2015

ANALYTICAL METHOD DEVELOPMENT FOR THE DETECTION AND ANALYSIS OF PROTEIN CARBONYLS

Chelsea M. Coffey
Virginia Commonwealth University

Follow this and additional works at: https://scholarscompass.vcu.edu/etd
Part of the Analytical Chemistry Commons

© The Author

Downloaded from https://scholarscompass.vcu.edu/etd/3737

This Dissertation is brought to you for free and open access by the Graduate School at VCU Scholars Compass. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of VCU Scholars Compass. For more information, please contact libcompass@vcu.edu.
ANALYTICAL METHOD DEVELOPMENT FOR THE
DETECTION AND ANALYSIS OF PROTEIN CARBONYLS

A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy at Virginia Commonwealth University

By:

Chelsea Coffey

Advisor: Dr. Scott Gronert

Department of Chemistry

Virginia Commonwealth University

Richmond, Virginia

May 2015
Acknowledgement

First and foremost, I would like to express my gratitude to my advisor, Dr. Scott Gronert, for his constant support over these last five years. I have the utmost respect for him as a professor, mentor, scientist, and person. His relentless guidance and support helped make this research possible. I will be forever grateful for the scientist he has helped me to become and will always treasure the experiences gained in his research group.

I would also like to thank my committee members, Dr. Maryanne Collinson, Dr. Matthew Hartman, and Dr. Andrew Ottens. I am honored to name you as members of my committee and am grateful for all of the advice provided over the years.

To my fellow group members: a big thank-you goes out to our research scientist, Dr. David Simpson, who was always willing and able to offer guidance and assistance. I would also like to thank Dr. Mark Davis, Keyanna Conner, and Malissa Grose for their constant support. Lastly, I owe my deepest gratitude to Dr. Suresh Narayanasamy, who will always have a special place in my heart. Thank you for being my lab mate, mentor, and best friend.

I would also like to thank Dr. Michael Hunnicutt, whose passion and enthusiasm for science inspire me. I’m grateful for all I have learned from this amazing instructor, despite his allegiance to dUKe.

Last, but not least, words cannot express the gratitude and love I have for my family. To my mom, dad, brother, and better half, thank you for continuously loving and supporting me. I would not be where I am or who I am today without your influence. I love you with all of my heart.
# Table of Contents

List of Tables .......................................................................................................................... viii
List of Figures ............................................................................................................................ ix
List of Schemes ........................................................................................................................... xii
List of Abbreviations ................................................................................................................ xiii
Abstract ....................................................................................................................................... xv

Chapter 1 – Introduction ........................................................................................................... 1

1.1 Introduction ....................................................................................................................... 2
1.2 Pro-oxidants and Antioxidants ....................................................................................... 3
1.3 Protein Oxidation ............................................................................................................. 6
1.4 Protein Carbonylation ...................................................................................................... 11
   1.4.1 Protein Carbonylation Mechanisms ........................................................................ 12
   1.4.2 Carbonylation and Aging ....................................................................................... 15
   1.4.3 Carbonylation and Disease .................................................................................... 16
   1.4.4 Detecting Carbonylation ....................................................................................... 16
1.5 Mass Spectrometry .......................................................................................................... 18
   1.5.1 Ionization ................................................................................................................ 19
   1.5.2 Mass Analyzer ........................................................................................................ 20
   1.5.3 Detector .................................................................................................................. 22
   1.5.4 Data Analysis ........................................................................................................ 23
   1.5.5 Quantitative Proteomics ...................................................................................... 24

Chapter 2 – Developing Analytical Methods Using Carbonylated Human Serum Albumin (HSA) as a Model Protein ........................................................................................................... 25

2.1 Abstract ............................................................................................................................. 26
2.2 Introduction ....................................................................................................................... 27
2.3 Experimental Design ....................................................................................................... 31
   2.3.1 Materials ................................................................................................................ 31
3.1 Abstract...............................................................................................................................66
3.2 Introduction..........................................................................................................................67
3.3 Experimental Design...........................................................................................................71
  3.3.1 Materials .......................................................................................................................71
  3.3.2 Carboxylating HSA.........................................................................................................71
  3.3.3 Protein Biotinylation and Enrichment .............................................................................72
  3.3.4 Biotin Quantitation Assay...............................................................................................72
  3.3.5 Trypsin Digestion............................................................................................................73
  3.3.6 LC-MS/MS .....................................................................................................................73
  3.3.7 Data Analysis ................................................................................................................74
3.4 Results..................................................................................................................................75
  3.4.1 Labeling Efficiency..........................................................................................................75
  3.4.2 Identification of Modification Sites.................................................................................76
3.5 Discussion............................................................................................................................81
  3.5.1 Labeling Efficiency..........................................................................................................81
  3.5.2 Spectral Counting...........................................................................................................81
  3.5.3 Identification of Modification Sites.................................................................................82
  3.5.4 Limitations ...................................................................................................................89
  3.5.5 SpectraST ......................................................................................................................91
3.6 Conclusion ..........................................................................................................................96

Chapter 4 – Application: Investigation of Protein Carboxylation in Human Plasma
Collected From Chronic Kidney Disease (CKD) Patients on Dialysis ........................................97
4.1 Abstract...............................................................................................................................98
4.2 Introduction..........................................................................................................................99
4.3 Experimental Design..........................................................................................................103
  4.3.1 Materials ......................................................................................................................103
  4.3.2 Plasma Preparation .......................................................................................................103
4.3.3 Protein Labeling .................................................................................. 104
4.3.4 Protein Digestion ................................................................................. 104
4.3.5 Mass Spectrometry .............................................................................. 105
4.3.6 Database Searches and Analysis ......................................................... 105
4.3.7 Western Blotting .................................................................................. 106
4.3.8 Biotin Quantitation Assay ................................................................. 107
4.3.9 FTC Assay ............................................................................................ 108

4.4 Results ...................................................................................................... 109
4.4.1 Western Blotting .................................................................................. 109
4.4.2 Biotin Quantitation Assay ................................................................. 113
4.4.3 FTC Assay ............................................................................................ 114
4.4.4 Mass Spectrometry .............................................................................. 118

4.5 Discussion ................................................................................................ 120
4.5.1 Analytical Assays ............................................................................... 120
4.5.2 Other Factors to Consider .................................................................. 125

4.6 Conclusion ................................................................................................ 127

Chapter 5 – Application: Investigation of Protein Carbonylation in Human
Plasma/Serum Collected From Trauma Patients ............................................. 128
5.1 Abstract .................................................................................................... 129
5.2 Introduction ............................................................................................... 130
5.3 Experimental Design ............................................................................... 134
5.3.1 Materials ............................................................................................. 134
5.3.2 Preparation of Plasma/Serum ............................................................. 134
5.3.3 Protein Biotinylation .......................................................................... 135
5.3.4 ProteoMiner Depletion ...................................................................... 136
5.3.5 Western Blotting ................................................................................ 136
5.3.6 Affinity Enrichment and Tryptic Digestion ........................................ 137
5.3.7 iTRAQ Labeling.................................................................137
5.3.8 Mass Spectrometry.............................................................138
5.3.9 Database Searches and Analysis ........................................139
5.3.10 FTC Assay ........................................................................139

5.4 Results ..................................................................................141
  5.4.1 Western Blotting: Patients W, X, Y, and Z .........................141
  5.4.2 FTC Assay: Patient X ........................................................144
  5.4.3 iTRAQ Quantitation: Patients W and X ..............................146
  5.4.4 ProteoMiner Depletion and iTRAQ Quantitation: Patients Y and Z .... 150

5.5 Discussion ..............................................................................154
  5.5.1 Western Blotting ...............................................................154
  5.5.2 FTC Assay: Patient X ........................................................154
  5.5.3 iTRAQ: Patients W and X ...............................................155
  5.5.4 ProteoMiner and iTRAQ: Patients Y and Z .......................156

5.6 Conclusion ..............................................................................158

References ....................................................................................159
Appendices ....................................................................................178
List of Tables

