desorption ionization

Matrix-enhanced surface-assisted laser/desorption ionization (ME-SALDI) is a less common method that combines MALDI and SALDI [42,43]. Both organic matrix and SALDI substrates are used in this technique. It is proposed that the SALDI material is responsible for absorption of the laser radiation and transfer of energy to the analyte molecules, while the organic matrix desorbs the analytes and serves as a proton source for ionization of the analytes [42]. Additionally, the organic matrix absorbs extra laser energy, preventing analyte fragmentation. ME-SALDI has been successfully applied to metabolite imaging and small molecule analysis [43].
Figure 1.6. Illustration of MALDI mechanism.
1.5 Research focus

The work described in the dissertation details investigations into new chromatographic separation techniques as well as research for the development of new substrates for laser desorption/ionization mass spectrometry. Chapter 2 evaluates the benefits of enhanced-fluidity liquid chromatography for strong cation-exchange and HILIC separations, using amino acids as the probe compounds for these fundamental studies. The work in Chapter 3 continues to assess enhanced-fluidity mobile phases as a “green” alternative to traditional HILIC mobile phases, this time using gradient programming to separate RNA nucleosides and nucleotides. Finally, the work in Chapter 4 examines nanofibrous electrospun materials as substrates for SALDI and ME-SALDI experiments, particularly for detection of large polymer and protein samples. Additionally, further work was completed to characterize and optimize the morphology of these substrates. Together, this work provides new insights into the analytical fields of chromatography and mass spectrometry.
1.6 References


CHAPTER 2: ENHANCED-FLUIDITY LIQUID CHROMATOGRAPHY FOR MIXED-MODE HYDROPHILIC INTERACTION/STRONG CATION-EXCHANGE SEPARATION OF AMINO ACIDS

2.1 Introduction

The earliest demonstration of ion-exchange chromatography (IEC) dates back to 1850, when Thompson and Way discovered that soil acts as an ion-exchanger to separate ammonia from a fertilizer solution [1,2]. The invention of synthetic ion-exchange resins [3] in the mid-1930s and the development of high-performance liquid chromatography (HPLC) have since furthered the advancement of IEC, and today it continues to have numerous applications in chromatographic separations. In particular, IEC has proven useful for high-performance liquid chromatography (HPLC) separations of charged biological compounds. These include, but are not limited to, separations of amino acids/amines [4,5,6], peptides/proteins [7,8], carbohydrates [9,10], glycoproteins [11], and nucleotides [12,13]. Separation of these compounds is particularly important within the pharmaceutical industry and in fields such as metabolomics/proteomics, so further improvements to these separations would be highly beneficial.

Recently, supercritical fluid chromatography (SFC) with ion-exchange columns has been explored as an alternative to improve upon HPLC ion-exchange methods, with limited success in chiral separations [14,15]. In particular, SFC has seen a resurgence of
interest in which the mobile phase used is actually a subcritical liquid. This makes the term “SFC” a bit of a misnomer, since the mobile phase is no longer truly “supercritical.” As a result, some have attempted to distinguish what is truly subcritical fluid chromatography (SubFC) from conventional SFC, while others continue to label chromatography in the subcritical range as “SFC.” Regardless, the advantages of SFC that are typically exploited (high diffusivity and low viscosity of the mobile phase) are present whether the fluid is supercritical or subcritical [16]. Most commonly this mobile phase is CO$_2$, possibly with a small amount of organic modifier (acetonitrile, methanol, ethanol, etc.) to increase the polarity and solvent strength of the mixture. However, two major setbacks are often encountered in SFC/SubFC when trying to analyze a sample of highly polar or ionic compounds. First, ionic analytes often have limited solubility in supercritical CO$_2$, even with significant organic modifier addition [17]. Second, assuming solubility of these analytes is achieved, elution times from the column are often long. Elution of ionic compounds has been achieved through the addition of ion-pair reagents to supercritical mobile phase mixtures [18,19]; however, SFC still has not emerged as a competitive technique amongst ion-exchange separations.

Enhanced-fluidity liquid chromatography (EFLC) offers solutions to these drawbacks while maintaining the advantages inherent to SFC/SubFC. Enhanced-fluidity liquid (EFL) mobile phases are liquid mixtures to which high proportions of a liquefied gas have been added [20]. Like traditional SubFC mobile phases, these mobile phases are subcritical liquids, but use CO$_2$ as a modifier rather than the primary solvent. Even with lower CO$_2$ proportions, these mobile phases offer enhanced diffusivities and lower
viscosities than solvent mixtures with no added CO$_2$, which increases solute mass transfer and reduces system back pressure. For example, adding 0.3 mol fraction CO$_2$ to a 70/30 methanol/water mixture increased the diffusion coefficient of benzene by an order of magnitude and decreased the mobile phase viscosity by 50% [21]. This often leads to improved chromatographic performance in terms of efficiency, resolution, and separation time. Additionally, these improvements are gained without significant loss of polarity from the original mobile phase [22], which allows for analysis of highly polar or ionic compounds. Thus, by combining conventional SubFC with EFLC, the entire solvent range from 0-100% organic solvent is spanned. To date, EFLC has offered improved separations in reversed-phase [23], normal-phase [24], size-exclusion [25], chiral [26], and HILIC [27]. However, EFL mobile phases have not been studied in separations governed by an ion-exchange mechanism.

The focus of this study is to examine the usefulness of EFLC for isocratic chromatographic separations in which ion-exchange is a primary retention mechanism. Ten neutral side chain amino acids were chosen as probe analytes (Figure 2.1). These are polar compounds not easily separated under typical SFC/SubFC conditions, as Thurbide et al. demonstrated that even 30% methanol modifier in carbon dioxide could not elute tryptophan (a relatively nonpolar amino acid) from a normal-phase column [28]. Addition of small proportions of water can help alleviate this problem, but typically only 5% (v/v) water can be added before mobile phase miscibility issues arise [16]. As a result, amino acids and other polar, ionizable compounds are usually separated via other HPLC methods. “Mixed-mode” hydrophilic interaction chromatography (HILIC)/strong
cation-exchange (SCX) HPLC columns are commonly used to enhance selectivity [7,29,30,31,32,33]. Consequently, a HILIC/SCX column was used in this EFLC study. In HILIC, hydrophilic interactions contribute to retention when the mobile phase is polar organic with a small portion of aqueous cosolvent [34]. This mechanism is not yet well-understood, but is generally postulated to be a result of analyte partitioning between the bulk mobile phase and a water-layer on the surface of the hydrophilic stationary phase [35]. However, more recent work has suggested a combination of adsorption and partitioning is likely [36]. Regardless, retention of solutes increases with solute polarity.

The reasons for choosing these analytes were three-fold. First, a need for accurate analysis of these amino acids, peptides, and proteins exists in a myriad of scientific fields. As the building blocks of these compounds, amino acids will help to yield a fundamental understanding of the behavior of these compounds under EFLC conditions. Second, ion-exchange chromatography is a common method for amino acid/peptide/protein HPLC analysis, so direct comparisons between LC and EFLC experiments should be easily attainable. Cation-exchange or anion-exchange can be used, however cation-exchange methods are more prevalent due the fact that the isoelectric points of most amino acids are > 6 and many silica-based columns are only stable at pH < 8. Finally, these compounds are difficult to analyze via SFC, due to their polarity and charged end groups [28,37]. Derivatization may be necessary to enhance solubility [38], or ion-pair reagents may be required [39]. Previous attempts to separate underivatized amino acids via SFC/SubFC ion-exchange have shown minimal success, achieving partial resolution of only three or four amino acids [40,41]. Thus, considerable room for improvement exists.
Figure 2.1. Molecular structure of ten amino acids.
2.2 Materials and methods

2.2.1 HPLC/EFLC setup

An HPLC system was constructed from various commercially available components. An ISCO 260D syringe pump (Teledyne ISCO, Inc., Lincoln, NE, USA) was connected to a 6-port injector fitted with a 10 µL external sample loop. The injector was connected to a 200 mm × 4.6 mm PolySULFOETHYL A™ (Figure 2.2) HILIC/SCX column packed with 5 µm particles and 300 Å pore size (PolyLC, Inc., Columbia, MD, USA). This column was surrounded by a Shimadzu CTO-20A column oven (Shimadzu Corporation, Kyoto, Japan), used to control the column temperature. The column outlet was connected to a Varian 380-LC evaporative light scattering detector (ELSD) (Agilent Technologies, Inc., Palo Alto, CA, USA), using nitrogen as the evaporator gas. This allowed for direct analysis of the amino acids without derivatization. The evaporator gas flow rate was set to 2.50 mL/min, the nebulizer temperature was set to 30 ºC, and the evaporator temperature was set to 80 ºC.
Figure 2.2. Chemical functionality of PolySulfoethyl A<sup>TM</sup> column.
For EFLC experiments, the HPLC system was used as described above with two minor changes. First, a second ISCO 260D syringe pump was used to deliver the liquid carbon dioxide required for EFL mobile phases. Second, a fused silica capillary restrictor with an internal diameter of 30-50 µm (Polymicro Technologies, Inc., Phoenix, AZ, USA) was connected between the column outlet and the ELSD. The length/diameter of this capillary was adjusted to maintain a constant column outlet pressure of 100 bar with varying mobile phase mixtures and flow rates. A diagram of this entire setup is shown in Figure 2.3. Commercial instrumentation, such as an Agilent 1260 Infinity Analytical SFC system, can also be used for EFLC experiments provided both the CO₂ and liquid pumps can be set anywhere from 0 to 100% of the total flow [42]. Typically these instruments use back pressure regulators instead of capillary restrictors, but both serve the same purpose.
Figure 2.3. Diagram of instrumentation used for EFLC experiments.
2.2.2 Mobile phase preparation

The liquid mobile phases used were binary mixtures of acetonitrile/water (v/v) or methanol/water (v/v) buffered with formic acid/ammonium formate mixture. This buffer was chosen because its volatility makes it compatible with the ELSD. HPLC grade acetonitrile (Fisher Scientific, Fairlawn, NJ, USA) and methanol (Fisher Scientific, Fairlawn, NJ, USA) were used directly. Water was purified using a Barnstead Nanopure Infinity system (Thermo Scientific, Asheville, NC, USA) before use. 0.5 M ionic strength buffer solution of pH = 3.0 was prepared by adding pre-calculated amounts of ammonium formate (Fisher Scientific, Fairlawn, NJ, USA) and formic acid (Aldrich Chemical Company, Milwaukee, WI, USA) to water. The buffers were stored at 4 °C. Small volumes of these buffers were diluted in acetonitrile/water or methanol/water mixtures to achieve desired mobile phase ratios and buffer concentrations. The final liquid mobile phases were degassed using a Branson 2210 ultrasonic cleaner (Branson Ultrasonics Corporation, Danbury, CT, USA) and then filtered.

Enhanced-fluidity mobile phase preparation was identical to liquid mobile phase preparation until the addition of liquefied CO₂. In order to prepare these mobile phases, a desired volume of buffered organic/aqueous mobile phase was held in one syringe pump, while CO₂ was held in another syringe pump (pressurized to 100 bar). The syringe pumps were then connected via stainless steel tubing. With both valves on the syringe pumps closed, a headspace was created in the pump containing the liquid mobile phase to allow room for CO₂ to enter. The desired volume of CO₂ was then added to the pump containing the liquid mobile phase while maintaining the CO₂ pump pressure at 100 bar.
The resulting enhanced-fluidity liquid was mixed by cycling the syringe pump up and down and then kept under pressure and allowed to reach room temperature before use. By measuring volumes of both organic/aqueous mobile phase and CO₂ at 100 bar, the concentration of CO₂ can easily be expressed in either volume fraction or mole fraction. Additionally, since buffer concentration effects retention in ion-exchange separations, the amount of buffer added to the organic/aqueous mixture was calculated beforehand to account for any dilution that might occur as unbuffered CO₂ was added. This ensured that the final mobile phases had the same buffer concentration, regardless of the amount of CO₂ added. 0, 15%, and 30% volume fractions of CO₂ were studied, which correspond to 0, 0.09, and 0.20 mole fractions, respectively.

2.2.3 Sample preparation

Amino acid samples were prepared by dissolving L-alanine, L-asparagine, L-glutamine, L-tyrosine, L-tryptophan (Sigma-Aldrich Corporation, St. Louis, MO, USA), L-proline, L-valine (Amresco, Inc., Solon, OH, USA), L-threonine, L-leucine (Calbiochem, San Diego, CA, USA) and L-phenylalanine (Eastman Kodak, Rochester, NY, USA) in 90:10 acetonitrile:water or 90:10 methanol:water mixtures at individual concentrations of 200 µg/mL. Each sample was filtered through a 0.2 µm PTFE filter (Fisher Scientific, Pittsburgh, PA, USA) before injection onto the column. Samples were stored at 4 °C when not in use and were prepared fresh weekly.
2.2.4 Chromatography and data analysis

In order to directly compare LC and EFLC separations, all chromatographic parameters remained unchanged with the exception of the mobile phase and the capillary restrictor. The mobile phase buffer concentration was maintained at 15 mM unless otherwise noted. This concentration was high enough to maintain adequate peak shape, but low enough to produce acceptable retention times of all amino acids when the column was operating under ion-exchange mode. The mobile phase pH was maintained at 3.0 to promote cation-exchange interactions, since all amino acids studied carry some degree of positive charge at that pH (the pKₐ’s of the carboxylic acid groups vary from ~ 2-3).

All experiments were conducted with the syringe pump in constant flow mode, at a flow rate of 1.00 mL/min. At least 20 column volumes (40 mL) of mobile phase were used to equilibrate the column prior to injection in both LC and EFLC experiments. Care was taken to flush EFLC mobile phases out of column for overnight storage, in order to prevent gaseous CO₂ from developing and drying out the inside of the column. Chromatograms were recorded using EZ Chrom Version 6.7 (Scientific Software Inc., Pleasanton, CA, USA). Retention factor, efficiency, and resolution data analyses were performed in triplicate using PeakFit Version 4 (SPSS Inc., Chicago, IL, USA).

2.3 Results and discussion

2.3.1 Retention in LC mode: Effect of organic modifier

Prior to performing EFLC experiments, it was important to document the effect of organic modifier addition on amino acid retention, as this can introduce hydrophilic
interactions on top of ion-exchange interactions. Both acetonitrile and methanol concentrations were varied, although acetonitrile is a weaker eluent and by far the most common organic modifier in HILIC separations [35]. However, far greater amounts of CO$_2$ can be added to methanol/water mixtures than can be added to acetonitrile/water mixtures before miscibility issues arise [43], making methanol/water/CO$_2$ solvents better for EFLC mobile phases. Thus methanol/water retention studies were completed as well.

Hydrophilic interactions were present to a much higher extent in acetonitrile/water mixtures than in methanol/water mixtures. Only the most polar of the ten amino acids, L-glutamine and L-asparagine, exhibited significant increases in retention factor as the methanol concentration was increased. Concentrations higher than 80% methanol did not appear to enhance retention or resolution of the remaining amino acids at all (Figure 2.4). Additionally, the order of elution did not correlate with the relative polarity of the amino acids, as would be expected for a HILIC separation. This suggests that a cation-exchange mechanism is predominantly responsible for retention under methanol/water mobile phases. This is further supported by Figure 2.5, which shows the effect of buffer concentration on retention of the analytes. Decreasing the buffer concentration drastically increases the retention factor of all amino acids in methanol/water mobile phases, as would be expected for a cation-exchange mechanism. In fact, as the buffer concentration is lowered from 200 mM to 5 mM, each amino acid sees over a 400% increase in retention factor.
Figure 2.4. Effect of methanol content on retention of amino acids in HPLC. Mobile phase conditions: methanol/water with 15 mM ammonium formate buffer (pH = 3.0). Analytes: L-trp (♦), L-phe (■), L-leu (▲), L-tyr (▲), L-val (●), L-pro (▲), L-thr (—), L-ala (+), L-gln (▬), L-asn (♦).
Figure 2.5. Effect of buffer concentration on retention of amino acids in methanol/water mobile phases. Mobile phase conditions: 80:20 methanol:water buffered with ammonium formate to pH=3.0. Error bars are contained within the size of the data points. Analytes: L-trp (♦), L-phe (■), L-leu (▲), L-tyr (X), L-val (♦), L-pro (●), L-thr (─), L-ala (+), L-gln (—we), L-asn (♦).
On the other hand, the volume of acetonitrile present within a mobile phase had a
tremendous effect on analyte retention. The most retained amino acid (L-asparagine)
showed an increase in retention factor of 2300% from 60% to 85% acetonitrile, while
even the least retained (L-tryptophan and L-phenylalanine) still showed increases of over
300% (Figure 2.6). This suggests that a strong HILIC mechanism is present when
acetonitrile/water is used as the mobile phase. Figure 2.7 shows that an ion-exchange
mechanism is also present, but the hydrophilic interactions are so strong that the ion-
exchange interactions make up a much smaller portion of the total interactions. Retention
factors of the amino acids remain almost unchanged until buffer concentrations are lower
than 10 mM. From 200 mM to 5 mM, retention factors increased as little as 13.3% (L-
threonine) to as much as 32.8% (L-asparagine). Even at 200 mM buffer concentration, all
amino acids exhibited significant retention with 80% acetonitrile content, suggesting
HILIC is the dominant mechanism governing retention in acetonitrile/water mixtures.
**Figure 2.6.** Effect of acetonitrile content on retention of amino acids in HPLC. Mobile phase conditions: acetonitrile/water with 15 mM ammonium formate buffer (pH = 3.0). 