<table>
<thead>
<tr>
<th>Table 1-1</th>
<th>Factors related to various types of protein oxidation ........................................7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2-1</td>
<td>Fractions of biotinylated peptides detected with mass spectrometry before and after monomeric avidin column enrichment ........................................41</td>
</tr>
<tr>
<td>Table 2-2</td>
<td>Fractions of biotinylated peptides detected with mass spectrometry before and after monomeric avidin column enrichment ........................................45</td>
</tr>
<tr>
<td>Table 2-3</td>
<td>Fractions of carbonylated HSA spiked into plasma, labeled, and then detected with mass spectrometry before and after monomeric avidin column enrichment ........................................47</td>
</tr>
<tr>
<td>Table 2-4</td>
<td>Enrichment results at the protein level – mass spectrometry spectral counting results for Labels 1, 2, and 4 .................................................................48</td>
</tr>
<tr>
<td>Table 2-5</td>
<td>Average anisotropy (r) values along with standard deviations for three replicate instrumental analyses for both Standard Sets ........................................52</td>
</tr>
<tr>
<td>Table 3-1</td>
<td>Peptide Prophet Analysis: List of modified HSA peptides with mass shifts for modified cysteine (C), histidine (H), and lysine (K) ..................................................93</td>
</tr>
<tr>
<td>Table 3-2</td>
<td>SpectraST Analysis: List of modified HSA peptides with mass shifts for modified cysteine (C), histidine (H), and lysine (K) ..................................................93</td>
</tr>
<tr>
<td>Table 4-1</td>
<td>The CKD stages according to the National Kidney Foundation (KDOQI) ..................99</td>
</tr>
<tr>
<td>Table 4-2</td>
<td>Calculated biotin to protein ratios for two subsets of CKD patient samples ........113</td>
</tr>
</tbody>
</table>
# List of Figures

| Figure 1-1 | Protein oxidation is dependent on the actions of pro-oxidants, antioxidants, and proteases .......................................................... 5 |
| Figure 1-2 | Formation, detoxification, and reaction of $\alpha,\beta$-unsaturated aldehydes during LPO ............................................................ 14 |
| Figure 1-3 | A general diagram for an LTQ equipped with ESI ......................................................... 21 |
| Figure 2-1 | Structure of acrolein .................................................................................. 29 |
| Figure 2-2 | Biotin quantitation assay results ................................................................. 43 |
| Figure 2-3 | Labeling efficiencies visualized via Western blotting ..................................... 44 |
| Figure 2-4 | Principle of fluorescence anisotropy .............................................................. 49 |
| Figure 2-5 | HSA Standard Set 1 includes (A) Emission spectrum and (B) Corresponding calibration curve. HSA Standard Set 2 includes (C) Emission spectrum and (D) Corresponding calibration curve .......................................................... 50 |
| Figure 2-6 | HSA Standard Set 1 includes (A) Emission spectrum and (B) Corresponding calibration curve. HSA Standard Set 2 includes (C) Emission spectrum and (D) Corresponding calibration curve ........................................................................... 51 |
| Figure 2-7 | Structures and molecular weights for Labels 1-4 ............................................. 55 |
| Figure 2-8 | Peptide level: Plot depicting number of MS/MS spectra identified before and after enrichment of HSA derivatized with three different biotin labels ........................................................................ 57 |
| Figure 2-9 | Plot depicting number of MS/MS spectra identified following protein enrichment for Label 4-derivatized HSA ........................................ 59 |
| Figure 3-1 | Biotin quantitation assay results ................................................................ 76 |
| Figure 3-2 | Percent of modified peptides for 0, 10, 25, 50, and 75 % carbonylated HSA spikes .................................................................................. 78 |
| Figure 3-3 | HSA amino acid sequence ........................................................................... 79 |
Figure 3-4  MS/MS spectrum of the peptide RH{528.24}PYFYAPELLFFAK in the +2 charge state, m/z 1145.84, with the oxidative modification on the histidine (H) residue .................................................................80

Figure 3-5  Spectral counting results for triplicate injections of 0, 10, 25, 50, and 75 % carbonylated HSA spikes.................................................................83

Figure 3-6  Comparison of modification sites identified between the alkoxyamine label versus the biotin hydrazide labels .........................................................85

Figure 3-7  Number of spectra for shared modification sites between the biotin hydrazide labels and the alkoxyamine label .......................................................86

Figure 3-8  Comparison of modified peptide spectra to unmodified peptide spectra for certain peptides .................................................................88

Figure 3-9  SpectraST spectrum for RH{528.24}PYFYAPELLFFAK in the +2 charge state .................................................................95

Figure 4-1  SAMPLE SET #1: Duplicate Western blot analyses of patient samples 907, 935, and 967 .................................................................111

Figure 4-2  SAMPLE SET #2: Duplicate Western blot analyses of patient samples 910, 930, and 952 .................................................................112

Figure 4-3  CKD Sample Set 1: emission spectra from three interday FTC assay analyses of patient samples 909, 922, and 951 .................................................................115

Figure 4-4  CKD Sample Set 2 emission spectra from: A. First sample preparation on Day 1. B. Second sample preparation on Day 2. C. First sample preparation reanalyzed on Day 3. D. Second sample preparation reanalyzed on Day 3. .................................................................116

Figure 4-5  Average anisotropy (r) values and standard deviations for triplicate analyses of patient samples control 909, HD 922, and PD 951 .................................................................117

Figure 4-6  Average percentage of methionine/histidine oxidation for three LC-MS/MS analyses of sample numbers 907, 924, 935, 962, and 967 .................................................................119

Figure 5-1  Illustration depicting the acute phase response .................................................................132

Figure 5-2  Western blot analyses of trauma patient samples W and X .................................................................142

Figure 5-3  Western blot analyses of trauma patient samples Y and Z .................................................................143

Figure 5-4  Emission spectra from triplicate FTC assay analyses of Patient X .................................................................145
Figure 5-5  A. Components of an iTRAQ label. B. Product following reaction between iTRAQ label and a peptide (indicated by amide bond).........................146

Figure 5-6  Carbonylated protein levels for ten proteins that exhibited the most change over the course of eight days for Patient W .........................148

Figure 5-7  Carbonylated protein levels for ten proteins that exhibited the most change over the course of six days for Patient X .........................149

Figure 5-8  ProteoMiner concept depicting equalization of proteins ................................151

Figure 5-9  Carbonylated protein levels for ten proteins that exhibited the most change over the course of four days for Patient Y .........................152

Figure 5-10 Carbonylated protein levels for ten proteins that exhibited the most change over the course of four days for Patient Z .........................153
List of Schemes

Scheme 1-1 Initiation (1), propagation (2-3), and termination (4) steps of the lipid peroxidation process .................................................. 9

Scheme 1-2 Carbonylation of arginine, lysine, and proline to form glutamic and aminoadipic semialdehydes ........................................... 13

Scheme 2-1 Acrolein-based Michael addition forms adducts on cysteine, histidine, and lysine ................................................................. 39

Scheme 2-2 Reaction between a hydrazide and an aldehyde to form a hydrazone ................................................................................. 40

Scheme 2-3 Tautomerization of HABA upon addition of avidin with a subsequent red shift ................................................................. 42

Scheme 2-4 The labeling of carbonylated proteins with FTC ................................................................. 48

Scheme 3-1 Products resulting from reaction of the alkoxyamine label with a protein carbonyl and subsequent enrichment, reduction, and alkylation ......................................................... 70

Scheme 4-1 Reaction between a protein carbonyl and DNPH to form the derivatized product ................................................................. 109

Scheme 4-2 Chemiluminescence reaction .................................................................................. 110