Analytes: L-trp (♦), L-phe (■), L-leu (▲), L-tyr (♦), L-val (♦), L-pro (●), L-thr (─), L-ala (+), L-gln (──), L-asp (♦).
Figure 2.7. Effect of buffer concentration on retention of amino acids in acetonitrile/water mobile phases. Mobile phase conditions: 80:20 acetonitrile/water buffered with ammonium formate to pH=3.0. Error bars are contained within the size of the data points. Analytes: L-trp (♦), L-phe (■), L-leu (▲), L-tyr (♦), L-val (♦), L-pro (●), L-thr (─), L-ala (+), L-gln (←), L-asn (♦).
2.3.2 Retention in EFLC mode: Effect of CO$_2$ addition

Methanol/water EFL mobile phases with varying proportions of CO$_2$ were studied to determine the effect of CO$_2$ on retention behavior. Figure 2.8 shows the retention behavior of the ten amino acids as the volume percent of CO$_2$ was varied from 0-30%. Increasing the volume percent of CO$_2$ past 40% (0.3 mol fraction) resulted in mobile phase miscibility issues. Phase diagrams of methanol/water/CO$_2$ mixtures support this result [43], as only 0.4 mol fraction CO$_2$ can be added to methanol/water mixtures containing 10% (v/v) water before phase separation occurs. The majority of the analytes showed a slight increase in retention factor as the volume percent of CO$_2$ was increased. The magnitude of this increase ranged from 5.9% (L-leucine) to 43.0% (L-asparagine). This behavior of increased retention with increasing CO$_2$ content has also been observed in the retention of polar nucleosides [27] and nucleotides [44] in an EFL-HILIC separation using other stationary phases, so it is possible that this increase in retention is due to reduced eluent strength of the mobile phase. Carbon dioxide is known to exhibit nonpolar solvent strength between hexane and carbon tetrachloride [45], which would decrease the HILIC eluent strength of a methanol/water mixture.
Figure 2.8. Variation of amino acid retention as a function of CO$_2$ added to mobile phase. Mobile phase conditions: 75:25 methanol:water (pH = 3.0). Analytes: L-trp (♦), L-phe (■), L-leu (▲), L-tyr (×), L-val (♦), L-pro (●), L-thr (—), L-ala (+), L-gln (▬), L-asn (♦).
Three of the analytes (L-phenylalanine, L-tryptophan, and L-tyrosine), however, exhibited a decrease in retention as more CO₂ was added. The magnitude of these decreases ranged from -0.8% (L-tyrosine) to -6.0% (L-tryptophan). While these decreases are slight, it is clear that no increase in retention was observed for these analytes. Interestingly, these are the most nonpolar of the amino acids studied, and therefore the least likely to be retained strongly via a HILIC mechanism. Further analysis of the mechanisms present is detailed later in section 2.3.6.

2.3.3 Efficiency

The variation in chromatographic efficiency, N, was studied for mobile phases with different proportions of CO₂ for a given methanol/water ratio (Figure 2.9). The addition of 30% by vol. CO₂ (0.2 mol fraction) has been shown to increase the diffusivity of solutes in methanol/water mixtures by almost a factor of 3 [22], which should lower band dispersion at or above the optimum flow velocity. At a flow rate of 1.0 mL/min, EFLC mixtures showed increased efficiencies for all ten amino acids relative to their LC counterparts. The magnitude of this increase was greatest for those compounds which exhibited only small changes in retention as a result of CO₂ addition. Those compounds that showed a larger increase in retention typically exhibited a smaller increase in efficiency, with the exception of L-glutamine. This can be attributed to the fact that band dispersion is directly proportional to the retention factor of the analyte when mobile phase mass transfer is the controlling force [46].
Figure 2.9. Effect of CO$_2$ addition on chromatographic efficiency. Mobile phase conditions: 75:25 methanol:water (pH = 3.0) with 0% (■), 15% (■), and 30% (■) CO$_2$ by volume and 80:20 acetonitrile:water (pH=3.0) (■). Flow rate = 1.0 mL/min. Error bars correspond to ± one standard deviation, n = 3.
Additionally, van Deemter plots for the amino acids illustrate three key advantages of methanol/water/CO$_2$ mobile phases over methanol/water mobile phases: higher optimum flow velocity, lower minimum plate height, and shallower slope above the optimum flow rate. Plots of plate height, $H$ vs. linear flow velocity, $u$ for L-proline and L-asparagine are shown in Figure 2.10. These advantages indicate this EFLC mobile phase can offer some of the benefits typically seen in SFC/SubFC experiments, while still maintaining the polarity that HPLC mobile phases can offer. While the optimum flow rates herein (~1.25-1.75 mL/min) are lower than typical optimum flow rates in SFC (3.0 mL/min) for a column of these dimensions, they are still significantly higher than those in HPLC (1.0 mL/min). Additionally, moving above the optimum flow rate of these EFLC mobile phases decreases efficiency of the separation at a much slower rate (Table 2.1). All of these advantages can lead to improved separations of highly polar or ionic compounds not typically possible via SFC or SubFC.
Figure 2.10. van Deemter plots for L-proline (orange circles) and L-asparagine (black diamonds) under LC-methanol/water (hollow markers), LC-acetonitrile/water (patterned markers), and EFLC (shaded markers) conditions. LC conditions: 75:25 methanol:water (15mM NH$_4^+$ formate, pH=3.0) and 80:20 acetonitrile:water (15mM NH$_4^+$ formate, pH=3.0). EFLC conditions: 75:25 methanol:water (15mM NH$_4^+$ formate, pH=3.0) with 30% by vol. CO$_2$. 
Table 2.1. Slopes of $H$ vs $u$ curves above the optimum flow velocity for both LC and EFLC conditions, calculated using least-squares linear regressions. R-squared values are indicated in parenthesis.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>LC (methanol/water)</th>
<th>EFLC (methanol/water/CO$_2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slope (sec)</td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td>342.8 (0.99)</td>
<td>148.2 (.97)</td>
</tr>
<tr>
<td>Phe</td>
<td>297.3 (0.98)</td>
<td>74.1 (0.96)</td>
</tr>
<tr>
<td>Tyr</td>
<td>247.3 (0.99)</td>
<td>329.6 (0.99)</td>
</tr>
<tr>
<td>Leu</td>
<td>744.1 (0.99)</td>
<td>264.1 (0.99)</td>
</tr>
<tr>
<td>Val</td>
<td>548.5 (0.99)</td>
<td>266.8 (0.97)</td>
</tr>
<tr>
<td>Thr</td>
<td>720.7 (0.99)</td>
<td>523.5 (0.99)</td>
</tr>
<tr>
<td>Trp</td>
<td>1067.1 (0.99)</td>
<td>192.3 (0.99)</td>
</tr>
<tr>
<td>Ala</td>
<td>583.0 (0.99)</td>
<td>272.1 (0.96)</td>
</tr>
<tr>
<td>Gln</td>
<td>351.5 (0.98)</td>
<td>48.6 (0.95)</td>
</tr>
<tr>
<td>Asn</td>
<td>233.6 (0.96)</td>
<td>149.3 (0.96)</td>
</tr>
</tbody>
</table>
Figure 2.9 and Figure 2.10 clearly illustrate that the chromatographic efficiencies of methanol/water/CO$_2$ EFLC mixtures are drastically higher than their methanol/water LC counterparts. However, they also indicate that acetonitrile/water mixtures perform comparably or even slightly better than methanol/water EFLC mixtures with 30% CO$_2$ added, at least in terms of efficiency. However, if one considers the efficiency of the separation along with the separation time, it becomes evident that the EFLC mixture offers the best efficiencies in the shortest amount of time. Figure 2.11 shows these efficiency/time (N/t) ratios for each of the optimized mobile phases. With the exception of L-tryptophan, the N/t ratio for each of the amino acid peaks is highest for the methanol/water/CO$_2$ mixture.
Figure 2.11. Efficiency to retention time ratio (N/t) for optimized mobile phases. Mobile phase conditions: 75:25 methanol:water (15mM NH$_4^+$ formate, pH = 3.0) with 0% (■) and 30% (■) CO$_2$ by volume, and 80:20 acetonitrile:water (15mM NH$_4^+$ formate, pH = 3.0) (■). Flow rate = 1.0 mL/min. Error bars correspond to ± one standard deviation, n = 3.
2.3.4 Resolution

The resolution, $R$, of the 10 amino acids was greatly improved under enhanced-fluidity conditions in comparison to methanol/water LC conditions. As seen in Figure 2.4, changing methanol content has only a very small effect on the retention of the amino acids. While decreasing the buffer concentration (Figure 2.5) does have a significant effect on retention, its effect is similar across the range of amino acids. Additionally, the buffer concentration must be maintained high enough to maintain adequate peak shape. As a result, even the optimized methanol/water mobile phase cannot isocratically resolve all ten amino acids. Figure 2.12 shows chromatograms under “optimized” LC and EFLC mobile phase conditions, where the goal is to achieve baseline resolution of all amino acids in the shortest time possible. Under optimized methanol/water LC conditions, only seven identifiable peaks could be seen, many with only partial resolution between them. Upon the addition of 15% CO$_2$ to that same LC mobile phase, 9 amino acid peaks could be identified, however L-alanine and L-tryptophan still co-elute. Continued addition of CO$_2$ up to 30% improved resolution even further. Under these EFLC conditions, all 10 amino acids were baseline-resolved. This is a clear improvement over using a methanol/water LC mobile phase, and results from a combination of increased retention for some of the analytes and improved efficiencies. Additionally, these chromatograms demonstrate that ionic species can in fact be analyzed via EFLC. Ionic analyte solubility, symmetrical peak shape, and reasonable retention times are demonstrated, areas in which traditional SFC and SubFC struggle to compete with LC ion-exchange methods. Finally,
this separation is also a significant improvement over previous SFC methods for underivatized amino acid separation [40].

Figure 2.13 illustrates the effect of CO$_2$ addition on the resolution of each pair of adjacent amino acid peaks. In general, the highest resolution was obtained at 30% CO$_2$ for each pair. However, the resolution between two pairs (L-proline and L-phenylalanine, L-threonine and L-tryptophan) actually decreased as CO$_2$ was added. This can be attributed to the fact that both L-phenylalanine and L-tryptophan showed no retention increases as more CO$_2$ was added to the mobile phase, while both L-proline and L-threonine showed increases in retention. However, this decrease in resolution is of no concern since both pairs of amino acids had resolutions well over 1.0, even at 30% CO$_2$. 
Figure 2.12. Separation of 10 amino acids with varying CO$_2$ content in methanol/water mobile phases. Mobile phase conditions: 75:25 methanol:water (pH = 3.0), flow rate = 1.0 mL/min. CO$_2$ concentrations were (A) LC-0% (B) EFLC-15% and (C) EFLC-30% by volume. Analytes: L-pro (1), L-phe (2), L-tyr (3), L-leu (4), L-val (5), L-thr (6), L-trp (7), L-ala (8), L-gln (9), L-asn (10).
Figure 2.13. Effect of CO$_2$ addition on resolution of amino acid pairs. Mobile phase conditions: 75:25 methanol:water (pH = 3.0) with 0% (■), 15% (■), and 30% (■) CO$_2$ by volume. Flow rate = 1.0 mL/min. Error bars correspond to ± one standard deviation, n = 3.
2.3.5 Comparison with acetonitrile/water LC mobile phases

While the previous results show that methanol/water/CO₂ EFLC mobile phases offer improved chromatographic performance over their methanol/water LC counterparts, it is also worth comparing to other optimized LC mobile phases. Acetonitrile is the most commonly used organic solvent for HILIC/SCX separations because it is a weak eluent, although it is expensive and environmentally unfriendly. Therefore, an alternative “greener” mobile phase (such as methanol/water/CO₂) that performs comparably well could be useful.

Figure 2.14 shows a chromatogram of the optimized isocratic acetonitrile/water separation, along with the optimized methanol/water and methanol/water/CO₂ separations. The ten amino acids can in fact be resolved isocratically by an 80:20 acetonitrile/water mixture buffered to pH = 3.0, with high efficiencies. The acetonitrile/water mobile phase with high HILIC interactions exhibits higher efficiencies than the methanol/water LC mobile phase (Figure 2.9). This is expected, since HILIC separations are typically highly efficient provided ion-exchange contributions are small. Adding CO₂ to the methanol/water mixture increases efficiency of the separation, most certainly due to increased diffusivity and increased HILIC interaction. Consequently, van Deemter plots for this EFLC mixture begin to approach those of acetonitrile/water (Figure 2.10) as well. However, the acetonitrile/water LC separation (60 min) takes over twice as long as the optimized EFLC methanol/water/CO₂ mixture (25 min) at the same flow rate. Looking back to Figure 2.6, it can be seen that lower proportions of acetonitrile would decrease the overall analysis time, but at the expense of resolution. Three pairs of
compounds are barely baseline resolved at 80% acetonitrile (Figure 2.14), and at smaller concentrations their peaks begin to overlap. It should be noted that an acetonitrile gradient could possibly improve the separation time of the amino acids without sacrificing resolution or efficiency. However, use of a gradient would also be possible in EFLC if programmable syringe pumps and an inline mixer are used [42].

Also worth noting are the differences in the order of elution seen between the three sets of conditions. In the LC acetonitrile/water case, elution appears to correlate strictly with polarity of the amino acids. In the LC methanol/water case, however, this correlation is much weaker. L-tryptophan, L-proline, and L-threonine all showed significant changes in retention relative to the other amino acids. Finally, addition of CO₂ further changed the elution order for the methanol/water/CO₂ EFLC case. These changes in selectivity suggest different retention mechanisms, which will be discussed in Section 2.3.6. However, clearly this EFLC mobile phase can offer faster isocratic separation, higher resolution, and different selectivity than either methanol/water or acetonitrile/water LC mobile phases for these amino acids.
Figure 2.14. Separation of 10 amino acids with varying mobile phase compositions. (A) 80:20 acetonitrile:water (pH=3.0), (B) 75:25 methanol:water (pH = 3.0), and (C) 75:25 methanol:water (pH = 3.0) with 30% vol. CO₂. Buffer concentration was 15mM in each mobile phase, and flow rate was 1.0 mL/min. Analytes: L-pro (1), L-phe (2), L-tyr (3), L-leu (4), L-val (5), L-thr (6), L-trp (7), L-ala (8), L-gln (9), L-asn (10).
2.3.6 Retention mechanism study – van’t Hoff plots

As illustrated by the differences in amino acid retention between the three optimized methods, it is clear that the retention mechanisms under LC and EFLC conditions are very different. Therefore, a brief study of the differences is presented here. The van’t Hoff equation (Equation 2.1) describes the relationship between column temperature, $T$, and analyte retention factor, $k$, where $R$ is the universal gas constant, $\Delta H$ and $\Delta S$ are enthalpy and entropy of transfer between the mobile phase and stationary phase, and $\Phi$ is the phase ratio.

$$\ln k = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} + \ln \Phi$$

(Equation 2.1)

The phase ratio, $\Phi$, can be estimated from the total column porosity, $\varepsilon_t$, according to Equation 2.2 [47].

$$\Phi = \frac{1-\varepsilon_t}{\varepsilon_t}$$

(Equation 2.2)

By plotting $\ln k$ vs. $1/T$, a van’t Hoff plot can be generated for a given analyte across a temperature range. This plot will be linear if the change in heat capacity for the solute transfer is zero, and values for $\Delta H$ and $\Delta S$ can be calculated from the slope and intercept of the line. This is common for separations where retention is governed by a single mechanism. However, deviations from linearity are often observed for mixed retention mechanisms.