Scheme 4-3 Redox processes of methionine (Met) ....................................................................... 124
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-HNE</td>
<td>4-hydroxynonenal</td>
</tr>
<tr>
<td>4-ONE</td>
<td>4-oxo-2-nonenal</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ACR</td>
<td>Acrolein</td>
</tr>
<tr>
<td>AGEs</td>
<td>Advanced glycation end products</td>
</tr>
<tr>
<td>APPs</td>
<td>Acute Phase Proteins</td>
</tr>
<tr>
<td>APR</td>
<td>Acute Phase Response</td>
</tr>
<tr>
<td>ARP</td>
<td>Aldehyde reactive probe</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium chloride dehydrate</td>
</tr>
<tr>
<td>CAPD</td>
<td>Continuous Ambulatory Peritoneal Dialysis</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CEMs</td>
<td>Channel electron multipliers</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate</td>
</tr>
<tr>
<td>CID</td>
<td>Collision-induced dissociation</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic Kidney Disease</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular Disease</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DNPH</td>
<td>2,4-dinitrophenylhydrazine</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECD</td>
<td>Electron capture dissociation</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>ESRD</td>
<td>End Stage Renal Disease</td>
</tr>
<tr>
<td>ETD</td>
<td>Electron transfer dissociation</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FP</td>
<td>False Positive Rates</td>
</tr>
<tr>
<td>FTC</td>
<td>Fluorescein-5-thiosemicarbazide</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular Filtration Rate</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>GuHCl</td>
<td>Guanidine Hydrochloride</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HABA</td>
<td>4'-hydroxyazobenzene-2-carboxylic acid</td>
</tr>
<tr>
<td>HACA</td>
<td>6-hydroxycaproic acid</td>
</tr>
<tr>
<td>HAVA</td>
<td>5-hydroxy-2-aminovaleric acid</td>
</tr>
<tr>
<td>HD</td>
<td>Hemodialysis</td>
</tr>
<tr>
<td>HOCl</td>
<td>Hypochlorous acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Pressure Liquid Chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>IAA</td>
<td>Iodoacetamide</td>
</tr>
<tr>
<td>ICAT</td>
<td>Isotope coded affinity tags</td>
</tr>
<tr>
<td>ICR</td>
<td>Ion cyclotron resonance</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>iTRAQ</td>
<td>Isobaric tags for relative and absolute quantification</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>KDOQI</td>
<td>Kidney Disease Outcomes Quality Initiative</td>
</tr>
<tr>
<td>LC</td>
<td>Long Chain</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography tandem mass spectrometry</td>
</tr>
<tr>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Lethal dose, 50%</td>
</tr>
<tr>
<td>LOOH</td>
<td>Lipid hydroperoxides</td>
</tr>
<tr>
<td>LPO</td>
<td>Lipid peroxidation</td>
</tr>
<tr>
<td>LTQ</td>
<td>Linear quadrupole ion trap</td>
</tr>
<tr>
<td>mA</td>
<td>Milliamps</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption ionization</td>
</tr>
<tr>
<td>MCO</td>
<td>Metal catalyzed oxidation</td>
</tr>
<tr>
<td>MCX</td>
<td>Mixed-Mode Cation Exchange</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>MDRD</td>
<td>Modification of Diet in Renal Disease study</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>MeSH</td>
<td>Medical Subject Headings</td>
</tr>
<tr>
<td>MetSO</td>
<td>Methionine sulfoxide</td>
</tr>
<tr>
<td>MSR</td>
<td>Methionine sulfoxide reductase</td>
</tr>
<tr>
<td>NaBH&lt;sub&gt;3&lt;/sub&gt;CN</td>
<td>Sodium Cyanoborohydride</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>PD</td>
<td>Peritoneal Dialysis</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PSMs</td>
<td>Peptide-Spectrum Matches</td>
</tr>
<tr>
<td>PTMs</td>
<td>Post-translational modifications</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acids</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive Nitrogen Species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SC</td>
<td>Spectral counting</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate – polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SILAC</td>
<td>Stable isotope labeling by amino acids</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid phase extraction</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-Buffered Saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-Buffered Saline with Tween-20</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TG</td>
<td>Tris/glycine buffer</td>
</tr>
<tr>
<td>TGS</td>
<td>Tris/glycine/SDS buffer</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3’,5,5’-tetramethylbenzidine</td>
</tr>
<tr>
<td>TMT</td>
<td>Tandem mass tags</td>
</tr>
<tr>
<td>TOF</td>
<td>Time-of-flight</td>
</tr>
<tr>
<td>TPP</td>
<td>Trans-Proteomic Pipeline</td>
</tr>
</tbody>
</table>
Abstract

ANALYTICAL METHOD DEVELOPMENT FOR THE DETECTION AND ANALYSIS OF PROTEIN CARBONYLS

By Chelsea Coffey, Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2015.

Director: Prof. Scott Gronert
Department of Chemistry

Oxidative stress can result in changes to many biomolecules and also affect their activities. We are interested in protein carbonylation, a type of unnatural oxidation which has been associated with numerous degenerative disease states and is also a consequence of the natural aging process. Protein carbonyls are stable species, but countless analytical barriers exist in terms of their identification. Thus, the main goal of this work was to develop and optimize analytical methods that could be used to help us better understand which, where, and how proteins are being carbonylated.

Initial studies involved method validation for carbonylating, tagging, and enriching the model protein human serum albumin (HSA). We have developed a reproducible method of
producing carbonylated protein in vitro in which HSA is treated with acrolein to carbonylate cysteines, histidines, and lysines. Protein carbonyls are compatible with various affinity labels and enrichment techniques. We strived to learn more about the efficiencies of various biotin affinity labels and avidin enrichment techniques using quantitative assays and mass spectrometry. Results showed a preference for different affinity labels based on their chemical properties and suggested that monomeric columns are selective for particular peptides. Most recently, method development and validation work was done involving a cleavable biotin tag that enables both enrichment and identification of protein carbonylation modification sites. This affinity tag offered the highest labeling efficiency of all tags tested in the past and greater coverage of modification sites than biotin hydrazide reagents.

We applied our analytical methods to two sets of human blood samples. The first sample set was plasma taken from chronic kidney disease (CKD) patients. No carbonylation patterns were elucidated, but this project marked the beginning of blood analyses in which existing protocols were adapted to blood samples. The second sample set was serum/plasma taken from patients with traumatic injuries. We effectively applied our analytical methods to these sample sets and were able to visualize and quantitate temporal protein carbonylation patterns via Western blotting and iTRAQ-based mass spectrometry experiments. ProteoMiner experiments proved successful in that we were able to identify a larger and more diverse amount of carbonylated proteins via mass spectrometry.
Chapter 1 - INTRODUCTION
1.1 INTRODUCTION

“Drink green tea; it has antioxidants!” is a phrase commonly heard today, but most likely not fully comprehended. Antioxidants are necessary to help combat the pro-oxidants in a person’s body and maintain a balance between the two species. If this balance is not maintained, for instance the amount of pro-oxidants exceeds the amount of antioxidants, then oxidative stress occurs. This negatively affects protein structure and function and has been linked to the aging process and progression of various degenerative diseases.1 Four years ago, Madian et al. demonstrated a decrease in protein carbonylation, detected by selected reaction monitoring (SRM) analysis with a nanoUPLC-QSTAR mass spectrometer, in diabetic rats living on a green tea-based diet.2 Thus, scientific proof exists to support the claim that green tea is indeed a useful therapy for increased oxidation levels, particularly in relation to disease-associated oxidative stress.

The human body is one of the most fascinating machines, but, just like any other piece of equipment, fades over time due to daily wear and tear. Aging is inevitable; thus, protein oxidative damage is inevitable. We cannot stop the aging process, but it is possible to develop therapies to treat oxidation. Not only could this potentially minimize and/or delay the effects of aging, but might also serve as treatment for various age-related diseases. Much is still unknown about the causal-effect relationship concerning protein oxidation and how to treat it. Regardless, with ever improving methods and technology, researchers continue to investigate where, how, and why proteins are being oxidatively modified as they work to prevent and/or treat oxidative stress-related aging and diseases.
1.2 PRO-OXIDANTS AND ANTIOXIDANTS

Oxidative stress hinges on the balance (redox potential) between the amount of pro-oxidants and antioxidants in a biological system. While oxygen is essential in metabolic and energy-based processes in most biological species, it can also be very damaging. In the simplest of terms, pro-oxidants are electron acceptors and antioxidants are electron donors. In a biological system antioxidants generally donate a hydrogen or abstract an oxygen; pro-oxidants generally donate an oxygen. Pro-oxidants, also known as reactive oxygen species (ROS) or reactive nitrogen species (RNS), are capable of oxidizing and impairing proteins, lipids, carbohydrates, and DNA. These pro-oxidants can be radical species, which possess one or more unpaired electrons, or nonradical species. The radical species include: superoxide anion (O$_2^•$), hydroxyl (HO’), peroxyl (ROO’), alkoxyl (RO’), and nitric oxide (NO’). and are extremely reactive due to their ability to donate or abstract an electron. Nonradical species are equally damaging and include: hydrogen peroxide (H$_2$O$_2$), hypochlorous acid (HOCl), peroxides (ROOH), aldehydes (HCOR), ozone (O$_3$), and peroxynitrite (ONOOH). Many pro-oxidant species are produced during daily metabolism. For instance, about 5% of molecular oxygen (O$_2$) is repeatedly reduced to form H$_2$O$_2$, OH’, and hydroxide anion (OH’) in the electron transport chain. Conversely, antioxidants inhibit oxidation in many different ways, depending on the type of pro-oxidant and antioxidant. Some antioxidants include: proteins like ceruloplasmin, transferrin, and ferritin; enzymes like superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-S-transferase (GST), methionine sulfoxide reductase (MSR), and glutathione reductase; vitamins A, C, and E; and metal ions like Zn$^{2+}$, Mg$^{2+}$, and Mn$^{2+}$. 
Both the environment and our bodies contribute to the production of ROS. Externally, different types of radiation and pollutants generate ROS, while internally, ROS are generated via daily metabolic activities. The diagram depicted in Figure 1-1, redrawn from Stadtman and Levine, shows the various pro-oxidants and anti-oxidants and the events that can occur due to imbalance. ROS are generally very reactive with brief half-lives ranging anywhere from nanoseconds to hs. Both reactivity and half-life length are affected by biological conditions, for instance pH and interfering species. Half-life does not necessarily constitute reactivity; ROS with longer half-lives can travel to produce oxidative damage at distant sites. On the other hand, ROS with extremely short half-lives cause oxidative damage near ROS production sites. The fast reactivity of ROS not only make them difficult to study, but also demonstrates the difficulty in preventing oxidative damage. Thus, in this dissertation, we focused on oxidative damage to proteins, which provide more robust markers of oxidative stress.
Figure 1-1. Protein oxidation is dependent on the actions of pro-oxidants, antioxidants, and proteases.
1.3 PROTEIN OXIDATION

As depicted in Figure 1-1, if proteases are unable to degrade oxidized proteins to peptides or individual amino acids, then these oxidized proteins remain in the system. By definition, protein oxidation is the covalent modification of the protein resulting directly from ROS or indirectly from interaction with secondary oxidative stress-related products. Each protein has a distinct biological function\(^1\) and its modified counterpart is stable for up to weeks at a time.\(^8\) This, along with the fact that native proteins are readily available via blood,\(^9\) makes modified proteins ideal species to study rather than the short lived ROS. Oftentimes, a loss of function is also observed with protein oxidation. For instance, metal catalyzed oxidation of fibrinogen leads to its inability to form clots\(^10\) and HOCl based-oxidation of the protease inhibitor \(\alpha-1\) antitrypsin leads to lung tissue degradation in emphysema patients.\(^11\) However, since there are a variety of oxidation mechanisms and a majority of amino acids can be oxidatively modified, many types of modified proteins can be formed. A compilation of these modifications can be found in Table 1-1, reproduced from Shacter.\(^1\) Various types of modified proteins have also been linked to aging\(^7,12-14\) and disease (an extensive list of biomarkers can be found in a review by Dalle-Donne et al.).\(^15\)
Table 1-1. Factors related to various types of protein oxidation.

<table>
<thead>
<tr>
<th>MODIFICATION TYPE</th>
<th>AMINO ACID(S) OXIDIZED</th>
<th>PRO-OXIDANT(S)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disulfides, glutathiolation</td>
<td>Cys</td>
<td>All, ONOO'</td>
</tr>
<tr>
<td>Methionine sulfoxide</td>
<td>Met</td>
<td>All, ONOO'</td>
</tr>
<tr>
<td>Carboxyls (aldehydes &amp; ketones)</td>
<td>Lys, Arg, Pro, Thr, etc.</td>
<td>All</td>
</tr>
<tr>
<td>Oxo-histidine</td>
<td>His</td>
<td>γ-Ray, MCO, O2</td>
</tr>
<tr>
<td>Dityrosine</td>
<td>Tyr</td>
<td>γ-Ray, MCO, O2</td>
</tr>
<tr>
<td>Chlorotyrosine</td>
<td>Tyr</td>
<td>HOCl</td>
</tr>
<tr>
<td>Nitrotyrosine</td>
<td>Tyr</td>
<td>ONOO'</td>
</tr>
<tr>
<td>Tryptophanyl modifications</td>
<td>Trp</td>
<td>γ-Ray</td>
</tr>
<tr>
<td>Hydro(pero)xy derivatives</td>
<td>Val, Leu, Tyr, Trp</td>
<td>γ-Ray</td>
</tr>
<tr>
<td>Chloramines, deamination</td>
<td>Lys</td>
<td>HOCl</td>
</tr>
<tr>
<td>Lipid peroxidation adducts (MDA, HNE, etc.)</td>
<td>Lys, Cys, His</td>
<td>γ-Ray, MCO</td>
</tr>
<tr>
<td>Amino acid oxidation adducts</td>
<td>Lys, cys, His</td>
<td>HOCl</td>
</tr>
<tr>
<td>Glycoxidation adducts</td>
<td>Lys</td>
<td>Glucose</td>
</tr>
<tr>
<td>Cross-links, aggregates, fragments</td>
<td>Numerous</td>
<td>All</td>
</tr>
</tbody>
</table>

*All = γ-Ray, MCO, HOCl, ozone, O2; ONOO' = peroxynitrite; MCO = metal catalyzed oxidation; HOCl = hypochlorous acid

Only two of these mechanisms are involved extensively in this work and will be discussed in further detail: metal catalyzed oxidation (MCO) and lipid peroxidation (LPO). Since the early 1980s, Stadtman & Levine have studied MCO and determined it to be one of the most prevalent biological oxidation mechanisms. The most abundantly produced ROS are hydrogen peroxide and alkylperoxides, both of which are fairly harmless. Upon reaction with the transition metal iron (Fe (II)), hydroxyl radicals or alkoxy radicals can be formed as shown in Equations 1 and 2, respectively. These radicals generally modify proteins in a site specific manner, oxidizing approximately 1-3 amino acids at the locations of metal binding on the protein. This chemistry is commonly known as the Fenton reaction. MCO is often accomplished in model
systems using a combination of oxygen (O$_2$), ascorbate, and iron (III) chloride (FeCl$_3$) as pioneered by Levine.$^{10,19}$

\[
\text{H}_2\text{O}_2 + \text{Fe(II)} \rightarrow \text{HO}^+ + \text{OH}^- + \text{Fe(III)} \quad \text{Eq (1)}
\]

\[
\text{ROOH} + \text{Fe(II)} \rightarrow \text{RO}^+ + \text{OH}^- + \text{Fe(III)} \quad \text{Eq (2)}
\]

Unlike the direct oxidation induced by MCO, the reaction of lipid peroxidation products with protein amino acids is an indirect form of oxidative modification.$^{1,20,21}$ Most cell membranes are composed of lipids, which also play a role in managing cell functions.$^{22}$ In LPO, ROS react with lipids, which undergo hydrogen abstraction and oxygen insertion to produce compounds such as lipid peroxyl radicals, lipid hydroperoxides (LOOH), and/or secondary oxidation products.$^{20,21}$ These secondary oxidation products react with amino acids to form oxidatively modified proteins. Polyunsaturated fatty acids (PUFAs) are the lipids most suitable for LPO because they contain bis-allylic methylene groups, from which the hydrogens are more easily removed.$^{20}$ However, cholesterol, glycolipids, and phospholipids are also candidates for LPO modification.

LPO progresses through three different steps: initiation, propagation, and termination. An overview of the process was recreated from Ayala et al. and is depicted below in Scheme 1-1.$^{21}$ In the initiation step, a pro-oxidant (R$^*$) captures an allylic hydrogen from the lipid, which leaves a carbon-centered lipid radical. In the propagation step, an oxygen molecule (O$_2$) reacts with the lipid radical to create a lipid peroxy radical (LOO$^*$). The lipid hydroperoxide (LOOH) is formed when LOO$^*$ captures an allylic hydrogen from a second lipid, at which point this second lipid
now contains a radical and the process can continue in a repetitious fashion. In the termination step, LOO’ captures a hydrogen from an antioxidant to form a nonradical product.$^{20-23}$

**Scheme 1-1.** Initiation (1), propagation (2-3), and termination (4) steps of the lipid peroxidation process.
Of most interest in our work is the formation of the aldehyde-containing secondary oxidation products, which most commonly include: malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), 4-oxo-2-nonenal (4-ONE), acrolein (ACR), propanal, and hexanal. These aldehydes result from fragmentation of an ester-containing LOOH and are particularly dangerous because they have longer life spans and thus can travel to create oxidative damage in distant locations. The electrophilic properties of the α,β-unsaturated aldehydes (4-HNE, 4-ONE, and ACR) make them extremely reactive toward nucleophiles, such as sulfhydryl groups in cysteines and amino groups in lysines/histidines.

The majority of oxidative modifications regardless of the source are irreversible, with the exception of oxidation of sulfur-containing amino acids. Modification of the sulfur in both methionine and cysteine are reversible. Upon reaction with ROS, methionine is oxidized to form S- and R-stereoisomers of methionine sulfoxide (MetSO), commonly referred to as MetA and MetB, respectively. Two types of MetSO reductases, MsrA and MsrB, serve to return the oxidized amino acid back to its native form of methionine. Oxidation of cysteine can result in three products: sulfenic, sulfinic, and sulfonic acids, each of which contains one, two, or three additional oxygens, respectively. Sulfenic acid serves as an intermediate to form sulfinic or sulfonic acid and is reactive toward other thiols, resulting in disulfide bridges. Native cysteine can be reformed through reduction of disulfides by glutathione or thioredoxin or reduction of sulfinic acid by sulfiredoxin. This phenomenon is discussed further in Chapter 4 of this dissertation.
1.4 Protein Carbonylation

Only eight years ago, the term ‘protein carbonylation’ was added to the PubMed Medical Subject Headings (MeSH) used to refine searches, and deemed a “standard marker for oxidative stress”. However, well before then, oxidative carbonylation had been studied as a valuable marker for various conditions and diseases and as a consequence of the natural aging process. Since aging accompanies the onset of numerous degenerative diseases, differentiating unique oxidative carbonylation patterns can be very challenging. Following methionine and cysteine oxidation, carbonylation is the third most prevalent post-translational modification and is a major concern due to the association of increased levels of oxidative carbonylation with aging and disease.