Figure 2.15 - Figure 2.17 display van’t Hoff plots for the optimized acetonitrile/water and methanol/water LC mobile phases, as well as the optimized methanol/water/CO$_2$ EFLC mobile phase. Both LC mobile phases showed a highly linear
correlation, illustrating that the mechanism of analyte transfer may remain unchanged under the considered temperature range. This would suggest a single mechanism is likely responsible for retention of the amino acids. Another possibility exists, however. Gritti and Guiochon have demonstrated that in rare cases, the linear behavior of van’t Hoff plots may merely be fortuitous. For example, in a reversed-phase separation with a heterogeneous stationary phase, linearity was still observed despite multiple adsorption sites [48]. In this case, linearity does not indicate constant values of $\Delta H$, $\Delta S$ and $\Phi$ across a temperature range, but rather changing values that compensate one another to yield an overall linear trend in the van’t Hoff plot. This is certainly a possibility in HILIC separations as well, as further study of HILIC phases by Gritti et al. indicate that retention can be governed by a combination of adsorption and partitioning interactions [36]. Since retention factor is a measurement of the sum of these interactions, it may be that linearity is a result of compensation of $\Delta H$, $\Delta S$ and $\Phi$ values as they change across the temperature range.

If the underlying assumptions of the van’t Hoff equation are true, then it is evident that the calculated $\Delta H$ and $\Delta S$ values were quite different between the two mobile phase mixtures (Table 2.2). The values for $\Delta H$ were significantly more negative when changing from acetonitrile to methanol and most showed a more negative change in $\Delta S$. HILIC separations often have shallow van’t Hoff slopes, and adsorption processes like ion-exchange typically have negative entropy change as analytes lose degrees of freedom when adsorbed to the stationary phase. When considering these results along with those from Figure 2.4 - Figure 2.7, our initial hypothesis regarding the governing mechanisms
is further validated. It is most likely that both optimized LC separations are governed by a single, but different, retention mechanism. In the acetonitrile/water case, where increasing organic content results in significantly increased retention, a HILIC mechanism is most likely responsible. In the methanol/water case, where organic content has a smaller effect on retention, the driving mechanism is more than likely strong cation-exchange.

Interestingly, the van’t Hoff plot for the optimized EFLC methanol/water/CO₂ mobile phase (Figure 2.17) did not show a high degree of linearity over the same temperature range. It should be noted that this is not uncommon in SFC or SubFC [49,50], even for retention governed by a single mechanism, usually for one of two reasons. Either $\Delta H$ varies with mobile phase density (which is dependent on temperature), and/or $\Phi$ varies due to variable adsorption of mobile phase constituents on the stationary phase over the given temperature range. Both of these variations typically occur as a result of the high compressibility of the supercritical carbon dioxide present in the mobile phase. It is quite possible that this is the reason that nonlinearity is observed for ELFC conditions as well. However, it is also known that SubFC and EFLC mixtures are less compressible than SFC mobile phases, since the proportion of compressible CO₂ is much smaller, in this case 0.2 mol fraction. Therefore, it is also possible that the phase ratio remains almost entirely constant across the temperature range studied. This has been demonstrated before in SubFC mobile phases with high portions of organic modifier [51]. Given the differences in retention and selectivity of the amino acids observed between the LC and EFLC mobile phases, the non-linearity may simply be due to a mixed-mode
mechanism. Further studies are needed to determine which exactly the case is, but regardless it is clear that significant differences exist between the methanol/water LC and methanol/water/CO\textsubscript{2} EFLC methods.

If we do assume the phase ratio to be constant, it is clear that the $\Delta H$ and $\Delta S$ values are clearly temperature dependent, making estimation of the values from the van’t Hoff plot under EFLC conditions difficult. The van’t Hoff curves were split into low-temperature (25-35ºC) and high-temperature (40-50ºC) regions, and linear regressions were performed for each of them. This yielded two separate values for both $\Delta H$ and $\Delta S$, one at low temperature and one at high temperature. These values (Table 2.2) were quite different from one another for a given amino acid, illustrating that mechanism of analyte transfer does not remain constant as temperature is varied. This indicates that a mixed-mode retention mechanism may be responsible for the separation. In both the high temperature and low temperature EFLC cases, both the $\Delta H$ and $\Delta S$ values show an increasing trend as the polarity of the amino acid increases. As stated earlier, shallower van’t Hoff slopes and increased $\Delta S$ values are commonly seen in separations governed by a HILIC mechanism. Therefore, the degree of HILIC interaction relative to ion-exchange interaction is likely increasing as the polarity of the analytes increase. Previously, our group has also demonstrated that addition of CO\textsubscript{2} decreases the eluent strength of methanol/water mixtures in HILIC separations [27,44], resulting in increased retention of polar compounds. Since the addition of CO\textsubscript{2} in this case also increased the retention time of the more polar amino acids (Figure 2.8) it is proposed that hydrophilic interactions become significant upon the addition of CO\textsubscript{2} in this case as well. Most likely, these
interactions are superimposed upon the existing ion-exchange interactions, resulting in a mixed-mode HILIC/SCX retention mechanism. This is most likely responsible for the difference in selectivity and elution order seen between the EFLC and LC mobile phases.
Figure 2.15. van’t Hoff plot for LC – 80:20 acetonitrile:water (15mM NH$_4^+$ formate, pH = 3.0). The temperature range studied was 30-55°C. Solid lines indicate linear regressions. Analytes: L-trp (♦), L-phe (■), L-leu (▲), L-tyr (X), L-val (♦), L-pro (●), L-thr (—), L-alala (+), L-gln (—), L-asn (♦).
Figure 2.16. van’t Hoff plot for LC – 75:25 methanol:water (15mM NH$_4^+$ formate, pH = 3.0). The temperature range studied was 30-55°C. Solid lines indicate linear regressions. Analytes: L-trp (♦), L-phe (■), L-leu (▲), L-tyr (X), L-val (*), L-pro (●), L-thr (—), L-ala (+), L-gln (—), L-asn (♦).
Figure 2.17. van’t Hoff plot for EFLC – 75:25 methanol:water (15mM NH$_4^+$ formate, pH = 3.0) with 30% CO$_2$ by volume. The temperature range studied was 30-55°C. Solid lines indicate linear regressions. Analytes: L-trp (♦), L-phe (■), L-leu (▲), L-tyr (▲), L-val (★), L-pro (●), L-thr (—), L-ala (+), L-gln (—), L-asn (♦).
**Table 2.2.** Enthalpy and entropy of transfer of amino acids from the mobile phase to the stationary phase for each mobile phase composition studied, as determined from van’t Hoff plots. The uncertainty in each value is reported as ± one standard deviation.

| Amino acid | LC – acetonitrile/water | | LC – methanol/water | | EFLC – methanol/water/CO₂ (low temp.) | | EFLC – methanol/water/CO₂ (high temp.) |
|------------|--------------------------|-----------------|---------------------|---------------------|-----------------------------|-----------------------------|
|            | ΔH (kJ/mol)               | ΔS (J/mol·K)    | ΔH (kJ/mol)         | ΔS (J/mol·K)        | ΔH (kJ/mol)                 | ΔS (J/mol·K)                |
| Trp        | 2.8 ± 0.2                 | 21.4 ± 0.7      | -8.5 ± 0.2          | -9.2 ± 0.5          | 9.6 ± 0.8                   | -11.9 ± 2.5                 |
| Phe        | -1.3 ± 0.1                | 9.0 ± 0.2       | -5.7 ± 0.1          | -7.9 ± 0.2          | -7.2 ± 1.1                  | -9.2 ± 3.5                  |
| Leu        | -3.9 ± 0.1                | 2.2 ± 0.4       | -6.1 ± 0.2          | -4.6 ± 0.7          | -10.7 ± 0.8                 | -18.8 ± 2.7                 |
| Tyr        | 2.5 ± 0.1                 | 26.0 ± 0.3      | -5.9 ± 0.1          | -3.9 ± 0.4          | -4.6 ± 0.6                  | 1.5 ± 12.0                  |
| Val        | -4.8 ± 0.1                | 4.9 ± 0.4       | -5.4 ± 0.2          | -1.2 ± 0.7          | -7.7 ± 1.0                  | -7.9 ± 3.2                  |
| Pro        | -5.2 ± 0.1                | 4.3 ± 0.2       | -5.7 ± 0.2          | -8.4 ± 0.6          | -1.8 ± 0.1                  | 7.5 ± 0.4                  |
| Ala        | -1.8 ± 0.1                | 21.4 ± 0.4      | -6.9 ± 0.1          | -1.0 ± 0.4          | -4.5 ± 0.7                  | 5.4 ± 2.4                  |
| Thr        | -2.5 ± 0.1                | 17.3 ± 0.2      | -7.1 ± 0.1          | -6.6 ± 0.4          | -4.4 ± 0.5                  | 4.4 ± 1.6                  |
| Gln        | -0.7 ± 0.1                | 28.9 ± 0.3      | -8.8 ± 0.1          | -8.0 ± 0.2          | -0.5 ± 0.1                  | 4.0 ± 0.3                  |
| Asn        | -0.5 ± 0.1                | 31.4 ± 0.3      | -10.7 ± 0.1         | -12.9 ± 0.4         | -0.6 ± 0.8                  | 15.7 ± 2.4                 |

**Table 2.2.** Enthalpy and entropy of transfer of amino acids from the mobile phase to the stationary phase for each mobile phase composition studied, as determined from van’t Hoff plots. The uncertainty in each value is reported as ± one standard deviation.
2.3.7 Retention mechanism study - enthalpy-entropy compensation analysis

Enthalpy-entropy compensation (EEC) studies can provide additional information about the mechanism(s) controlling a separation (again assuming that β is constant). EEC shows the dependence of the enthalpy of transfer, $\Delta H$, on the entropy of transfer, $\Delta S$, and is described by Equation (2.3) where $\Delta G_{T_c}$ is the Gibbs free energy that characterizes the overall interactions of the analyte with the chromatographic system at the compensation temperature $T_c$.

$$\Delta H = T_c \Delta S + \Delta G_{T_c} \tag{2.3}$$

When EEC occurs, plots of $\Delta S$ vs. $\Delta H$ are linear, and the slope is called the compensation temperature, $T_c$. Any compounds demonstrating the same thermodynamic behavior will have equal $\Delta G_{T_c}$ at this temperature. Therefore, EEC can be used for grouping the amino acids with equal overall interactions at the compensation temperature $T_c$, even if their temperature dependencies may differ. By combining Equation 2.1 with Equation 2.3, a relationship between retention factor, $k_T$, and $\Delta H$ can be developed according to Equation 2.4.

$$\ln k_T = -\Delta H \left(\frac{1}{T} - \frac{1}{T_c}\right) - \frac{\Delta G_{T_c}}{RT_c} + \ln \Phi \tag{2.4}$$

If a plot of $\ln k_T$ vs. $-\Delta H$ is linear for a series of analytes, then a compensation temperature, $T_c$ exists and this is strong evidence that the same retention mechanism is controlling retention of the compounds.

Enthalpy-entropy compensation plots were made for the optimized LC and EFLC mobile phases (Figure 2.18 - Figure 2.21). If one mechanism is truly responsible for retention of the amino acids under LC conditions, one would expect both LC mobile
phases to exhibit linear behavior. Under methanol/water LC conditions, a moderate linear correlation was observed with an $R^2$ value = 0.86. This seems to affirm that cation-exchange is at least the strongest, if not the only, mechanism governing the methanol/water LC separation. However, the enthalpy-entropy compensation plot for acetonitrile/water at first appeared to have no discernable trend. This suggested that mixed retention mechanisms may in fact exist. However, if we exclude the four early eluting, less polar amino acids, then the remaining amino acids do exhibit a strong linear correlation of $R^2 = 0.94$. These six were the same amino acids that showed a great change in retention with increasing proportions of acetonitrile (Figure 2.6). Therefore, it is still highly likely that HILIC is the governing mechanism for these compounds. It may also be the governing mechanism for the remaining amino acids at concentrations higher than 80% acetonitrile, but no EEC data were collected at these concentrations.

Most importantly, the EEC plots under EFLC conditions appear to affirm a mixed-mode retention mechanism. There were no discernable trends in linearity, making it likely that a combination of retention mechanisms is responsible for retention of the amino acids. This, in combination with earlier results, suggests this is a probable explanation for the differences in selectivity seen between LC and EFLC conditions.
Figure 2.18. Enthalpy-entropy compensation plot at 25ºC for LC – 80:20 acetonitrile:water (15 mM NH₄⁺ formate, pH = 3.0).
Figure 2.19. Enthalpy-entropy compensation plot at 25°C for LC – 75:25 methanol:water (15 mM NH$_4^+$ formate, pH = 3.0).
Figure 2.20. Enthalpy-entropy compensation plot at 25°C for EFLC – 75:25 methanol:water (15mM NH₄⁺ formate, pH = 3.0) with 30% CO₂ by volume.
Figure 2.21. Enthalpy-entropy compensation plots at 40°C for EFLC – 75:25 methanol:water (15mM NH$_4^+$ formate, pH = 3.0) with 30% CO$_2$ by volume.
2.4 Conclusions

A baseline separation of ten neutral side chain amino acids was achieved using a methanol/water/CO\textsubscript{2} mobile phase under EFLC conditions. This isocratic separation offered higher resolution and efficiencies than methanol/water LC mobile phases at a flow rate of 1.0 mL/min, as well as slower decreases in efficiency as the flow rate was increased. Additionally, analysis time was half that required by an isocratic acetonitrile/water LC separation on the same column. EFLC conditions also afforded differences in selectivity, most likely due to a mixed-mode HILIC/SCX retention mechanism, whereas one mechanism tended to dominate the separation in either LC mode. Therefore, EFLC mobile phases can be a unique solution in resolving difficult pairs of compounds without changing the column. Finally, EFLC mobile phases have demonstrated that they can be used in the separation of ionic or ionizable compounds without encountering solubility or elution issues, while still offering increased diffusivity and lower viscosities than LC mobile phases.
2.5 References


CHAPTER 3: GRADIENT ENHANCED-FLUIDITY LIQUID HYDROPHILIC INTERACTION CHROMATOGRAPHY - A PATH TOWARDS “GREEN” SEPARATIONS

3.1 Introduction

In the past twenty years, there has been a significant push towards the development and implementation of environmentally sustainable chemistry. This practice, often referred to as “green chemistry,” is the design of chemical products and processes which reduce or eliminate the use and generation of hazardous substances [1] and has been defined by a set of 12 principles [2]. These principles serve as a guideline to minimize the environmental risk of any chemical process and can be applied across all disciplines, including high-performance liquid chromatography (HPLC).

The largest environmental concern in HPLC is its heavy use of toxic, organic solvents. A single analytical chromatograph with a conventional column (4.6 mm x 150 mm) produces upwards of 500 liters of solvent waste per year [3]. To offset this, “green” chromatographic methods can be designed to minimize the generation of hazardous solvent waste while maintaining chromatographic performance. Smaller scale systems with shorter column dimensions and reduced particle diameters can lessen the required solvent volume [4], however conventional systems are generally limited to columns at least 2.1 mm in diameter. Portions of solvents can also be recovered and reused;
however, this is generally only feasible for isocratic separations of clean samples. While many of these techniques can be successful, the most direct method of “greening” chromatography is simply to develop methods that utilize “green” solvents without compromising chromatographic performance. Solvent selection guides even exist to aid chromatographers in making “green” solvent choices for their separations [5,6].

Hydrophilic interaction chromatography (HILIC) is an area of chromatography that could benefit greatly from “greener” methods, as it is increasingly becoming the preferred chromatographic mode for the separation of highly polar and ionizable compounds. As mentioned in Chapter 2, HILIC separations are achieved via a partitioning mechanism in which analyte partitions between a water-enriched layer on the surface of a polar stationary phase and a highly organic mobile phase [7]. As a result, polar compounds preferentially partition into the water-rich layer and are well-retained. Additionally, the presence of high amounts of volatile organic solvents in the mobile phase make HILIC well suited for liquid chromatography – mass spectrometry (LC-MS) analysis. Together, these advantages have made HILIC methods extremely popular for difficult separations of polar biological compounds [8].

Unfortunately, HILIC methods typically rely heavily on acetonitrile as a mobile phase component. Acetonitrile is already one of the most common solvents used throughout high-performance liquid chromatography because of its highly desirable chromatographic properties, including low viscosity, high solvating power, and low UV cutoff. Additionally, its aprotic character makes it a very weak eluent in HILIC separations, yielding excellent retention of polar compounds. As a result, the vast
majority of HILIC separations are performed using acetonitrile as the organic portion of the mobile phase. However, when compared to other commonly used chromatographic solvents, acetonitrile is much more expensive and much less environmentally friendly. Given the recent drive towards sustainable chemistry and the increasing popularity of HILIC separations, development of quality HILIC methods using “greener” solvents would be highly beneficial.

A few attempts have been made to minimize/eliminate acetonitrile from HILIC mobile phases for the purpose of “greener” chromatography, with limited success. Typically primary alcohols are used as a substitute, however the polar protic nature of these solvents results in disruption of the water layer [9] and drastically decreased retention relative to acetonitrile [10,11,12]. Others have performed “reversed HILIC” separations, which use small portions of acetonitrile with large amounts of aqueous mobile phase [13]. However, this is really just aqueous reversed-phase chromatography using a HILIC stationary phase, and doesn’t work for highly polar analytes. Additionally, given the recent revival of supercritical fluid chromatography (SFC) due to its “green” character, supercritical fluids have been used as mobile phases with HILIC columns [14,15,16,17]. However, unlike true HILIC methods, water is not typically a component of the mobile phase.

Recently, our group [18,19,20] and others [21] have demonstrated the use of enhanced-fluidity liquids (EFLs) as “green” mobile phases in HILIC separations. This includes the results outlined in Chapter 2 of this work. By adding nonpolar CO₂ as a modifier in HILIC, the eluent strength of alcohol/water mobile phases can be decreased
significantly, yielding retention similar to traditional acetonitrile/water mobile phases. Even with these lower CO₂ proportions, these mobile phases also offer the chromatographic advantages of SFC mobile phases, including enhanced solute mass transfer and reduced system back pressure [22]. This leads to improved chromatographic performance in terms of efficiency, resolution, and separation time. Since the bulk of mobile phase is still organic solvent and water, this still allows for analysis of highly polar compounds. Furthermore, by limiting the mobile phase components to CO₂, water, and organic solvents like methanol or ethanol, the overall “greenness” of the method is greater than traditional acetonitrile-based HILIC separations.

While these benefits of enhanced-fluidity of liquid chromatography (EFLC) have been demonstrated for HILIC, they have only been demonstrated in isocratic separations. However, samples with wide retention factor ranges often require gradient elution in order to resolve all peaks within a reasonable analysis time. By performing a gradient with EFL mobile phases, the HILIC eluent strength of the mobile phase can be varied drastically, allowing for analysis of compounds with a wide range of polarities. As a proof of concept, a sample containing sixteen RNA nucleosides and nucleotides (Table 3.1) was chosen for analysis in this study. The characterization of nucleoside and nucleotide mixtures is important in numerous areas of science, such as milk analysis [23], cell component studies [24], and pharmaceutical analyses [25], so development of new analytical techniques would be useful. The optimized HILIC method herein marks the first time that “green” EFL mobile phases have been used for gradient separations with commercial SFC instrumentation.
Table 3.1. Nucleoside and nucleotide structures with corresponding logP values

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>LogP</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td><img src="image" alt="Structure of A" /></td>
<td>-1.02</td>
</tr>
<tr>
<td>AMP</td>
<td><img src="image" alt="Structure of AMP" /></td>
<td>-2.07</td>
</tr>
<tr>
<td>ADP</td>
<td><img src="image" alt="Structure of ADP" /></td>
<td>-3.35</td>
</tr>
<tr>
<td>ATP</td>
<td><img src="image" alt="Structure of ATP" /></td>
<td>-4.62</td>
</tr>
<tr>
<td>C</td>
<td><img src="image" alt="Structure of C" /></td>
<td>-1.94</td>
</tr>
<tr>
<td>CMP</td>
<td><img src="image" alt="Structure of CMP" /></td>
<td>-1.56</td>
</tr>
</tbody>
</table>

continued
Table 3.1 continued

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>LogP</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDP</td>
<td><img src="image" alt="CDP Structure" /></td>
<td>-4.25</td>
</tr>
<tr>
<td>CTP</td>
<td><img src="image" alt="CTP Structure" /></td>
<td>-5.52</td>
</tr>
<tr>
<td>G</td>
<td><img src="image" alt="G Structure" /></td>
<td>-1.72</td>
</tr>
<tr>
<td>GMP</td>
<td><img src="image" alt="GMP Structure" /></td>
<td>-2.28</td>
</tr>
<tr>
<td>GDP</td>
<td><img src="image" alt="GDP Structure" /></td>
<td>-3.55</td>
</tr>
<tr>
<td>GTP</td>
<td><img src="image" alt="GTP Structure" /></td>
<td>-4.82</td>
</tr>
</tbody>
</table>

continued
Table 3.1 continued

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>LogP</th>
</tr>
</thead>
<tbody>
<tr>
<td>U</td>
<td><img src="image" alt="Structure of U" /></td>
<td>-1.61</td>
</tr>
<tr>
<td>UMP</td>
<td><img src="image" alt="Structure of UMP" /></td>
<td>-1.58</td>
</tr>
<tr>
<td>UDP</td>
<td><img src="image" alt="Structure of UDP" /></td>
<td>-4.27</td>
</tr>
<tr>
<td>UTP</td>
<td><img src="image" alt="Structure of UTP" /></td>
<td>-5.54</td>
</tr>
</tbody>
</table>
3.2 Materials and methods

3.2.1 Instrumentation

The instrument used for the EFLC analysis was a 1260 Infinity Analytical SFC system (Agilent Technologies, Santa Clara, CA). The system was comprised of a Fusion A5 SFC control module (600 bar maximum), 1260 HiP degasser, 1260 SFC binary pump, 1260 SFC ALS auto-sampler, 1290 thermostatted column compartment (-10 °C - 100 °C), and a 1200 diode array detector (DAD) SL (190-950 nm). The wavelength for analyte detection in the DAD was set to 270 nm. During all data collection the pressure of the back pressure regulator (BPR) was maintained at 120 bar and the temperature was set at 60.0 °C. The temperature of the column inlet was maintained at 40.0 °C while the column outlet was maintained at 37.5 °C. A Shimadzu Scientific Instruments (Kyoto, Japan) HPLC instrument was used for the traditional liquid (ACN:H₂O and MeOH:H₂O) mobile phase analysis. The HPLC was composed of two LC-20AT pumps, an SIL-20A auto-sampler, a CTO-20A column oven, an SPD-20A UV/vis detector (also set to a wavelength of 270 nm), and a CBM-20A communications bus module. The column used for all analysis was a 4.6 mm x 150 mm XBridge™ Amide column packed with 3.5 µm particles (Waters, Milford, MA).

3.2.2 Chemicals

Supercritical fluid extraction grade CO₂ (99.999% purity) from Praxair, Inc. (Danbury, CT), HPLC grade methanol, HPLC grade acetonitrile, and HPLC grade o-phosphoric acid 85% were purchased from Fisher Scientific (Fair Lawn, NJ) and used as
received. (≥ 99%) Sodium phosphate monobasic dihydrate was purchased from Sigma-Aldrich (St. Louis, MO). H₂O was purified to 18.2 MΩ on a Barnstead Nanopure Infinity system (Thermo Scientific, Asheville, NC) before use. All nucleoside and 5’-nucleotide analytes were purchased as a kit from Sigma-Aldrich (St. Louis, MO), which contains (≥ 99%) adenosine (A), (≥ 99%) cytidine (C), (≥ 98%) guanosine (G), (≥ 99%) uridine (U), (≥ 99%) adenosine 5’-monophosphate sodium salt (AMP), (≥ 99%) cytidine 5’-monophosphate disodium salt (CMP), (≥ 99%) guanosine 5’-monophosphate disodium salt hydrate (GMP), (≥ 99%) uridine 5’-monophosphate disodium salt (UMP), (≥ 95%) adenosine 5’-diphosphate sodium salt (ADP), (≥ 95%) cytidine 5’-diphosphate sodium salt hydrate (CDP), (≥ 96%) guanosine 5’-diphosphate sodium salt (GDP), (95-100%) uridine 5’-(trihydrogen diphosphate sodium salt (UDP), (≥ 99%) adenosine 5’-triphosphate disodium salt hydrate (ATP), (≥ 95%) cytidine 5’-triphosphate disodium salt (CTP), (≥ 95%) guanosine 5’-triphosphate sodium salt hydrate (GTP), and (≥ 96%) uridine 5’-triphosphate trisodium salt hydrate (UTP). Analytes were dissolved in 80:20 (v:v) MeOH:H₂O and adjusted to pH=7.5 with dilute NaOH for stability purposes. All analyte solutions were prepared to a final concentration of 1.25 x 10⁻⁴ M unless otherwise indicated and placed into a Misonix ultrasonic cleaner (QSonica LLC, Newtown, CT) to aid in the solvation process. The injection volume for sample injection was 5 µL unless reported otherwise. When not in use, analyte solutions were stored at 4 °C to prolong stability.
3.2.3 Mobile phase preparation

The Agilent Technologies SFC system is capable of mixing liquefied CO\textsubscript{2} from pump A and organic/aqueous liquid mixtures from pump B at various (v:v) concentrations. The liquid contents delivered through pump B must be prepared prior to experimentation by mixing the desired amounts of organic solvent and aqueous buffer. The ionic strength of any buffer housed in reservoir B is reported with respect to total volume (prior to CO\textsubscript{2} addition from pump A). For example, in order to prepare 300 mL of an 80:20 (v:v) MeOH:H\textsubscript{2}O solution (containing 40 mM sodium phosphate buffer at pH=2.65), 24 mL of 500 mM sodium phosphate (pH=2.65) stock solution was mixed with an additional 36 mL of H\textsubscript{2}O and then combined with 240 mL of MeOH. Regular flushing of the system with 80:20 (v:v) MeOH:H\textsubscript{2}O was performed to aid in the removal of buffer salts from the instrument, as well as to remove any CO\textsubscript{2} from the column to prevent it from drying out with long term storage.

3.2.4 Data analysis

Data analysis for the ACN:H\textsubscript{2}O and MeOH:H\textsubscript{2}O systems was performed using PeakFit Version 4 software (SPSS Inc. Chicago, IL). The MeOH:H\textsubscript{2}O:CO\textsubscript{2} analysis was conducted with OpenLab software (Agilent Technologies). Peak widths, efficiencies, resolutions, and other chromatographic parameters were all measured using the half of peak height (w\textsubscript{1/2}) method.
3.3 Results and discussion

3.3.1 Buffer selection and optimization

Ionizable compounds, like nucleosides and nucleotides, often require buffering of the mobile phase in order to ensure that the analyte molecules maintain the same degree of ionization. If a mobile phase is not buffered, conversion between ionized and non-ionized forms can occur. This can lead to band broadening of chromatographic peaks since varying ionized forms can interact differently with a stationary phase. Figure 3.1 shows a chromatogram of the 16 nucleosides/nucleotides obtained using a MeOH:H2O:CO2 mobile phase without the addition of any buffer. The significant peak tailing present required the incorporation of buffer into the mobile phase for both the LC and EFLC systems.

While a buffer is necessary to ensure the nucleosides and nucleotides remain in the same ionized form, the type of buffer is especially important in this specific case. Phosphorylated compounds like nucleotides often suffer from strong chromatographic tailing caused by hydrogen-bond interactions with free silanols from the stationary phase [11] or chelation of metal ions from the connecting stainless steel tubing or column [26]. As a result, addition of phosphoric acid or phosphate buffers typically improves the observed peak tailing by competing with and minimizing these secondary phosphate interactions [26], whereas non-phosphate buffers do not. Furthermore, addition of these buffer salts has been show to increase the degree of HILIC interactions for nucleotides, which results in predictable retention that is correlated to analyte polarity/degree of phosphorylation [19] (e.g. nucleosides elute first, followed by monophosphate...
nucleotides, diphosphate nucleotides, and triphosphate nucleotides). For these reasons, phosphate buffers are essential for this particular separation.

Unfortunately, phosphate buffers have limited solubility in organic solvents, which make up a high portion of HILIC mobile phase composition. Several different phosphate buffer salts were studied, including potassium phosphate, ammonium phosphate, and sodium phosphate salts. The potassium phosphate salt was nearly insoluble in mobile phases containing less than 25% H₂O, and was eliminated as an option. Ammonium phosphate was slightly more soluble than potassium phosphate; however, heavy tailing still existed (Figure 3.2). Sodium phosphate exhibited similar solubility to ammonium phosphate but yielded better overall peak shape, and thus was used for the remainder of this study.
Figure 3.1. Chromatogram of 16 nucleoside/nucleotide analyte mixture (1.25 x 10^{-4} M) with MeOH:H_{2}O:CO_{2} mobile phase - Component A: CO_{2}, Component B: 80:20 (v:v) MeOH:H_{2}O. Gradient program is (0-1.50 min) 70% B, (1.50-10.00 min), 70-90% B with 1.00 mL/min flow rate. Analyte key: (1) A, (2) U, (3) G, (4) C, (5) AMP, (6) UMP, (7) CMP, (8) GMP, (9) ADP, (10) UDP, (11) CDP, (12) GDP, (13) ATP, (14) UTP, (15) CTP, (16) GTP.
**Figure 3.2.** Isocratic separation of A, AMP, ADP, and ATP analyte mixture \((1.25 \times 10^{-4} \text{ M})\) with 80:20 (v:v) MeOH:H\(_2\)O mobile phase containing 10 mM ammonium phosphate buffer (pH=2.00).
Because the solubility of phosphate buffers in organic solvents decreases as pH increases, only a small range of low pH values (2.00-3.15) could be tested. As a result, limited retention changes were seen as pH was altered, with the exception of the triphosphate nucleotides (Figure 3.3). However, drastic peak width changes were seen as a both buffer concentration and pH were altered. To determine the optimized pH for the sodium phosphate buffer, peak widths of the analytes were compared at several different pH values (Figure 3.4). While the peak widths of the nucleosides and monophosphate nucleotides remained largely unchanged, the peak widths of the diphosphate and triphosphate nucleotides decreased as the pH increased. This was attributed to the increased dihydrogen phosphate ion content that is present in higher pHs at the same total buffer concentration ($pK_{a1}=2.15$). These phosphate ions compete with the phosphate containing analytes for interactions with free silanols or the metal column, and therefore minimize the secondary interactions responsible for peak tailing. While it appears that pH=3.15 produces the lowest peak widths, it should be noted here that the amount of CO$_2$ that can be added to the mobile phase not only depends on the MeOH:H$_2$O ratio, but also upon the pH or ionic strength of the buffer used. At pH=3.15, the desired amount of CO$_2$ could not be added before precipitation of buffer would occur, so pH=2.65 was chosen due to the fact that more CO$_2$ could be added with minimal loss in peak efficiency.
Figure 3.3. Variation of average retention factor ($k^*$) as a function of mobile phase pH.