Carbonylation, which involves the incorporation of an aldehyde or ketone onto an amino acid, is often used as a measure of oxidative stress. This type of modification is irreversible, meaning the chemical change is unaffected by antioxidant actions. Conversely, examples of reversible modifications include cysteine and methionine, both sulfur-containing residues and both subject to modification reversal by specific enzymes. The irreversibility of carbonylation is both a positive and negative. In terms of disease and aging, the irreversible nature causes an excess of unrepairable and dysfunctional proteins leading to further complications. On the other hand, the reactivity of the modification allows for a variety of labeling, enrichment, and detection techniques to be used. Following derivatization of the carbonyl, the stability of the moiety permits extended storage under proper conditions (−80°C). For these reasons, carbonylation is one of the most commonly studied types of markers for oxidative stress.
1.4.1 Protein Carbonylation Mechanisms

Revolutionary work by Swallow, Garrison, and Scheussler & Schilling demonstrated that HO’ was responsible for the formation of different types of oxidized products, which implied that protein carbonylation can occur via a variety of mechanisms. Direct carbonylation occurs when hydroxyl radicals oxidize amino acid side chains, generally those of arginine, lysine, proline, and threonine, to form aldehydes or ketones. Indirect carbonylation occurs when cysteines, histidines, or lysines react with secondary products from the lipid peroxidation process (4-HNE, ACR, MDA) to form aldehyde-containing oxidation products. Through the alpha-amidation pathway, hydroxyl radicals can also attack the protein backbone by capturing a hydrogen atom, leaving a carbon-centered radical that can react with oxygen or other carbon-centered radicals to form single or crosslinked carbonylation products. Lastly, the reaction between reducing sugars and lysine amino groups can produce carbonylated species known as advanced glycation end products (AGEs). The first two mechanisms concerning direct and indirect carbonylation were explored in this dissertation.

The hydroxyl radicals involved in direct oxidation are often derived from either ionizing radiation or MCO. The most prominent carbonylation products observed from direct oxidation are glutamic semialdehyde, derived from arginine or proline, and aminoadipic semialdehyde, derived from lysine. These products have been observed in model proteins such as glutamine synthetase, bovine serum albumin, ribonuclease A, and lysozyme as well as in biological samples such as rat liver. Reactions for the carbonylation of arginine, lysine, and proline to form glutamic and aminoadipic semialdehydes were recreated from Requena et al. and are depicted in **Scheme 1-2**. Although not pictured, the carbonylation derivative of threonine is known as 2-amino-3-ketobutyric acid.
Scheme 1-2. Carbonylation of arginine, lysine, and proline to form glutamic and aminoadipic semialdehydes (figures in gold). Blue represents oxidation, red represents leaving groups, and green represents reduction.
Indirect carbonylation proceeds through Michael addition of secondary lipid peroxidation products ($\alpha,\beta$-unsaturated aldehydes) to the sulfur of cysteine, or the amine of histidine and lysine. Visible in Figure 1-2, fragmentation of lipid hydroperoxides (LOOH) leads to the generation of reactive aldehyde-containing products. As illustrated by 4-HNE, these reactive species can either react with proteins to form carbonylation products or be detoxified by glutathionylation, oxidation via aldehyde dehydrogenase, or reduction via alcohol dehydrogenase.\textsuperscript{52}

**Figure 1-2.** Formation, detoxification, and reaction of $\alpha,\beta$-unsaturated aldehydes during LPO. PUFA = polyunsaturated fatty acids; ROS = reactive oxygen species; PRX, GRX = peroxiredoxin, glutathione peroxidase; PUFA-OOH = lipid hydroperoxides; 4-HNE = 4-hydroxynonenal; GSTA4 = glutathione S-transferase alpha 4. (Redrawn from Grimsrud et al.)
1.4.2 Carbonylation and Aging

Recent research indicates that carbonylation affects nearly one-third of all proteins in a fully aged animal. That means one-third of proteins in matured species are potentially nonfunctional, which also play a role in the catalytic inactivation of enzymes. These conclusions originate from the “free radical theory of aging” proposed by Denham Harman in 1954, which postulates that the actions of free radicals promote aging and concomitant oxidation of biomolecules. Intuitively, Harman proposed that inhibiting free radicals would presumably increase an animal’s life span. Since then, correlations between aging and carbonylation have been identified by many researchers: Oliver & Levine (1987), Sohal, Sohal, & Orr (1995), Bertlett & Stadtman (1997), and Stadtman (2006). One of the most popular representations of this trend was published by Stadtman and Levine in 2000. The plot depicts a trend line correlating age and carbonyl content for human, fly, and rat samples, with the most prominent correlation in the last third of the animal’s lifetime.

A comprehensive list of 179 carbonylated proteins identified during aging in an assortment of species can be found in the review by Cabiscol et al. The main purposes of compiling such a list were to classify the carbonylated proteins by physiological function and also cross reference these proteins across species. In doing so, it was determined that heat shock proteins and those associated with protein metabolism were most conducive to age-related carbonylation, and that various proteins, such as those involved in glucose metabolism, were carbonylated in a variety of species. This information indicates which physiological functions are most affected by the aging process and confirmed by identification in various organisms.
1.4.3 Carbonylation and Disease

Today, Harman’s “free radical theory of aging” could be changed to the Free Radical Theory of Aging and Disease, considering the overwhelming evidence correlating oxidation with disease progression. As with aging, an increase in ROS-derived oxidative carbonylation has been observed with the progression of countless diseases and conditions. An extensive, though not complete, list of diseases and conditions associated with carbonylation was published by Levine and Stadtman in 2006. Naturally, diseases and conditions associated with aging demonstrate compound carbonylation, yet the causal/effect relationship between carbonylation, aging, and disease has not been fully elucidated. The following are diseases/conditions that have been linked to oxidative carbonylation and published frequently and recently (within the last 15 years): Alzheimer’s disease, caloric restriction/exercise, diabetes, ischemia-reperfusion/stroke, sepsis, Parkinson’s disease, and kidney disease.

1.4.4 Detecting Carbonylation

To this day, carbonyl detection and analysis remains a significant analytical challenge. However, much progress in analytical methodology has been made over the years as evidenced by pioneering contributions from Esterbauer et al., Stadtman & Levine, Shacter, Dalle-Donne et al., Butterfield, and Regnier et al., to name a few. Improvements in technology and instrumentation have also played a vital role in the advancement of methodologies.

In general, analytical analyses of carbonylated proteins can be divided into two categories: gel-based and gel-free. Sample preparation differs for these types of analyses, but both commonly utilize mass spectrometry as the final detection method. Gel-based methods often involve derivatizing carbonyl groups with an affinity tag, performing one-dimensional (1-
D) or two-dimensional (2-D) Western blotting, and detecting with an antibody specific for the affinity tag. This technique allows for the visualization of an array of modified proteins, but complications including sensitivity and non-specific binding are often encountered. In-gel digestions of particular protein bands can be performed in preparation for identification by mass spectrometry. Gel-free methods involve a variety of assays and techniques for measuring protein carbonylation. Thanks to the early work by Green, affinity tags and enrichment systems utilizing the strong association between avidin and biotin are among the most popular sample preparation tools today. Regnier and coworkers popularized biotin hydrazide as an affinity label and monomeric avidin as an enrichment technique in the early 2000s. Since then, variations of these reagents have been used in an assortment of studies. One of the earliest and most popular assays was the DNPH assay developed by Levine et al. It involved derivatization of protein carbonyls with 2,4-dinitrophenylhydrazine (DNPH) and monitoring the molecule’s absorption around 370 nm using a spectrophotometer.

While each technique has its pros and cons, there still remains a universal struggle with many analytical factors such as: complex sample matrices, reproducibility, sensitivity, specificity, limit of detection, etc. More detailed information regarding these analytical challenges and particular proteomic techniques will be discussed throughout this dissertation.
1.5 MASS SPECTROMETRY

As mentioned previously, proteins are important to study because each possesses a unique function that correlates to metabolic activity and/or disease states. The term proteomics appeared in the 1990s as scientists took interest in identifying and quantifying various proteins produced via the central dogma of biology: DNA→RNA→proteins. This was similar to genomics work at the time involving gene classification studies. With the production of 100,000+ proteins from the human genome, one can imagine the immensity of classifying all human proteins in both natural and diseased states. Proteins are active in all living organisms, thus proteomics studies are not limited to human samples, further expanding the scope of these studies. A more refined field is redox proteomics, in which classification studies focus on the effects of oxidation of proteins. Throughout this dissertation, our interest is in redox proteomics due to our focus on protein carbonylation.