Mobile phase component A: CO$_2$, mobile phase component B: 80:20 (v:v) MeOH:H$_2$O containing 25 mM sodium phosphate buffer at pH=2.00 (■), pH=2.65 (■), and pH=3.15 (■). Gradient program: (0-1.00 min) 70% B, (1.00-6.67 min) 70-90% B with 1.50 mL/min flow rate.
**Figure 3.4.** Peak widths for 16 nucleoside/nucleotide analytes (100 ppm) as a function of pH. Mobile phase component A: CO$_2$, component B: 80:20 (v:v) MeOH:H$_2$O containing 25 mM sodium phosphate buffer at pH=2.00 (■), pH=2.65 ( ), and pH=3.15 ( ). Gradient program used is the same as in Figure 3.3.
The ionic strength of the buffer system also plays an important role in the separation. As increased amounts of phosphate buffer become dissolved in the H₂O layer a lessened amount of peak tailing and peak broadening is observed. However, there is a finite amount of sodium phosphate buffer that can be dissolved in solution before precipitation occurs. The dissolved buffer salts not only must stay in solution while in the mobile phase pump reservoir, but also upon addition of CO₂ to the mobile phase. Peak widths were measured for mobile phases containing 10 mM, 25 mM, and 40 mM sodium phosphate buffers (Figure 3.5). Peak widths decrease as the ionic strength of the buffer increase, especially for the diphosphate and triphosphate nucleotides. Ultimately, the 40 mM sodium phosphate buffer system yielded narrower peak widths over the 10 mM and 25 mM concentrations tested, but concentrations higher than 40 mM resulted in solubility issues.
Figure 3.5. Peak widths for 16 nucleoside/nucleotide analyte mixture (100 ppm) as a function of buffer concentration. Mobile phase component A: CO$_2$, component B: 80:20 (v:v) MeOH:aqueous phosphate buffer at pH=2.65 with an ionic strength of 10 mM (■), 25 mM (■), and 40 mM (■) in the mobile phase. Gradient program used is same as in Figure 3.3.
3.3.2 Traditional HILIC mobile phases

Prior to EFLC mobile phase optimization, the characteristics and performance of traditional HILIC solvents (ACN:H₂O and MeOH:H₂O) for the separation of the 16 nucleosides and nucleotides were evaluated. As expected, the weak eluent strength of ACN in comparison to MeOH for nucleosides/nucleotides under HILIC conditions quickly became evident. As ACN content is increased past 65%, retention of the nucleosides/nucleotides increases drastically (Figure 3.6). This retention increase is not observed as MeOH content is increased (Figure 3.7). In fact, 90% MeOH content is required to achieve the same retention as 65% ACN, which is not even adequate to separate A, AMP, ADP, and ATP, let alone 16 nucleosides and nucleotides. Table 3.2 illustrates the ranges in retention factors for varying ACN and MeOH content for the 16 nucleosides and nucleotides grouped according to degree of phosphorylation: nucleosides, monophosphate nucleotides, diphosphate nucleotides, and triphosphate nucleotides. Clearly, ACN content can be increased to achieve significant retention (\(k > 2\)) for compounds within each class. On the other hand, increasing MeOH content only resulted in adequate retention of the most polar compounds, the diphosphate and triphosphate nucleotides. This demonstrates that while it is a “greener” option than ACN, MeOH is not a suitable replacement in this particular case.
Figure 3.6. Effect of ACN content on retention factor of A (♦), AMP (■), ADP (▲), and ATP (+). Remaining mobile phase content was 100 mM sodium phosphate (pH=2.65). Note: Some retention factors of ADP and ATP are off scale (>25) in (B) – see Table 3.2.
Figure 3.7. Effect of MeOH on retention factor of A (♦), AMP (■), ADP (▲), and ATP (+). Remaining mobile phase content was 100 mM sodium phosphate (pH=2.65).
Table 3.2. Retention factors for nucleosides (A, U, C, G), monophosphate nucleotides (AMP, UMP, CMP, GMP), diphosphate nucleotides (ADP, UDP, CDP, GDP), and triphosphate nucleotides (ATP, UTP, CTP, GTP) under isocratic conditions using various ACN:100 mM sodium phosphate (pH=2.65) and MeOH:400 mM sodium phosphate (pH=2.65) mobile phases.

<table>
<thead>
<tr>
<th>Mobile Phase Mixture</th>
<th>ACN:buffer (v:v)</th>
<th>Retention Factor ($k$)</th>
<th>ACN:buffer (v:v)</th>
<th>Retention Factor ($k$)</th>
<th>ACN:buffer (v:v)</th>
<th>Retention Factor ($k$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(A,U,C)</td>
<td>AMP,UMP,CMP,GMP</td>
<td>(ADP,UDP,CDP,GDP)</td>
<td>(ATP,UTP,CTP,GTP)</td>
<td>(A,U,C)</td>
<td>AMP,UMP,CMP,GMP</td>
</tr>
<tr>
<td>50:50</td>
<td>0.40-0.55</td>
<td>0.57-0.71</td>
<td>0.72-0.81</td>
<td>0.87-0.98</td>
<td>0.40-0.55</td>
<td>0.57-0.71</td>
</tr>
<tr>
<td>55:45</td>
<td>0.40-0.76</td>
<td>0.68-1.04</td>
<td>0.94-1.28</td>
<td>1.23-1.57</td>
<td>0.40-0.76</td>
<td>0.68-1.04</td>
</tr>
<tr>
<td>60:40</td>
<td>0.48-1.04</td>
<td>0.98-1.61</td>
<td>1.52-2.16</td>
<td>2.13-2.82</td>
<td>0.48-1.04</td>
<td>0.98-1.61</td>
</tr>
<tr>
<td>65:35</td>
<td>0.61-1.53</td>
<td>1.54-2.77</td>
<td>2.71-4.11</td>
<td>4.14-5.84</td>
<td>0.61-1.53</td>
<td>1.54-2.77</td>
</tr>
<tr>
<td>70:30</td>
<td>0.82-2.58</td>
<td>2.79-5.83</td>
<td>5.93-10.49</td>
<td>10.30-16.46</td>
<td>0.82-2.58</td>
<td>2.79-5.83</td>
</tr>
<tr>
<td>80:20</td>
<td>0.41-0.62</td>
<td>0.48-0.80</td>
<td>0.99-1.71</td>
<td>1.92-3.47</td>
<td>0.41-0.62</td>
<td>0.48-0.80</td>
</tr>
<tr>
<td>85:15</td>
<td>0.46-0.76</td>
<td>0.56-1.02</td>
<td>1.40-2.70</td>
<td>3.32-6.50</td>
<td>0.46-0.76</td>
<td>0.56-1.02</td>
</tr>
<tr>
<td>90:10</td>
<td>0.51-0.90</td>
<td>0.64-1.26</td>
<td>1.96-4.21</td>
<td>5.95-13.74</td>
<td>0.51-0.90</td>
<td>0.64-1.26</td>
</tr>
</tbody>
</table>
3.3.3 Impact of CO$_2$ on retention

As indicated in the previous sections, MeOH:H$_2$O mixtures clearly cannot produce adequate retention of the nucleosides and monophosphate nucleotides. However, by adding CO$_2$ to MeOH:H$_2$O mixtures, the eluent strength of the mobile phase can be significantly decreased. This type of retention behavior has been observed previously for separations governed by a HILIC mechanism [18,19,20,27]. The increase in analyte retention as a function of added CO$_2$ is demonstrated in Figure 3.8 for adenosine and its phosphate nucleotides. The most drastic increases are observed for the most polar compounds (ADP and ATP), however significant increases are seen for A and AMP as well. Table 3.3 summarizes the changes in retention factors for each class of compounds. Retention factors within each class show an increasing trend as an increasing proportion of CO$_2$ is added to the mobile phase system. Again, while the most drastic change is for the most polar compounds (the diphosphate and triphosphate nucleotides), a significant change is observed even in the nucleosides and monophosphate nucleotides.

The above behavior suggests that when CO$_2$ is used as the weak solvent, retention behavior is similar to that seen when ACN is added to H$_2$O. In fact, an evaluation of the solvent strengths of the mobile phases (in which ACN, MeOH, and CO$_2$ are considered as weak eluents) illustrates exactly that. For a HILIC separation, the relationship between the retention factor, $k$, and the volume fraction, $\varphi$, of the stronger eluent (H$_2$O) in the mobile phase is often estimated by Eq. (1)

$$\log k = \log k_{org} - S\varphi$$ (1)
where $k_{org}$ is the hypothetical retention factor of the analyte when using the weaker solvent as the eluent and $S$ is the slope of the plot of $\log k$ vs $\phi$ when fitted to a linear regression model [28]. The $S$ value can be used as a measure of eluent strength, with greater $S$ values indicating greater change in retention per change in volume fraction of strong solvent. Figure 3.9 - Figure 3.11 shows plots of $\log k$ vs $\phi$ for the adenosine containing analytes for ACN:H$_2$O, MeOH:H$_2$O, and MeOH:H$_2$O:CO$_2$ mobile phases. H$_2$O was treated as the strong eluent for the ACN:H$_2$O and MeOH:H$_2$O separations, and 80:20 (v:v) MeOH:H$_2$O was treated as the strong eluent in the MeOH:H$_2$O:CO$_2$ case. This allowed examination of the effect of ACN, MeOH, and CO$_2$ on retention individually.

The $S$ values obtained from the $\log k$ vs $\phi$ plots are summarized in Table 3.4. From these values, it is evident that the addition of CO$_2$ to 80:20 (v:v) MeOH:H$_2$O develops a solvent strength intermediate to that of ACN and MeOH. As a result, by adding CO$_2$ to MeOH:H$_2$O mixtures, the solvent strength of the overall mixture begins to approach that of high ACN content mobile phases.

The impact of the mobile phase solvent strength depends significantly on the polarity of the analytes. By examining the average retention factors of analytes under isocratic solvent conditions for these mobile phases, isoeulotropic nomograms (Figure 3.12) were developed for the each class of compounds (nucleosides, monophosphate nucleotides, diphosphate nucleotides, and triphosphate nucleotides). These nomograms indicate the relative percentages of weak eluent (ACN, MeOH, or CO$_2$) needed to obtain similar solvent strength. Decreases in solvent strength that are not possible even using
90:10 (v:v) MeOH:H₂O mixtures, become possible simply by adding CO₂ to an 80:20 (v:v) MeOH:H₂O mobile phase. This effect is increased as the polarity of the analytes is increased. For example, 30% CO₂ added to an 80:20 (v:v) ratio MeOH:H₂O mobile phase results in solvent strength equivalent to 65:35 (v:v) ACN:H₂O mixtures for nucleosides, but a 77:23 (v:v) ACN:H₂O mixture for triphosphate nucleotides. Therefore, MeOH:H₂O:CO₂ mixtures are potential candidates for “green” HILIC separations in which MeOH:H₂O mobile phases fail to achieve adequate retention. Furthermore, the large variation in solvent strength over a small CO₂ range makes these solvents particularly useful for gradient separations.
Figure 3.8. Effect of CO$_2$ content on retention factor of A (♦), AMP (■), ADP (▲), and ATP (+). Mobile phase component A: CO$_2$, mobile phase component B: 80:20 (v:v) MeOH:H$_2$O with 40 mM sodium phosphate buffer, pH=2.65.
**Table 3.3.** Retention factor ranges for nucleosides (A, U, C, G), monophosphate nucleotides (AMP, UMP, CMP, GMP), diphosphate nucleotides (ADP, UDP, CDP, GDP), and triphosphate nucleotides (ATP, UTP, CTP, GTP) under isocratic conditions using 80:20 (v:v) MeOH:H₂O (40 mM sodium phosphate, pH=2.65) mobile phases with various amounts of CO₂ added.

<table>
<thead>
<tr>
<th>% CO₂</th>
<th>A,U,C,G</th>
<th>AMP,UMP, CMP,GMP</th>
<th>ADP,UDP, CDP,GDP</th>
<th>ATP,UTP, CTP,GTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>5%</td>
<td>0.21-0.29</td>
<td>0.30-0.63</td>
<td>0.81-1.66</td>
<td>1.96-4.08</td>
</tr>
<tr>
<td>10%</td>
<td>0.24-0.49</td>
<td>0.40-0.87</td>
<td>1.18-2.57</td>
<td>3.01-6.69</td>
</tr>
<tr>
<td>15%</td>
<td>0.28-0.60</td>
<td>0.57-1.25</td>
<td>1.78-3.93</td>
<td>4.67-10.59</td>
</tr>
<tr>
<td>20%</td>
<td>0.28-0.76</td>
<td>0.68-1.69</td>
<td>2.35-5.67</td>
<td>6.56-15.84</td>
</tr>
<tr>
<td>25%</td>
<td>0.40-0.95</td>
<td>1.04-2.46</td>
<td>3.68-8.97</td>
<td>10.38-26.11</td>
</tr>
<tr>
<td>30%</td>
<td>0.49-1.32</td>
<td>1.58-4.10</td>
<td>6.30-17.14</td>
<td>19.06-54.21</td>
</tr>
</tbody>
</table>
Figure 3.9. Log $k$ vs. $\phi$ plots for 100 ppm of A (♦), AMP (■), ADP (▲), and ATP (+) for ACN:H$_2$O mobile phases. Mobile phase component A: ACN, mobile phase component B: 100 mM sodium phosphate (pH=2.65). Lines represent linear regressions.
Figure 3.10. Log $k$ vs. $\varphi$ plots for 100 ppm of A (♦), AMP (■), ADP (▲), and ATP (+) for MeOH:H$_2$O mobile phases. Mobile phase component A: MeOH, mobile phase component B: 400 mM sodium phosphate (pH=2.65). Lines represent linear regressions.
Figure 3.11. Log $k$ vs. $\varphi$ plots for 100 ppm of A (♦), AMP (■), ADP (▲), and ATP (+) for MeOH:H$_2$O:CO$_2$ mobile phases. Mobile phase component A: CO$_2$, mobile phase component B: 80:20 (v:v) MeOH:H$_2$O (40 mM sodium phosphate, pH=2.65). Lines represent linear regressions.
**Table 3.4.** $S$ value ranges for nucleosides (A, U, C, G), monophosphate nucleotides (AMP, UMP, CMP, GMP), diphosphate nucleotides (ADP, UDP, CDP, GDP), and triphosphate nucleotides (ATP, UTP, CTP, GTP) with varying mobile phases.

<table>
<thead>
<tr>
<th>Mobile Phase</th>
<th>A,U,C,G</th>
<th>AMP,UMP, CMP,GMP</th>
<th>ADP,UDP, CDP,GDP</th>
<th>ATP,UTP, CTP,GTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACN:H$_2$O</td>
<td>2.1-3.5</td>
<td>4.1-5.0</td>
<td>5.3-6.1</td>
<td>6.1-6.8</td>
</tr>
<tr>
<td>MeOH:H$_2$O</td>
<td>0.63-1.2</td>
<td>1.0-1.8</td>
<td>2.5-3.5</td>
<td>3.9-4.8</td>
</tr>
<tr>
<td>MeOH:H$_2$O:CO$_2$</td>
<td>1.8-2.5</td>
<td>2.5-3.1</td>
<td>3.2-4.0</td>
<td>3.6-4.4</td>
</tr>
</tbody>
</table>
Figure 3.12. Isoelutropic nomogram comparing percentage of weak eluent needed to obtain identical solvent strength for (A) nucleosides, (B) monophosphate nucleotides (C) diphosphate nucleotides, and (D) triphosphate nucleotides. The strong eluent is H₂O for ACN and MeOH and 80:20 (v:v) MeOH:H₂O for CO₂.
Figure 3.12 continued

(C) Decreasing Eluent Strength

(D) Decreasing Eluent Strength
3.3.4 EFLC gradient optimization

The separation of the mixture of 16 nucleosides/nucleotides was optimized using EFLC mobile phases, with the goal of achieving the highest resolution for all of the analytes in the shortest amount of time. Due to the large range in polarity of the nucleosides and nucleotides, a reverse CO$_2$ gradient program was implemented. As illustrated in Section 3.3.3, the retention behavior of each group of compounds varies drastically depending on the amount of CO$_2$ present in the mobile phase. High portions of CO$_2$ were required initially to obtain adequate retention and resolution of the less polar nucleosides and monophosphate nucleotides, followed by a decrease in CO$_2$ content to elute the more polar diphosphate and triphosphate nucleotides.

The optimized separation of the mixture using an EFLC mobile phase is shown in Figure 3.13. The gradient program contains two isocratic holds and two linear gradients. Isocratic holds and gradients have been used previously for the analysis of nucleotides with several different types of columns under HILIC conditions [29,30,31,32]. The initial isocratic hold was essential to resolve the nucleosides prior to beginning a gradient to separate the remaining nucleotides. The gradient program for the optimized EFLC separation is described below, using CO$_2$ as solvent A and 80:20 (v:v) MeOH:H$_2$O with 40 mM sodium phosphate (pH=2.65) as solvent B with a total flow rate of 1.00 mL/min. Initially, 71% B is held (0-1.50 min) in order to adequately retain and resolve adenosine and uridine. Solvent B is then increased from 71% to 82% (1.50-2.75 min) to begin eluting cytidine, guanine, AMP, and UMP. Solvent B is then held at 82% (2.75-4.50 min) until these compounds elute before it is further increased to 90% (4.50-7.50 min) to begin
elution of the highly polar diphosphate and triphosphate nucleotides. Finally, B is held at 90% until all analytes are eluted from the column. To the best of our knowledge, this is the first time that all sixteen RNA nucleosides/nucleotides were separated with $R \geq 1.3$ in under twenty minutes.

3.3.5 Chromatographic comparison of optimized mobile phases

The performance of traditional MeOH:H$_2$O and ACN:H$_2$O HILIC mobile phases was examined relative to the performance of the EFLC MeOH:H$_2$O:CO$_2$ mobile phase, using similar buffer conditions. Both traditional HILIC mobile phases were optimized to provide the best separation of the 16 analytes within a similar time frame as the optimized EFLC separation (≤ 20 min). Figure 3.13 shows these optimized separations using ACN:H$_2$O, MeOH:H$_2$O, and MeOH:H$_2$O:CO$_2$ mobile phases at a flow rate of 1.0 mL/min. The optimized MeOH:H$_2$O separation is less than 15 minutes; however, the quality of the separation is hindered by the fact that almost no retention of the nucleosides and monophosphate nucleotides can be obtained. As a result, multiple analytes coelute. No amount of added MeOH content could provide adequate retention of the nucleosides and monophosphate nucleotides, and peak shape of the diphosphate nucleotides and triphosphate nucleotides is very poor. The ACN:H$_2$O system produces much better retention and peak shape, but is unable to resolve two analyte pairs (CMP and UDP, CDP and UTP). It may be possible to resolve all of the compounds by delaying or lengthening the gradient, but this would obviously come at the expense of increased
Clearly the best overall resolution in the time frame allowed is obtained using the MeOH:H$_2$O:CO$_2$ mobile phase (Figure 3.14).

Interestingly, both the ACN:H$_2$O and MeOH:H$_2$O methods also exhibited different retention order than the EFL mobile phase (Figure 3.15). This can also be an advantage of using an EFL mixture, since it can offer different selectivity than traditional methods. For example, compound pairs that have similar retention in ACN:H$_2$O (in this case CMP/GMP, and CDP/ATP) may have very different retention in MeOH:H$_2$O:CO$_2$, and vice versa. Theoretically, an EFLC or traditional HILIC method could be chosen depending on which compounds are of highest interest or importance. In short, the EFL mobile phase provided the best separation of the three mobile phases in the shortest time, with better resolution, and with different selectivity than either of the traditional HILIC mobile phases.

Others have also studied the separation of nucleosides/nucleotides using other HILIC columns with traditional HILIC mobile phases, either with a subset of the RNA nucleoside/nucleotide mixture or with additional biologically similar analytes, such as DNA nucleosides/nucleotides. A review article recently summarized these attempts [33]. Zhou et al. developed a HILIC separation of 11 RNA nucleoside/nucleotides with four intermediates in 26 minutes ($R_s \geq 1.3$) using a titania column with ACN:H$_2$O mobile phases [31]. However, their study did not include the four RNA nucleosides or CDP. Padivitage et al. was able to separate sixteen nucleotides (replacing the nucleosides with thymidine and its mono- di- and tri- phosphate nucleotides), but with a long analysis time (60 minutes) and multiple co-eluting pairs (CMP and GMP, CDP and ATP ) [32]. Yang
*et al.* separated monophosphate- and diphosphate nucleotides using HILIC with an amide column and ACN:H$_2$O mixtures, but this was combined with mass spectrometric detection making chromatographic resolution unnecessary [29]. Additionally, nucleosides and triphosphate nucleotides were not included in their study. Even with the benefit of a mass spectrometer and fewer compounds, the analysis time for Yang’s method was still 20 minutes. Clearly the optimized EFLC separation herein is markedly improved relative to previously developed HILIC methods, in terms of both analysis time (16 min) and resolved nucleoside/nucleotide compounds (16). Additionally it is a significant improvement of previous EFLC work in our group, which separated 15 RNA nucleoside/nucleotides isocratically in just under an hour [34]. By using gradient elution, the analysis time was decreased by almost a factor of 4, while still resolving all 16 compounds.
Figure 3.13. Optimized separation of the 16 nucleoside/nucleotide analyte mixture (1.25 x 10^{-4} M) with (A) ACN:100 mM sodium phosphate (pH=2.65) (0-7.5 min, 30% B, 7.5-15 min, 30-40% B, hold 40% B), (B) MeOH:400 mM sodium phosphate (pH=2.65) (0-4 min, 10% B, 4-6 min, 10-20% B, hold 20% B), and (C) MeOH:H$_2$O:CO$_2$ with gradient and sodium phosphate as described in Section 3.3.4. Analyte key is the same as Figure 3.1. Mobile phase gradients indicated with dashed lines.
Figure 3.14. Resolution data for (■) ACN:H$_2$O, (■) MeOH:H$_2$O and (■) MeOH:H$_2$O:CO$_2$ optimized separations (mobile phase conditions listed in Figure 3.13). Dashed line represents R=1.0.
Figure 3.15. Variation in elution order (from top to bottom) for nucleosides/nucleotides under different mobile phase conditions.
3.3.6 Efficiency and resolution

As illustrated in Chapter 2, the advantages gained by using EFLC are not limited to the changed polarity of the mobile phase, but also include higher analyte diffusivity and a lower viscosity solution in comparison to HPLC. This combination of improved mass transport properties allows for increased flow rates without significant loss in chromatographic efficiency. While it is not fair to directly compare the effective plate heights between these conditions (since most analytes were eluted using a gradient program), we can compare the efficiencies of the analytes eluted prior to the beginning of the gradient program. In this case, A, C, G, U, and AMP all elute prior to the gradient reaching the column in each optimized separation (ACN:H₂O, MeOH:H₂O, and MeOH:H₂O:CO₂). Figure 3.16 shows the observed efficiencies of each of these compounds under each set of optimized mobile phase conditions. It is evident that the EFLC MeOH:H₂O:CO₂ mixture produces greater efficiencies, particularly in comparison to MeOH:H₂O mobile phases. The heavy peak broadening and tailing seen when using viscous MeOH:H₂O mobile phases (Figure 3.13B) is significantly improved simply by adding CO₂ to the mobile phase, to the point where MeOH:H₂O mobile phases containing 30% CO₂ produce efficiencies greater than even ACN:H₂O mobile phases.

For comparison of analytes which elute under gradient mobile phase conditions, Neue previously illustrated that the square of peak volume ($p^2_0$) vs mobile phase flow rate (pseudo van Deemter plots) is useful, provided that the gradient volume is held constant [35]. Figure 3.17 shows pseudo van Deemter curves for the adenosine containing analytes (A, AMP, ADP, and ATP) with the ACN:H₂O, MeOH:H₂O, and MeOH:H₂O:CO₂
optimized gradient separations. As the flow rate is increased, \( p_v^2 \) (which is proportional to peak width squared) increases as well for all analytes with all mobile phase gradients. However, the rate at which \( p_v^2 \) (and peak width squared) increases is smaller for the EFLC mobile phase than for either the MeOH:H\(_2\)O or ACN:H\(_2\)O mobile phase.

Because this rate of decrease in efficiency is smaller, the flow rate of the optimized EFLC separation can be increased to 2.0 mL/min before the resolution of peaks AMP and UMP falls under \( R_s=1.0 \) (Figure 3.18). This effectively means that separation of these 16 RNA analytes can be completed in less than 10 minutes of analysis time (Figure 3.19). All other pairs of compounds still have resolution greater than 1.3 at this point so if resolution of AMP and UMP is not important, the flow rate could be increased further to 3.0 mL/min before instrument back pressure limits (600 bar) would be reached.
Figure 3.16. Efficiency data for early-eluting peaks (those which elute prior to the gradient reaching the column) for (■) MeOH:H₂O:CO₂, (■) MeOH:H₂O and (■) ACN:H₂O optimized separations (mobile phase conditions listed in Figure 3.13).
Figure 3.17. *Pseudo* van Deemter plots for 100 ppm of (A) A, (B) AMP, (C) ADP, and (D) ATP for the MeOH:H₂O:CO₂ (♦), MeOH:H₂O (■), and ACN:H₂O (▲) optimized mobile phases. Peak volumes were measured at various flow rates under optimized mobile phase conditions in Figure 3.13, adjusting gradient parameters accordingly.
Figure 3.17 continued

C

slope = 0.037
R² = 0.93

slope = 0.036
R² = 0.98

slope = 0.0088
R² = 0.98

Flow Rate (mL/min)

D

slope = 0.35
R² = 0.99

slope = 0.024
R² = 0.99

slope = 0.018
R² = 0.98

Flow Rate (mL/min)
Figure 3.18. Resolution of neighboring analyte pairs under optimized EFLC gradient conditions (Section 3.3.4) using 0.50 mL/min (■), 1.00 mL/min (■), 1.50 mL/min (■), and 2.00 mL/min (■) flow rates, adjusting gradient parameters accordingly. The dashed line marks a resolution of 1.0.
Figure 3.19. Separation of the 16 nucleoside/nucleotide analyte mixture (1.25 x 10^{-4} M) with (A) 0.50 mL/min, (B) 1.00 mL/min, (C) 1.50 mL/min, and (D) 2.00 mL/min flow rates, using optimized EFLC gradient conditions (Section 3.3.4). Analyte key is the same as Figure 3.1.
Figure 3.19 continued
3.3.7 Evaluation of method “greenness”

Each method was evaluated from an environmental, health, and safety standpoint. While MeOH and CO$_2$ are considered “greener” alternatives to ACN, the amount of each solvent used must also be taken into consideration. In order to assess the “greenness” of the optimized LC and EFLC methods, HPLC environmental assessment tool (HPLC-EAT) scores [36] were calculated for a single run under each set of optimized mobile phase conditions: ACN:H$_2$O, MeOH:H$_2$O, and MeOH:H$_2$O:CO$_2$. These scores take into consideration the environmental, health, and safety issues for all solvents within an HPLC method. The HPLC-EAT score for a given method is calculated using Eq. (2)

$$HPLC-EAT = (SI)_1 m_1 + (HI)_1 m_1 + (EI)_1 m_1 + (SI)_2 m_2 + (HI)_2 m_2 + (EI)_2 m_2 + ... + (SI)_n m_n + (HI)_n m_n + (EI)_n m_n$$

(2)

where $SI$, $HI$, and $EI$ are safety, health, and environmental impact factors, respectively (calculated for each solvent according to Koller et. al. [37]), $m$ is the mass of solvent used, and $n$ is the number of solvents. Lower scores indicate more environmentally friendly methods. The scores were tabulated for a single run (at 1 mL/min) using each method, and are reported in Table 3.5. Not only does the optimized MeOH:H$_2$O:CO$_2$ EFLC method use less total organic solvent per run, it also has a total HPLC-EAT score (25.44) that is less than half that of the ACN:H$_2$O method (61.24). Additionally, the individual safety, health, and environmental impact scores are all lower for the EFLC method compared to the ACN:H$_2$O method. As expected, the EFLC method has comparable HPLC-EAT scores to that of the optimized MeOH:H$_2$O separation, but the EFLC separation offers vastly superior chromatographic performance. When examining
all three methods from both a chromatographic and environmental standpoint, it is clear that the MeOH:H₂O:CO₂ method is by far the best option.
Table 3.5. Method parameters and HPLC-EAT scores for optimized ACN:H₂O, MeOH:H₂O, and MeOH:H₂O:CO₂ separations. Optimized conditions are shown in Figure 3.13.

<table>
<thead>
<tr>
<th>Method</th>
<th>Organic Solvent (g)</th>
<th>Safety Impact (SI)</th>
<th>Health Impact (HI)</th>
<th>Environ. Impact (EI)</th>
<th>HPLC-EAT Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACN:H₂O</td>
<td>13.47</td>
<td>36.61</td>
<td>14.27</td>
<td>10.36</td>
<td>61.24</td>
</tr>
<tr>
<td>MeOH:H₂O</td>
<td>9.53</td>
<td>17.79</td>
<td>3.98</td>
<td>2.97</td>
<td>24.74</td>
</tr>
<tr>
<td>MeOH:H₂O:CO₂</td>
<td>9.27</td>
<td>18.29</td>
<td>4.10</td>
<td>3.05</td>
<td>25.44</td>
</tr>
</tbody>
</table>
3.4 Conclusions

For the first time, EFL MeOH/H₂O/CO₂ mobile phases were demonstrated to be a viable “green” alternative to traditional ACN:H₂O mobile phases in gradient HILIC separations. By adding small portions of CO₂ to MeOH:H₂O mixtures, a solvent strength similar to ACN:H₂O can be easily obtained. Furthermore, addition of CO₂ to mobile phases allows for fine tuning of mobile phase polarity for the separation of analytes with wide polarity ranges when paired with gradient elution programming. These EFL mobile phases offered higher efficiency, resolution, and speed of analysis compared to ACN:H₂O and MeOH:H₂O mobile phases, using an optimized separation of 16 RNA nucleosides/nucleotides as a proof of concept. This separation yielded resolution greater than 1.3 for all nucleosides and nucleotides with an analysis time of approximately 16 minutes, and could be completed in approximately 8 minutes before resolution dropped below 1.0. Finally, these EFL solvents performed better than an equivalent ACN:H₂O separation in terms of environmental impact, making them ideal candidates for “green” HILIC separations.
3.5 References


CHAPTER 4: ELECTROSPUN NANOFIBERS AS SUBSTRATES FOR SURFACE-ENHANCED LASER DESORPTION/IIONIZATION MASS SPECTROMETRY ANALYSIS OF LARGE MOLECULES

4.1 Introduction

Matrix-assisted laser desorption/ionization (MALDI) is a widely used soft-ionization technique for analysis of nonvolatile and thermally labile large biomolecules and organic polymers [1,2]. When paired with mass spectrometry (MS), this analytical method is referred to matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). In MALDI-MS, samples are prepared by mixing the analytes of interest with an organic matrix and spotting the mixture on a target plate. When the sample dries, cocrystallization of the matrix and analyte molecules occurs. A laser is then used to ionize the sample. The mechanism of ionization is still not fully understood [3], but generally it is postulated that the organic matrix molecules (which are usually weak, UV- absorbing, organic acids) absorb the laser energy and transfer a portion of that energy to the analytes, which desorb and vaporize. The matrix also serves as a proton donor and/or receptor, and is therefore able to ionize the sample in both positive and negative ionization modes [4]. After desorption/ionization, the analyte ions are accelerated into the mass analyzer of the mass spectrometer, which is commonly a time-of-flight (TOF) instrument.
While MALDI-MS is certainly a widely useful technique, it is not without flaws. In the process of preparing a dried-droplet sample using organic matrices, complex processes such as solvent drying and analyte/matrix cocrystallization occur on the MALDI target plate. During these processes, many factors can lead to inhomogeneous distribution of analyte crystals. This makes the quality of a MALDI mass spectrum highly dependent on the spot that is irradiated by the laser. Areas that produce the greatest signal to noise ratio (S/N) are referred to as “sweet spots,” and must be searched for manually which can be both difficult and time consuming. The cause of these “sweet spots” has been the subject of discussion, but it appears to be the result of an inhomogeneous incorporation of analyte within the crystals of the matrix [5]. Regardless, “sweet spot” formation and poor shot-to-shot reproducibility are inherent disadvantages of MALDI. As a result, attempts to develop MALDI methods with homogenous samples and reproducible signals have been studied extensively [6,7,8,9].