Within the last decade, mass spectrometry has become the foremost technique used in large, complex proteomics studies due to instrumentation improvements concerning mass accuracy, sensitivity, and resolution. The two most popular mass spectrometry-based proteomics methods are “top-down” analysis and “bottom-up” analysis. Top-down proteomics is used for the characterization of entire proteins, which are first fractionated, ionized in the gas phase, and then fragmented in the mass spectrometer. While this type of analysis is thought to offer a more comprehensive look at the entire protein and post-translational modifications (PTMs) it may carry, it also suffers from limited sensitivity and analysis of complex protein mixtures. Although improvements in resolution continue to fuel these types of analyses, bottom-up proteomics generally remains the analysis of choice. In this method,
large mixtures of proteins are digested to form peptides, which are then separated chromatographically and analyzed with liquid chromatography tandem mass spectrometry (LC-MS/MS). Since smaller species are more easily fractionated, ionized, and fragmented, bottom-up methods, or shotgun proteomics as it is commonly known, can be applied to a larger variety of more complex protein samples.\textsuperscript{97,99} All of the work in this dissertation involved bottom-up proteomics methods.

1.5.1 Ionization

About 26 years ago, mass spectrometry broke into the world of biological compound analysis thanks to the revolutionary work by Franz Hillenkamp, Koichi Tanaka, John Fenn, and their colleagues. Since then, mass spectrometry has climbed its way to the top of biological scientific techniques, where it remains to this day. In the simplest of terms, a mass spectrometer contains an ion source, a mass analyzer, and a detector. Combined, these components allow for selective determination of analyte concentration, elemental composition, and/or structural information; however, ions must be in the gas phase in order to be separated and detected. Since ions are charged species, the mass-to-charge (m/z) ratio is determined instead of the direct mass.\textsuperscript{98}

Biological sample analysis was limited due to the gas phase requirement until the mid-1980s when two novel soft ionization techniques were proposed: matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI).\textsuperscript{103–107} The first practical experiments demonstrating the use of MALDI were published by Hillenkamp\textsuperscript{106,107} in 1985 and Tanaka\textsuperscript{105} in 1988, roughly the same time as Fenn’s initial ESI reports between 1985-1989.\textsuperscript{103,104} Both achievements were honored with a Nobel Prize in 2002.\textsuperscript{108,109} As of today, ESI and matrix-
assisted laser desorption ionization (MALDI) are the two most commonly used soft ionization techniques for biomolecules.\textsuperscript{110}

MALDI-MS analyses involve the addition of an organic-based matrix to a sample, which is then deposited on an analysis plate. The spot dries, forming a crystalline structure, which is next irradiated with a laser. The heating of the laser causes fast sublimation of the sample and matrix, and ultimately ionization.\textsuperscript{109} Despite MALDI’s ever growing success, we employed ESI ionization techniques for our analyses. The meaning of ESI implies exactly how it sounds; electrospray utilizes a needle carrying a charge of several kilovolts (1-6 kv) to disperse the liquid solution into small, charged droplets without causing fragmentation. As the droplets travel from the needle to the mass spectrometer, the solvent evaporates, eventually leaving only a charged species in the gas phase. The nebulization and evaporation processes are both enhanced using nitrogen (N\textsubscript{2}) gas. The charged species pass through a heated capillary (100-300°C), which serves to finalize desolvation and transfer the ions into the mass spectrometer.\textsuperscript{103,104,108} Other benefits of ESI include the capacity to work at low sample concentrations (µM range) and low flow rates (1-20 µL/min), both of which can be reduced with the use of nanospray ionization (nano-ESI). Using a capillary instead of a needle, lower flow rates of 20-50 nL/min can be achieved, which is amenable to work at nM concentrations. Nano-ESI also decreases solvent and salt interferences.\textsuperscript{108}

\subsection*{1.5.2 Mass Analyzer}

Following ionization, ions travel toward the mass analyzer, also known as the m/z analyzer. This device allows for spacial or temporal separation of the ions by their m/z using electric and/or magnetic fields, after which abundances can be determined by the detector.\textsuperscript{98} The
The most common mass analyzers used in proteomics include: time-of-flight (TOF), quadrupole, ion trap, and ion cyclotron resonance (ICR). While each offers various advantages and disadvantages, often times multiple analyzers will be connected in tandem to multiply the individual advantages. This was the case in our work, which involved a linear quadrupole ion trap (LTQ). The LTQ became commercially available in the early 2000s, but quadrupole ion traps (Q-traps) date back to the early 1950s. Paul first proposed the Q-trap in 1953, and it took about 40 years before their use with biological samples commenced. In typical LTQ instrumentation, the ESI-generated ions are focused into the ion traps using quadrupoles. Inside the ion trap, an electric potential is applied to a central ring electrode, drawing the ions toward the trap center. Upon application of an increasing Rf voltage, the ions are ejected from the trap based on their mass to charge; increasing mass to charge correlates with greater instability and thus ejection. Ion traps offer sensitivity and robustness, but low mass accuracy due to their limited trapping abilities. A diagram of a typical linear quadrupole ion trap adapted from the work by Schwartz and coworkers is pictured in Figure 1-3. The depiction also includes electrospray ionization and is representative of the instrumentation used in this dissertation.

![Diagram of an LTQ equipped with ESI](Image)

**Figure 1-3.** A general diagram for an LTQ equipped with ESI. Ideal vacuum pressures are presented at the top in torr.
Peptide fragmentation is of particular importance in proteomics analyses and data interpretation. The three main fragmentation methods used in proteomics are electron capture dissociation (ECD), electron transfer dissociation (ETD), and collision-induced dissociation (CID). ECD was first proposed in 1998 by McLafferty et al.\textsuperscript{118} and involves exposing protonated proteins/peptides to thermal electrons. Protein/peptide capture of electrons is an exothermic process that results in fragmentation of the protein or peptide along the backbone.\textsuperscript{98} ECD has generally been confined to ICR analyzers,\textsuperscript{118,119} making the technique costly due to the expense of the instrumentation.\textsuperscript{120} Six years later, ECD was manipulated to create ETD by Syka et al.\textsuperscript{119} In ETD, radical anions are used to transfer electrons to protonated peptides to induce fragmentation. ECD and ETD are typically employed when working with larger species and thus can be more conducive to PTM analyses. However, they can also serve as complements to CID techniques. In CID, peptides are exposed to neutral gas (often helium) and those with three or less protons are usually fragmented upon collision, producing unique fragmentation patterns consisting of b- and y- ions. On the other hand, ECD and ETD produce fragmentation patterns consisting of c- and z- ions. These fragmentation patterns correlate to cleavages along the peptide backbone and assist with peptide identification. Unlike ECD, both ETD and CID are compatible with complex samples and a variety of instruments, including ion traps.\textsuperscript{120}

1.5.3 Detector

As can be seen in Figure 1-3, the detectors reside on opposite sides of the ion trap. The most important goal of a detector is signal amplification. Following mass to charge separation in the mass analyzer, ions enter the detector(s) and, upon interaction with a charged surface, produce secondary particles. The summation of these particles for each mass to charge represents an amplified signal, but remains proportional to the original ion count. Desired detector traits
include: fast, efficient amplification, sensitivity, accuracy, and long lifetime.\textsuperscript{98,121} Three of the most popular detectors that offer most if not all of these traits are faraday cups,\textsuperscript{122,123} electron multipliers,\textsuperscript{124,125} and channel electron multipliers.\textsuperscript{126} Our instrumentation utilized two channel electron multipliers (CEMs), also referred to as array detectors. The CEM consists of a conductive tube, closed on one end and open on the other. The closed end consists of an endcap containing hundreds of channels per square inch while the open end allows for ions and secondary particles to exit. A voltage is applied to the tube. As the ions strike the various channels, secondary electrons are produced. As these electrons progress down the channels, they continue to hit the walls, producing even more secondary electrons until they emerge from the tube. The amount of amplification is referred to as the gain, and in this case with CEM, is about $10^7$-$10^8$ electrons. The electrical signal can then be interpreted with data analysis software to produce a more useable and interpretable output.\textsuperscript{98,121}

### 1.5.4 Data Analysis

Most often in mass spectrometry-based proteomics, multiple scans/fragmentation events occur for each sample during a mass spectrometry analysis. This results in thousands of tandem mass spectra for each sample. Since this is too many spectra for manual confirmation, computational software has been developed to perform the task. Most software relates to one of two types of data analyses: \textit{de novo} sequencing and database searching, both of which were developed in the late 1990s. \textit{De novo} sequencing involves direct elucidation of peptide sequences from the tandem mass spectra. Database searching, which we used in our work, involves comparing experimental mass spectra to theoretical mass spectra from all peptides comprising a protein sequence.\textsuperscript{127,128} Various database searching software programs exist, most notably
SEQUEST\textsuperscript{129} and MASCOT,\textsuperscript{130} both of which enable database comparisons, but differ in their scoring of peptide matches.\textsuperscript{127}