Surface-assisted laser desorption/ionization (SALDI) was developed as a matrix-free alternative to MALDI in order to remedy “sweet spot” formation. First reported by Sunner et. al. [10], SALDI uses inorganic substrates rather than organic matrices to absorb and transfer energy from the laser. A number of different materials have successfully been employed as SALDI substrates without the use of a matrix and without sweet spot formation, including carbon [10,11], silicon [12,13], and metals [14,15]. The type and micro- or nanostructure of these materials is extremely important, as they can greatly impact the quality of SALDI mass spectra [16,17,18]. For example it is well known that bulk materials often do not generate signals that their nanostructured materials do [17].
Relative to MALDI-MS, SALDI-MS also theoretically provides greater sensitivity based on the fact that more analytes can be desorbed from the particle surfaces in each shot: $10^7$ - $10^9$ analyte molecules per nanoparticle versus $10^3$ - $10^5$ matrix required molecules per analyte [15].

While SALDI methods do offer some advantages over MALDI, they too are not without flaws. For example, absorption of the laser energy by the substrate is sometimes so strong that excess heat is often generated, leading to greater analyte fragmentation and lower signal and resolution. Liquids, such as glycerol, can be used to effectively dissipate this energy/heat [10,19], but the use of liquids also increases pressure in the ion source which can cause decreased ionization efficiency and produce increased background signal [20]. Additionally, the nanostructure of the SALDI substrate can sometimes be problematic, particularly if nanoparticles are used [10]. These small particles can easily become dislodged from the target plate and contaminate the ion source or damage the instrument. This is especially troublesome for conductive materials like graphite or metals, which can cause electrical discharges or short circuits within the instrument [10,20].

Although a variety of analytes have been studied using matrix-free laser desorption/ionization methods, only a few have examined SALDI applications for analysis of large synthetic polymers and proteins. In fact, SALDI-MS has been limited by its inability to ionize high molecular weight synthetic polymers [21] and proteins [22,23]. To date, the largest protein that has been analyzed by SALDI is immunoglobulin G (IgG) with a weight of 150 kDa [23].
Recently our group developed novel SALDI substrates using electrospun polymer and carbon nanofibers [24]. Initial studies show that these substrates demonstrate remarkable success in achieving high ionization efficiency, high S/N ratios, and high shot-to-shot reproducibility without the use of a matrix for a number of small biomolecules and synthetic polymers. Additionally, these substrates have been used in combination with organic matrices in a technique called matrix-enhanced surface-assisted laser/desorption ionization (ME-SALDI) for further signal enhancement. As mentioned earlier, the nanostructure of these SALDI substrates is crucial for high S/N ratios. These electrospun nanofibers have an average diameter of hundreds of nanometers and arranged in a woven mat, which prevents any dislodging of the substrate material into the instrument.

The work outlined in this chapter examines these electrospun nanofibrous substrates for SALDI analyses of large synthetic polymers and protein samples. Additionally, studies were undertaken to determine the necessary morphology of these substrates in order to obtain optimum ionization efficiency and S/N ratios. The polymeric nanofibers were prepared from commercially available, low-cost polymers: polyacrylonitrile (PAN), polyvinyl alcohol (PVA), and an epoxide-based photoresist (SU-8). SU-8 was used as a precursor to obtain carbon nanofibers via pyrolysis. Polystyrene (PS), polyethylene glycol (PEG), bovine serum albumin (BSA), human transferrin (TF), and immunoglobulin G (IgG) were used as probe analytes.
4.2 Materials and methods

4.2.1 Chemicals

Polyacrylonitrile (99% +, \(M_w = 150,000\)), polyvinyl alcohol (99% + hydrolyzed, \(M_w = 89,000-98,000\)) were purchased from Sigma-Aldrich (St. Louis, MO). SU-8 2100, an epoxide-based photoresist, was purchased from MicroChem Corporation (Newton, MA). Analytical grade dimethylformamide (DMF), methanol (MeOH), acetonitrile (ACN), and tetrahydrofuran (THF) were obtained from Fisher Scientific (Fairlawn, NJ). Cyclopentanone and glutaraldehyde (70% in water) were obtained from Sigma-Aldrich (St. Louis, MO). Sinapinic acid (>99.0%), 2,5-dihydroxybenzoic acid (>99.0%, DHB), sodium trifluoroacetate (>98%), silver trifluoroacetate (>99.99%), and trifluoroacetic acid (99%, TFA) were used as matrices and cationizing agents, and were also obtained from Sigma-Aldrich (St. Louis, MO). Analytes studied include polystyrene with \(M_w = 5120\), polyethylene glycol with \(M_w = 3400\), bovine serum albumin (99%), human transferrin (98%), and immunoglobulin G (>95%), and all were obtained from Sigma-Aldrich (St. Louis, MO).

4.2.2 Instrumentation

The electrospinning setup including a Spellman CZE 1000PN30 high voltage power supply (Hauppauge, NY) and a Harvard Pump Elite 11 programmable syringe pump (Holliston, MA). A Plexiglas® enclosure was used to contain the electrospinning environment (syringe tip to collector plate). Microscopic images of electrospun material were obtained using a Hitachi High Technologies (Pleasanton, CA) S-3400 scanning
electron microscope (SEM). Prior to SEM analysis, the samples were sputter coated with gold for 2 min at 10 µA to make them conductive. Average fiber diameters were analyzed using Image J software (Available at http://www.rsbweb.nih.gov/ij/index.html), and were determined from 100 fiber measurements.

Mass spectra were collected using a Bruker (Billerica, MA) Microflex MALDI-TOF mass spectrometer in positive ion mode. A nitrogen laser at 337 nm with a pulse width of 3 ns was used. The ion acceleration potential was maintained at 20 kV. Laser power was maintained slightly above the ionization threshold for each analyte (ranging from 10-30 µJ) in order to obtain the spectra and optimize S/N ratios. For samples under 12 kDa, reflector mode was used. For samples above 12kDa, linear mode was used. Each spectrum was a summation of 50 laser shots unless otherwise stated. FlexAnalysis™ software was used for data analysis, with the CENTROID method used for quantification of S/N ratios.

4.2.3 Substrate preparation

The electrospinning setup was modified so that a Bruker Daltonics (Ypsilanti, MI) MSP 96 MicroScout commercial stainless steel target plate could be used as the collector plate (Figure 4.1). This allowed for deposition of the substrates directly onto the target plate. When samples required SEM analysis, stainless steel shim coil (Maudlin Products, Kemah, TX) cut to the same size as the commercial target plate was used instead. A 10% PAN in DMF (w/w) solution was prepared and electrospun using previously optimized conditions [25]: 20 kV applied voltage, 20 cm distance between syringe tip and collector,
25 μL/min flow rate, and 30% relative humidity. The PAN solution was prepared by heating to 50°C to dissolve the polymer. PVA was electrospun using a slight modification to a previously developed *in situ* crosslinking procedure [26]. Briefly, an 8% (w/w) PVA in H2O solution was prepared, and heated to 80°C to dissolve the polymer. Prior to electrospinning, glutaraldehyde (GA:PVA, mol:mol, 90:1) and HCl (GA:HCl, mol:mol, 6:1) were then added to the PVA solution to initiate the cross-linking reaction. The mixture was stirred for 5 minutes, transferred to a syringe, and then immediately electrospun using the following conditions: 20 kV applied voltage, 20 cm distance between syringe tip and collector, 500 μL/hr flow rate, and 30% relative humidity. After 30 min, the solution was too viscous to electrospin as a result of extensive crosslinking.

SU-8 was used as a carbon substrate precursor. Both 70% and 75% SU-8 in cyclopentanone (v/v) solutions were prepared and electrospun. Initially, previously optimized parameters were used [27], but later the applied voltage and distance from tip to collector was varied to change the fiber diameter. After electrospinning, the obtained SU-8 substrates were cross-linked under a UV flood light (Sunray 400SM, Uvitron International West, Springfield, MA) for 10 minutes. The cross-linked SU-8 was then placed into a tube furnace (Lindberg/Blue M, Model: STF55346C-1, Asheville, NC) and heated under forming gas (5% H2 in N2). A ramp rate of 2°C/min was used for final temperatures of 450°C and 600°C, and a rate of 1°C/min was used for a final temperature of 750°C. The final pyrolysis temperatures were held for 5 hours and then cooled down to room temperature.
Figure 4.1. Electrospinning setup modified for SALDI substrate preparation on a commercial MALDI target plate.
4.2.4 Sample preparation

Polystyrene with sodium trifluoroacetate (1:1, w/w) was dissolved in acetonitrile. Polyethylene glycol with sodium trifluoroacetate (1:1, w/w) was dissolved in methanol. BSA and TF were dissolved in deionized water with 1% TFA (v/v). Sinapinic acid was dissolved in 50:50 ACN:H₂O with 1% TFA (v/v) and DHB was dissolved in methanol. When matrices were used in sample spotting, equal volumes of analyte and matrix solution were mixed and then spotted onto the target plate. If solubility issues arose between analyte and matrix solutions, then the multiple-layer spotting method was used for sample preparation [28]. The volume of solution spotted was 0.1 µL unless otherwise noted. Matrix and analyte concentrations were varied to study the effect of the matrix to analyte ratio. After use, the electrospun substrates were cleaned from the target plate. The target plate was then reused after a thorough rinse with MeOH, ACN, and H₂O.

4.3 Results and discussion

4.3.1 Preparation of electrospun polymeric SALDI substrates

Our group’s previous work demonstrated the effectiveness of electrospun polymeric substrates in SALDI analysis of small biological molecules and synthetic polymers [26]. The electrospinning process can quickly and easily generate random fibrous networks with different chemical functionalities and nanostructured morphologies. Additionally, these robust materials can be electrospun directly to a stainless steel target plate, without the use of a binder or adhesive. PAN, PVA, and SU-8 photoresist (Figure 4.2) are all polymers that have shown considerable SALDI potential in their electrospun
nanofibrous forms, despite their low UV absorptivity. This is believed to be a result of analyte partitioning into the unique microporous structure of the polymer chains [26]. As a result, they were chosen as a starting point for SALDI experiments with larger molecular weight proteins. Each of these electrospun materials has varying fiber morphology and average fiber diameter (Figure 4.3 and Table 4.1), which is known to have a significant impact on the quality of SALDI mass spectra [16,17,18].

4.3.2 SALDI of high molecular weight proteins

SALDI has typically been limited by its inability to ionize high molecular weight synthetic polymers [21] and proteins [22,23]. For that reason, it is worth determining the capabilities of our electrospun SALDI substrates for these analytes. Bovine serum albumin (BSA, molecular weight = 66 kDa) and human transferrin (TF, molecular weight = 80 kDa) were used as sample polymers. SALDI spectra were obtained for both BSA and TF on PAN and PVA substrates; however, signals were weak and only occurred at very low concentrations of protein (< 2 mg/mL). Additionally, the SU-8 substrate provided no signal for the proteins. Interestingly, higher concentrations resulted in less desorption/ionization of the protein molecules and lower or no overall signal. This has been reported before, and was attributed to lower entanglement of higher molecular weight proteins/polymers at lower concentrations [29]. SALDI spectra for BSA and TF are shown in Figure 4.4 and Figure 4.5, respectively. Similar fragmentation patterns were observed relative to other SALDI and MALDI experiments with BSA and TF, but the degree of protonation was significantly less [23,30]. The strongest signal in our
experiments was typically from the adduct ion, \([M+H]^+\), whereas in other SALDI and MALDI experiments, doubly and triply protonated ions, \([M+2H]^{2+}\) and \([M+3H]^{3+}\), yielded higher signals. Immunoglobulin G (IgG, molecular weight = 150 kDa) was also examined as an analyte, however no signals were obtained on either substrate for any concentration tested.

While the observed protein signals and the concentration range at which signal was observed were small, both the PAN and PVA substrates showed good shot-to-shot reproducibility for both proteins (Table 4.2). Signal was generated from 50 laser shots at 10 random spots within the sample, and the average S/N and the corresponding standard deviations were calculated accordingly. The relative standard deviations in S/N are similar to those observed in our group for synthetic polymer samples [26], although the overall S/N for the high molecular weight proteins is significantly lower. This is due to a combination of low sample concentration and decreased ionization relative to the synthetic polymer samples.
Figure 4.2. Chemical structures of (A) PAN, (B) PVA, and (C) SU-8 photoresist.
Figure 4.3. SEM images of electrospun (A) PAN, (B) PVA, and (C) SU-8 photoresist.
Figure 4.3 continued
Table 4.1. Average fiber diameter of electrospun polymer nanofibers, reported with standard deviation.

<table>
<thead>
<tr>
<th>Electrospun polymer</th>
<th>Fiber diameter* (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAN</td>
<td>320 ± 80</td>
</tr>
<tr>
<td>PVA</td>
<td>190 ± 50</td>
</tr>
<tr>
<td>SU-8</td>
<td>380 ± 110</td>
</tr>
</tbody>
</table>

*calculated from 100 fiber measurements
Figure 4.4. SALDI mass spectrum of 2 mg/mL BSA on PAN substrate.
Figure 4.5. SALDI mass spectrum of 1 mg/mL TF on PVA substrate.
Table 4.2. Average S/N for synthetic polymers [26] and proteins, with relative standard deviation (in parenthesis) indicating shot-to-shot reproducibility. SU-8 was used for PS (M_w = 5120) and PVA was used for TF.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>PS (25 mg/mL)</th>
<th>PS (50 mg/mL)</th>
<th>BSA (2 mg/mL)</th>
<th>TF (1 mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAN</td>
<td>50 ± 10 (20%)</td>
<td>40 ± 8 (20%)</td>
<td>5.3 ± 0.6 (11%)</td>
<td>7.5 ± 1.8 (24%)</td>
</tr>
<tr>
<td>PVA/SU-8</td>
<td>3.3 ± 0.4 (12%)</td>
<td>3.6 ± 0.6 (17%)</td>
<td>4.1 ± 0.7 (17%)</td>
<td>14.2 ± 2.2 (16%)</td>
</tr>
</tbody>
</table>
4.3.3 ME-SALDI of high molecular weight proteins

Although SALDI analysis of both BSA and TF protein was possible, ionization only occurred within a small concentration window (0.1-5 picomoles). Typically, this range is slightly larger for peptides and proteins (0.01-10 picomoles). Therefore, it is worth noting if these substrates offer any improvement in MALDI experiments, where a matrix is also present. When both a matrix and substrate are present, the technique is referred to as matrix-enhanced surface-assisted laser desorption/ionization (ME-SALDI) [31,32]. As seen in Figure 4.6, the overall signal and S/N is significantly enhanced when a matrix (sinapinic acid) is used in addition to the substrate. Additionally, the presence of the matrix increases fragmentation and ionization of the sample. However, it is clear that the presence of a PAN or PVA substrate offers minimal signal enhancement over a standard stainless steel target plate (Figure 4.7).

More importantly, the presence of PAN and PVA substrate improved the limit of detection in comparison to a traditional MALDI stainless steel target plate. For example, BSA had limits of detection (defined as 3 times S/N) of approximately 75 femtomoles and 250 femtomoles using a PAN and PVA substrate, respectively, each in combination with 40 mg/mL sinapinic acid as a matrix. When a stainless steel target plate and matrix were used without the presence of an electrospun nanofibrous substrate, the limit of detection was slightly higher (about 500 femtomoles). Figure 4.8 shows the spectrum generated by 0.1 μL of 0.05 mg/mL BSA (75 femtomoles) on a PAN substrate with 40 mg/mL sinapinic acid.
Figure 4.6. ME-SALDI spectra of (A) 2 mg/mL BSA on PAN substrate and (B) 1 mg/mL TF on PVA substrate. Matrix concentration = 20 mg/mL sinapinic acid.
Figure 4.7. S/N for varying concentrations of (A) BSA and (B) TF on stainless steel (■), PVA (■), and PAN (■) substrates. Matrix concentration = 20 mg/mL sinapinic acid.
Figure 4.8. ME-SALDI spectrum of 0.05 mg/mL BSA on PVA substrate. Matrix concentration = 40 mg/mL sinapinic acid.
4.3.4 Fabrication of nanofibrous carbon SALDI substrates

Nanofibrous carbon substrates, prepared via pyrolysis of SU-8 precursor, have also demonstrated success as SALDI substrates [26]. However, the limited initial studies suggest that both fiber diameter and degree of pyrolysis may play an important role in the degree of desorption/ionization that occurs. As a result, further studies are included on these topics herein.

Electrospun mats with varying fiber diameters were generated by electrospinning SU-8 with different electrospinning parameters. SU-8 concentration (75-80%), applied voltage (10-20 kV), and distance from syringe tip to collector (5-20 cm) were all variables that were changed to change the average fiber diameter. The flow rate was maintained at 5 μL/min. Because most polymers only electrospin within a narrow range of electric field strength, many combinations of parameters resulted in little to no fibers collected. For example, when the distance between the tip and collector is increased, the applied voltage must also increase in order to maintain similar electric field strength. In general, two trends were observed. First, increased voltage resulted in larger fiber diameters. This was attributed to the stronger acceleration of the charged jet reducing the flight time of the fibers, giving them less time to stretch before it is deposited on the collector plate [33]. Second, increased distance resulted in increased fiber diameter. This is attributed to the decreased electric field strength, resulting in less stretching of the fibers [33]. Therefore, increased voltage and increased distance resulted in the highest average fiber diameters.

Even with variation of the electrospinning parameters, only a relatively small range of average fiber diameters could be generated for the SU-8 polymer (270-520 nm).
Additionally, after cross-linking and pyrolysis of these mats, the average fiber diameters of an electrospun sample shrink due to the significant mass loss. This further minimizes the range of fiber diameters available for carbon substrate experimentation. Ultimately, 3 sets of conditions yielding varying different average fiber diameters were tested as SALDI substrates. These electrospinning conditions and their resulting fiber diameters pre- and post-pyrolysis to 600 °C are shown in Table 4.3. The fiber diameter averages that result from pyrolysis are presented in Table 4.4.

In order to study how the extent of pyrolysis impacts carbon substrate SALDI performance, three different pyrolysis temperatures were studied (450, 600, and 750ºC). Pyrolysis temperatures higher than 750ºC resulted in the substrate bubbling and peeling away from the stainless steel target, regardless of the temperature ramp rate. Even at 750ºC, parts of the substrate sometimes peeled away from the target, making only parts of the mat useful. Digital photographs (Figure 4.9) and SEM images (Figure 4.10) illustrate the differences between both the macroscopic and microscopic properties of these substrates as a function of pyrolysis temperature.
Table 4.3. Average fiber diameters for electrospun SU-8 under different electrospinning conditions.

<table>
<thead>
<tr>
<th>Electrospinning parameters</th>
<th>As-spun fiber diameter* (nm)</th>
<th>Post-pyrolysis (600°C) fiber diameter* (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80% SU-8, 5 cm, 10 kV</td>
<td>270 ± 70</td>
<td>160 ± 20</td>
</tr>
<tr>
<td>80% SU-8, 10 cm, 12 kV</td>
<td>380 ± 80</td>
<td>180 ± 30</td>
</tr>
<tr>
<td>75% SU-8, 15 cm, 15kV</td>
<td>520 ± 120</td>
<td>250 ± 50</td>
</tr>
</tbody>
</table>

*calculated from 100 fiber measurements
Table 4.4. Fiber diameter shrinkage as a result of pyrolysis for SU-8 fibers with an as-spun average fiber diameter of 270 nm.

<table>
<thead>
<tr>
<th>Pyrolysis Temperature (°C)</th>
<th>Average fiber diameter* (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>270 ± 70</td>
</tr>
<tr>
<td>450</td>
<td>220 ± 40</td>
</tr>
<tr>
<td>600</td>
<td>160 ± 20</td>
</tr>
<tr>
<td>750</td>
<td>150 ± 30</td>
</tr>
</tbody>
</table>

*calculated from 100 fiber measurements
Figure 4.9. Digital photographs of SU-8 nanofibers (A) as-spun, (B) pyrolyzed to 450°C, (C) pyrolyzed to 600°C, and (D) pyrolyzed to 750°C on a stainless target plate.
Figure 4.9 continued
Figure 4.10. SEM images of SU-8 nanofibers (A) as-spun, (B) pyrolyzed to 450°C, (C) pyrolyzed to 600°C, and (D) pyrolyzed to 750°C.
Figure 4.10 continued
4.3.5 Impact of fiber diameter on carbon substrate SALDI performance

The fiber diameter of the electrospun carbon SALDI substrates was determined to play a significant role in the desorption/ionization efficiency of analytes. Using varying concentrations of PEG and PS as probe analytes, three different fiber diameters (160, 180, and 250 nm) were examined. As mentioned earlier (Table 4.3), these substrates with varying fiber diameter were generated from different SU-8 electrospinning conditions followed by pyrolysis to 600 °C. Three different concentrations (10, 25, and 40 mg/mL) of PEG and PS were then spotted and analyzed on each of the three substrates. Average S/N measurements were calculated from 5 replicates of each concentration on each of the three substrates.

As expected, smaller diameter fibers performed much better than larger diameter fibers pyrolyzed to the same temperature. This was predicted due the fact smaller diameter nanofibers yield a larger surface area that is available for laser energy absorption and transfer. Figure 4.11 illustrates the differences in signal intensity for a sample of 40 mg/mL PEG on each of the three substrates. While the signal obtained from the carbon substrate with 250 nm average fiber diameter is significant, S/N and signal intensity is greatly improved for the 180 and 160 nm fiber diameter substrates. This trend held true across the entire range of concentrations for both the PEG (Table 4.5) and PS (Table 4.6) samples, although the PEG samples showed greater ionization efficiency and S/N relative to the PS samples. This suggests that smaller diameter nanofibers are preferred for SALDI analysis using nanofibrous carbon substrates.
Figure 4.11. Mass spectra of 40 mg/mL PEG (M_w = 3400) using carbon nanofibrous substrates with average fiber diameters of (A) 250 nm, (B) 180 nm, and (C) 160 nm. The final pyrolysis temperature was 600°C.
Table 4.5. S/N of SALDI spectra for PEG (M\textsubscript{w} = 3400) on carbon substrates with different average fiber diameters. The pyrolysis temperature was 600 °C.

<table>
<thead>
<tr>
<th>Average fiber diameter (nm)</th>
<th>10 mg/mL</th>
<th>25 mg/mL</th>
<th>40 mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>21 ± 5</td>
<td>29 ± 2</td>
<td>43 ± 9</td>
</tr>
<tr>
<td>180</td>
<td>58 ± 6</td>
<td>71 ± 3</td>
<td>92 ± 24</td>
</tr>
<tr>
<td>160</td>
<td>79 ± 11</td>
<td>108 ± 18</td>
<td>109 ± 32</td>
</tr>
</tbody>
</table>
Table 4.6. S/N of SALDI spectra for PS ($M_w = 5120$) on carbon substrates with different average fiber diameters. The pyrolysis temperature was 600 °C.

<table>
<thead>
<tr>
<th>Average fiber diameter (nm)</th>
<th>10 mg/mL</th>
<th>25 mg/mL</th>
<th>40 mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>8 ± 3</td>
<td>10 ± 2</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>180</td>
<td>38 ± 9</td>
<td>47 ± 11</td>
<td>61 ± 14</td>
</tr>
<tr>
<td>160</td>
<td>55 ± 10</td>
<td>64 ± 18</td>
<td>77 ± 17</td>
</tr>
</tbody>
</table>
4.3.6 Impact of pyrolysis temperature on carbon substrate SALDI performance

The extent of pyrolysis was also shown to have a significant impact on carbon SALDI substrate performance. Once again, three different concentrations (10, 25, and 40 mg/mL) of PEG and PS were then spotted and analyzed on each of three substrates: this time 270 nm SU-8 pyrolyzed to 450, 600, and 750 °C. Average S/N measurements were again calculated from 5 replicates of each concentration on each of the three substrates.

Since many forms of nanostructured carbon are already known to be excellent SALDI substrates, it was hypothesized that increased pyrolysis temperatures (and therefore increased carbon content) would improve SALDI performance. Additionally, the decreased fiber diameter and increased surface area that results from higher pyrolysis temperatures should further improve the desorption/ionization process. In fact, the opposite turned out to be true. Not only did increased pyrolysis temperatures lead to fragile, detached substrates (Figure 4.9), but these substrates also produced higher amounts of polymer fragmentation and lower S/N than substrates pyrolyzed to lower temperature, regardless of laser power. Figure 4.12 shows the deterioration of the signal from 40 mg/mL PEG as the degree of pyrolysis is increased. At 450 °C, a relatively smooth polymer distribution is observed, with high S/N. At 600 °C, high S/N is still observed, but some degree of polymer fragmentation can be seen in the low mass region. At 750 °C, large fragmentation and significantly decreased S/N are evident.

Once again, this pattern held true across all concentration ranges for both PEG (Table 4.7) and PS (Table 4.8). It is proposed that the combination of high surface area and high carbon content in the 750 °C substrate results in significant absorption of laser
energy and hard ionization of analytes. As a result, it appears that smaller nanofiber diameters are most beneficial for substrates pyrolyzed to lower temperatures (600 °C or less).
Figure 4.12. Mass spectra obtained from 40 mg/mL PEG ($M_w = 3400$) on carbon substrates processed to different final temperatures during pyrolysis.
Table 4.7. S/N of SALDI spectra for PEG (M<sub>w</sub> = 3400) on carbon substrates processed to different pyrolysis temperatures.

<table>
<thead>
<tr>
<th>Final pyrolysis temperature (°C)</th>
<th>10 mg/mL</th>
<th>20 mg/mL</th>
<th>40 mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>450</td>
<td>84 ± 6</td>
<td>103 ± 8</td>
<td>131 ± 12</td>
</tr>
<tr>
<td>600</td>
<td>79 ± 11</td>
<td>108 ± 18</td>
<td>109 ± 32</td>
</tr>
<tr>
<td>750</td>
<td>24 ± 4</td>
<td>40 ± 3</td>
<td>55 ± 6</td>
</tr>
</tbody>
</table>
Table 4.8. S/N of SALDI spectra for PS ($M_w = 5120$) on carbon substrates processed to different pyrolysis temperatures.

<table>
<thead>
<tr>
<th>Final pyrolysis temperature (°C)</th>
<th>10 mg/mL</th>
<th>20 mg/mL</th>
<th>40 mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>450</td>
<td>34 ± 8</td>
<td>53 ± 18</td>
<td>58 ± 14</td>
</tr>
<tr>
<td>600</td>
<td>11 ± 3</td>
<td>14 ± 4</td>
<td>17 ± 9</td>
</tr>
<tr>
<td>750</td>
<td>No signal</td>
<td>No signal</td>
<td>12 ± 3</td>
</tr>
</tbody>
</table>
4.4 Conclusions

Both polymer-based and carbon-based electrospun nanofibrous substrates have shown significant potential for SALDI analysis. This potential has been demonstrated for both synthetic polymers and high molecular weight proteins (up to 80 kDa). For carbon-based substrates, optimal performance was achieved when nanofiber diameters were small (~200 nm) and pyrolysis temperatures were relatively low (less than 600 °C). Additionally, combination of these substrates with traditional organic matrices (ME-SALDI) resulted in improved limit of detection relative to MALDI.
4.5 References


5.1 Research summary

Chapter 2 of this dissertation demonstrated the benefits of using enhanced-fluidity liquid chromatography (EFLC) for a mixed-mode hydrophilic-interaction/strong cation-exchange (HILIC/SCX) separation of ten neutral side chain amino acids. Baseline separation of 10 amino acids was achieved using a methanol/water/CO$_2$ mobile phase under EFLC conditions. This isocratic separation offered higher resolution and efficiencies than methanol/water LC mobile phases at a flow rate of 1.0 mL/min, as well as slower decreases in efficiency as the flow rate was increased. Additionally, analysis time was half that required by an isocratic acetonitrile/water LC separation on the same column. Differences in selectivity were also observed between LC and EFLC separation modes. Finally, EFLC mobile phases demonstrated that they can be used in the separation of ionic or ionizable compounds without encountering solubility or elution issues, while still offering increased diffusivity and lower viscosities than LC mobile phases.

Chapter 3 of this dissertation extended the work from Chapter 2 by applying EFLC methods to gradient HILIC separations. MeOH/H$_2$O/CO$_2$ mobile phases were demonstrated to be a viable “green” alternative to traditional ACN:H$_2$O mobile phases in gradient HILIC separations. Addition of CO$_2$ to mobile phases allowed for fine tuning of mobile phase polarity for the separation of analytes with wide polarity ranges when
paired with gradient elution programming. Furthermore, by adding small portions of CO₂ to MeOH:H₂O mixtures, a solvent strength similar to ACN:H₂O can be easily obtained. These EFL mobile phases offered higher efficiency, resolution, and speed of analysis for a separation of 16 RNA nucleosides and nucleotides, while also performing better than an equivalent ACN:H₂O separation in terms of environmental impact, making them ideal candidates for “green” HILIC separations.

Finally, Chapter 4 of this work shows the significant potential of both polymer-based and carbon-based electrospun nanofibrous substrates for SALDI analysis. This potential was demonstrated for both synthetic polymers and high molecular weight proteins (up to 80 kDa). For carbon-based substrates, optimal performance was achieved when nanofiber diameters were small (~200 nm) and pyrolysis temperatures were relatively low (less than 600 °C). Additionally, combination of these substrates with traditional organic matrices (ME-SALDI) resulted in improved performance relative to MALDI.

5.2 Enhanced-fluidity liquid chromatography - Future work

The work in Chapters 2 and 3 of this dissertation clearly demonstrate the benefits of enhanced-fluidity liquids (EFL) as mobile phases for hydrophilic interaction chromatography (HILIC) methods. Methanol/H₂O/CO₂ mixtures exhibited comparable or better chromatographic performance than traditional acetonitrile/water HILIC mobile phases, all while minimizing environmental impact. These methods proved extremely
useful for methods using both evaporative light scattering and UV detectors, where peak resolution is an absolute necessity for identification and quantification.

Unfortunately, many chemical samples are too complex to obtain full resolution of all analytes present. In these cases, liquid chromatography – mass spectrometry (LC-MS) methods are typically used, since mass spectrometric detection provides information about the chemical composition of the analytes as they elute off the column. HILIC methods are becoming increasingly popular for LC-MS methods, particularly in areas such as metabolomics [1] and proteomics [2] where analytes are highly polar and/or charged. The high organic content, low water content, and commonly used volatile buffers (ammonium acetate, ammonium formate, etc.) in typical HILIC mobile phases make them ideal candidates for mass spectrometric detection, especially in electrospray ionization (ESI) mode [3].

Enhanced-fluidity liquids should make excellent alternatives to the acetonitrile/water mobile phases in HILIC-ESI-MS. Supercritical fluid chromatography (SFC) has been successfully integrated with mass spectrometry using both atmospheric pressure chemical ionization (APCI) and ESI, although APCI is more common since SFC mobile phases are typically void of water [4]. However, EFL mobile phases contain more water than SFC mobile phases, and thus should be even more suitable for ESI-MS than SFC mobile phases. Additionally, the presence of CO₂ increases volatization of the EFL mobile phases relative to traditional LC-MS. Therefore, the logical next step in our EFLC research is to pair EFLC with ESI-MS as a “green” alternative to HILIC-ESI-MS, and to demonstrate its chromatographic capabilities in difficult glycomics or metabolomics.
separations. This should be possible using the Agilent 1260 SFC system discussed in Chapter 3, albeit with a few modifications.

5.3 Electrospun nanofibrous SALDI substrates – Future Work

As seen in Chapter 4, both polymeric and carbon substrates have been shown to produce quality SALDI spectra of a range of biological molecules. Furthermore, the diameter of the carbon nanofibers and the degree of pyrolysis were demonstrated to have a significant effect on the quality of the spectra obtained. As mentioned earlier it is well known that nanostructure of a SALDI material is very important [5], and the carbon nanofibrous substrate proved to be no exception. It would be extremely interesting to see what effect nanoporous fibers would have on the performance of electrospun carbon substrates. Highly porous carbon nanofibers have been prepared via a number of different methods [6,7,8], but all of the methods result in an extremely high surface area carbon material. This extra surface area has the potential to improve the performance of our nanofibrous carbon substrates.
5.4 References


through the thermal treatment of electrospun copolymeric nanofiber webs, Small 3 (2007) 91-95.
BIBLIOGRAPHY

Chapter 1


**Chapter 2**


**Chapter 3**


Chapter 4


**Chapter 5**