### 1.5.5 Quantitative Proteomics

Today, quantitation has become the hallmark of mass spectrometry-based proteomics experiments. Albeit difficult to accomplish, thanks to improvements in instrumental and sample preparation techniques, small differences in peptide and/or protein concentrations have been determined in relation to various states and stimuli. Up until the late-1990s, mass spectrometry exhibited widespread use for qualitative determination of a plethora of proteins in complex mixtures.\textsuperscript{131} In 1999, Gygi et al. introduced isotope coded affinity tags (ICAT)\textsuperscript{132} followed by various other quantitative techniques in the early-2000s. Quantitation in proteomics can be either relative or absolute. Relative quantitation consists of comparing protein amounts across analyses to create abundance ratios.\textsuperscript{131} Strictly relative techniques are often referred to as label-free quantitation methods with examples being spectral counting (SC)\textsuperscript{133} and peptide peak intensity measurements.\textsuperscript{134} These approaches assume that more material equals more peptide spectra or larger peaks, respectively. Absolute quantitation consists of determining precise amounts of protein present and these techniques can be used for relative quantitation as well. Since Gygi et al.’s work in 1999, stable isotope labeling has been produced in various formats: stable isotope labeling by amino acids (SILAC),\textsuperscript{135} ICAT,\textsuperscript{132} isobaric tags for relative and absolute quantitation (iTRAQ),\textsuperscript{136} and tandem mass tags (TMT).\textsuperscript{137} Quantitation techniques explored in this dissertation include SC used for simple model protein solutions and iTRAQ used for more complex biological samples.
Chapter 2 - DEVELOPING ANALYTICAL METHODS USING CARBONYLATED HUMAN SERUM ALBUMIN (HSA) AS A MODEL PROTEIN
2.1 ABSTRACT

There are countless analytical barriers that complicate proteomics experiments and this is apparent by the marked decline in protein biomarker elucidations over the past 25 years. In this chapter, our focus was on improving current analytical methods and implementing new procedures for the detection of protein carbonyls. Building upon previous studies in our lab, we worked on optimizing the biotin affinity tag used to label protein carbonyls and the enrichment techniques used to isolate biotin-labeled material. Experiments involved four different biotin tags and one enrichment product, which was incorporated at the protein and peptide level. We introduced and validated two new assays, the biotin quantitation assay and the FTC assay. The biotin quantitation assay, a UV-Vis spectroscopy-based assay, was used to assess labeling efficiency and the FTC assay, a fluorescence-based assay, was developed as a more sensitive alternative to the quantitation assay. Overall, we were able to determine the most efficient biotin label in terms of enrichment and mass spectrometry analyses and implement two new assays, which were later applied to biological sample sets.
2.2 INTRODUCTION

A biomarker is defined as a measurable analyte that reflects the progression of a condition or disease. In terms of diseases, biomarkers generally reveal the initial onset and evolution of diseases along with responses to therapies.\textsuperscript{138,140} Most often, clinical-based assays are the byproducts of biomarker discovery – cheap, fast, and easy tests that can be used by a variety of technicians to identify biomarkers in patient samples. Blood is the optimum specimen for such tests.\textsuperscript{138} It is abundant, easily obtainable, and filled with proteins, offering a real-time depiction of the body’s state of health.\textsuperscript{9,138} Studies involving blood are addressed in Chapters 4 and 5 of this dissertation, but those studies remained at the discovery phase. An initial discovery phase involves distinguishing differences in certain protein concentrations between control and treated samples or healthy and diseased subject samples from model systems or clinical studies, respectively.\textsuperscript{138} Identifying biomarkers is not necessarily difficult – it is not uncommon to elucidate a unique marker, in our case maybe a particular protein, specific to a certain condition. The difficulty resides in clinical validation, especially in regards to obtaining approval from the United States Food and Drug Administration (FDA). Over the past 20 years, rates for approving novel protein biomarkers have remained sluggish with approximately one biomarker approved per year according to regulations set forth by the FDA.\textsuperscript{96,138,139}

In this study, we were not attempting to identify biomarkers, rather we were striving to develop methods that could potentially be used in biomarker discovery work. While this type of work is generally not as complicated as clinical validation work, which we encountered while applying our methods to human blood samples in Chapter 4, we still experienced many analytical barriers in our method development studies. Some common experimental obstacles
included: lack of availability of specific reagents and tools, cost of said reagents and tools, instrument variability between runs, sensitivity, reproducibility, and sample availability. In terms of conceptual issues, many researchers make poor biomarker predictions and thus waste effort and supplies investigating these assumptions. In relation to clinical validation, there is also a lack of knowledge pertaining to the pipeline, a term used to describe the steps between the discovery phase and diagnostic implementation phase. Many pipeline requirements are overlooked or ignored, making the proposed methods invalid and unpublishable. Relating to biological concerns, issues persist with inconsistent processing of biological samples in terms of collection, storage, handling, and improper experimental design, particularly in extensive studies with large numbers of samples. Another factor complicating experimental designs is the more recent requirement for multiple markers to confirm the presence and/or progression of a disease. This list outlines the barriers faced in analytical method development work and clinical validation studies, but is certainly not comprehensive.

The following method development work involved the model protein human serum albumin (HSA). At ~66 kDa, HSA is made up of 585 amino acids in its secreted form. It is the most abundant protein in human plasma/serum, constituting roughly 60% of the protein mass. HSA serves to regulate colloid osmotic pressure, transport a variety of molecules, and combat oxidation as an antioxidant. Hence, not only is HSA readily available and cheap, it has been the focus of many investigations concerning carbonylation and antioxidant research. Of note, serum albumin is also the most prominently carbonylated protein in chronic kidney disease, end stage renal disease, and liver disease patients.

In our lab, we reproducibly created carbonylated serum albumin by conjugating it to one of the most reactive $\alpha, \beta$-unsaturated aldehydes, 2-propenal, more commonly known as acrolein.
(ACR). Its structure is depicted below in Figure 2-1. Because it is composed of an alkene linked to an electrophilic group, acrolein is considered to be a conjugated type-2 alkene. Its discovery dates back to the 19th century. In the mid-1820s, a French lipid chemist coined the term glycerin, also known as glycerol, following the production of soap from fats and alkali. In 1839, another chemist named Berzelius, classified a degradation product of glycerin as acrolein and, four years later, Redtenbacher was the first to prepare acrolein via distillation of glycerin. He made note of the molecule’s obnoxious smell and ability to instantly bring those exposed to tears. Over the years, scientists realized this small molecule was extremely toxic.

Acrolein is in the environment due to partial combustion of materials such as plastic, petrol, coal, and wood, excess heating of frying oil, for instance when cooking, and cigarette smoking. In biological systems, acrolein can be formed via oxidation of polyamine metabolites, during the lipid peroxidation process, or by organism-driven modification of certain allyl-containing compounds or cyclophosphamide, an anticancer drug. Studies have correlated acrolein with cell apoptosis and acute toxicity. Acrolein-induced apoptosis has been observed at low micromolar concentrations in human alveolar macrophages, human bronchial epithelial cells, and human keratinocytes. Exposure to acrolein can cause extreme irritation of the eyes and mucosa and contact with acrolein can cause critical burns. According to Ullman’s
Encyclopedia of Industrial Chemistry, the LD$_{50}$ = 46 mg/kg for oral ingestion by a rat and the LD$_{50}$ = 562 mg/kg for dermal contact of a rabbit.$^{24}$

Despite its toxicity, acrolein is useful in our work because it is reactive toward nucleophilic sites of proteins, particularly thiol groups in cysteines and amino groups in histidines and lysines.$^{24,150}$ Reaction with the thiol of cysteines rapidly forms the most stable protein adduct.$^{24}$ Carbonylated products are formed by Michael addition, as illustrated in Scheme 2-1 in the results section. Serum albumin contains 59 lysines, 16 histidines, and 35 cysteines (17 disulfide bridges), with the one free thiol at cysteine-34 being the most reactive towards acrolein modification.$^{24,142,146}$ Creating carbonylated HSA in a reproducible fashion allowed us to use the modified protein to develop methods aimed at improving the detection and analysis of protein carbonyls.
2.3 EXPERIMENTAL DESIGN

2.3.1 Materials

Urea, 10x phosphate buffered saline (PBS), water (HPLC), methanol (HPLC), acetonitrile (ACN), dithiothreitol (DTT), dimethyl sulfoxide (DMSO), trifluoroacetic acid (TFA), Tween® 20, Brilliant blue, bovine serum albumin (BSA) (Fraction V), and Biotinylated BSA were purchased from Fisher Scientific (Pittsburgh, PA). Human serum albumin (HSA), (+)-Biotin hydrazide, sodium cyanoborohydride (NaBH₃CN), sodium dodecyl sulfate (SDS), iodoacetamide (IAA), and calcium chloride dehydrate (CaCl₂) were purchased from Sigma-Aldrich (St. Louis, MO). Trichloroacetic acid (TCA) and guanidine hydrochloride (GuHCl) were obtained from Acros Organics (Fair Lawn, NJ). Sequencing grade modified trypsin and 3,3’,5,5’-Tetramethylbenzidine (TMB) substrate were obtained from Promega (Madison, WI). The BCA Protein Assay, Biotin Quantitation kits, Monomeric Avidin kits, EZ-Link Biotin-LC-Hydrazide, and EZ-Link Biotin-PEG₄-Hydrazide were purchased from Thermo Scientific (Rockford, IL). Aldehyde Reactive Probe (ARP) was purchased from Dojindo Laboratories (Rockville, MD). Oasis MCX extraction cartridges were purchased from Waters Corporation (Milford, MA). Mini PROTEAN® TGX™ gels, 10x Tris/Glycine/SDS (TGS) Buffer, 10x tris-buffered saline (TBS), 10x Tris/Glycine (TG) Buffer, Laemmli sample buffer, Mini Trans-Blot filter paper, Immun-Blot polyvinylidene fluoride (PVDF) Membranes for Protein Blotting, and Blotting Grade Blocker were obtained from Bio-Rad (Hercules, CA). Fluorescein-5-thiosemicarbazide, HCl salt (FTC) was purchased from Marker Gene Technologies, Inc. (Eugene, OR).
2.3.2 HSA Modification

HSA was reacted with acrolein at a 10:1 acrolein:protein ratio and incubated at room temperature with gentle shaking for 1 h. Initial experiments proceeded directly to labeling. For spike experiments, excess acrolein was removed via precipitation of the protein with 20% TCA (aq). Solutions were incubated on ice for 30 min and supernatant removed following centrifugation. Pellets were washed three times with 50/50 ethanol/ethyl acetate or ice cold acetone, air dried 5 min, and resuspended in 2% SDS/PBS. Following dissolution, carbonylated HSA concentrations were determined with the BCA Assay.

2.3.3 Labeling of Modified HSA

Biotin hydrazide (Label 1), EZ-Link Biotin-LC-Hydrazide (Label 2), and EZ-Link Biotin-PEG₄-Hydrazide (Label 3) were all prepared in DMSO at 50 mM. Aldehyde reactive probe (Label 4) was prepared in water at 50 mM. All labels were added to a final concentration of 5 mM and incubated at room temperature for 2 h using an Eppendorf ThermoMixer. To reduce and stabilize hydrazone bonds formed during labeling with Labels 1-3, NaBH₃CN was prepared in PBS at 30 mM and added to a final concentration of 15 mM. The samples were incubated at room temperature for 1 h. No reduction step was required when using Label 4. TCA precipitation was performed as detailed in Section 2.3.2. The supernatant was removed and the pellets resuspended in PBS, 5%SDS/PBS, or 8 M urea/PBS, depending on the experiment (biotin quantitation assay, Western blotting, or digestion, respectively).
2.3.4 Biotin Quantitation Assay

The extent of biotinylation was assessed following the protocol provided by Thermo Scientific Pierce. Biotinylated protein was added to a solution containing 4'-hydroxyazobenzene-2-carboxylic acid (HABA) conjugated to avidin. Biotin’s stronger affinity for avidin causes the displacement of HABA, invoking an absorbance change detectable with UV-Visible spectroscopy. Using the extinction coefficient of 34,000 M⁻¹ cm⁻¹, the known protein concentrations, and the change in absorbance before and after biotinylated protein addition, the biotin to protein ratios were calculated and the extent of biotinylation determined. Commercially available biotinylated BSA was used as a standard; the manufacturer reports nine equivalents of biotin per BSA molecule.

2.3.5 Western Blotting

Equal amounts of biotinylated protein carrying Labels 1, 2, or 4 (5 µg) were incubated with 95% Laemmli buffer and 5% 2-mercaptoethanol for 5 min at 95°C. The samples, along with a protein standard ladder, were loaded onto a 4-20% gradient polyacrylamide SDS-PAGE gel and run in TGS buffer at 100 V and 90 mA for 1 h and 30 min. Proteins were then transferred to a PVDF membrane and run in TG buffer at 100 V and 300 mA for 1 h and 15 min. Following immunoblot, the membrane was blocked with 5% non-fat dry milk in 1X TBS-T (1xTBS buffer with 0.1% Tween® 20) for 1 h and then incubated with Streptavidin Poly-HRP at a 1:10,000 dilution in 5% BSA and TBS-T overnight at 4°C. The membrane was washed with TBS-T and incubated with TMB substrate for 5 min to visualize protein bands.
2.3.6 Protein Digestion

Following labeling, 8 M urea was added to the protein solutions as a denaturant. To reduce disulfide bonds, DTT was added to a final concentration of 10 mM and samples were incubated on the ThermoMixer for 1 h at 37°C. To alkylate cysteines, IAA was added to a final concentration of 20 mM and samples were again incubated as described above and in the dark. During a third incubation, a second addition of DTT served to saturate unreacted IAA. Following dilution with PBS, CaCl$_2$ was added to a final concentration of 1 mM, and overnight trypsin digestion was performed at 37°C and 300 rpm on the ThermoMixer. Formic acid was added to halt digestion and the acidified peptide samples were concentrated with individual Oasis MCX columns using the protocol supplied by Waters Corporation. Three 1-mL fractions were eluted from each column and then all fractions were dried with a Speed Vac concentrator. After evaporation, dried peptides were dissolved in 95/5 0.1% aqueous TFA/ACN, which helped to increase sensitivity and protonation during the electrospray ionization portion of the mass spectrometry analysis. The solution concentrations were established using a BCA assay kit following the protocol provided by Thermo Scientific Pierce.

2.3.7 Monomeric Avidin Column Enrichment

Thermo Scientific Monomeric Avidin Kits were used for enrichment processes according to the procedure published by the manufacturers. In brief, columns were first washed with 4 column volumes of PBS, followed by 3 column volumes of Biotin Blocking and Elution Buffer (2 mM D-biotin in PBS) and 6 column volumes of Regeneration Buffer (0.1M glycine, pH 2.8). The columns were again washed with PBS. Up to 2 mL of biotinylated protein was added to the columns and incubated for 1 h. Nonbound proteins were removed by washing columns with six
column volumes of PBS. Biotinylated material was eluted by addition of up to six column volumes of Biotin Blocking and Elution Buffer. (Validation experiments suggested the majority of biotinylated material eluted within 8 mL – see Table A1 in Appendix 1). Following elution, columns were washed with 2 column volumes of Regeneration Buffer and 3 column volumes of 0.01% sodium azide in PBS, the same solution in which columns were stored in at 4°C.

2.3.7.1 Enrichment at Peptide Level

In order to imitate a more realistic, biological sample environment, biotinylated HSA (Labels 1-3) was spiked into a mixture of five proteins immediately prior to digestion at one tenth the concentration of the other proteins. The five proteins were: alcohol dehydrogenase, cytochrome C, lysozyme, ribonuclease A, and ubiquitin, and were chosen based on molecular weight, availability, and cost. Digestion proceeded as detailed in Section 2.3.6. Afterward, samples were divided into two aliquots: one portion was analyzed via mass spectrometry and the remaining fraction was enriched using monomeric avidin columns. Enriched samples were then purified via solid phase extraction (SPE), dried, and analyzed using mass spectrometry.

2.3.7.2 Enrichment at Protein Level

Once again, in order to create a more realistic, biological sample, carbonylated HSA was spiked into human serum at ranges between 5-20% (v/v), labeled with either Label 1, 2, or 4, and precipitated as detailed above. Biotinylated proteins were then enriched using monomeric avidin columns following the procedure published by the manufacturers. Proteins in the collected fractions were again precipitated with TCA, reconstituted in urea/PBS, and digested as detailed in Section 2.3.6.
2.3.8 Mass Spectrometry

Peptide solutions were analyzed using liquid chromatography – tandem mass spectrometry (LC-MS/MS) on a Thermo (San Jose, CA) LTQ XL linear ion trap mass spectrometer with a nanospray ionization source. Utilizing a Thermo (San Jose, CA) Finnigan Micro ASII autosampler and Surveyor Plus HPLC System, peptides were separated on a C-18 reverse-phase column (150 μm × 10 cm, 5 μm particles, 300 Å pores) from Column Technology (Fremont, CA). Triplicate injections, each 20 μL, were made for each sample. Gradient elution from the column was accomplished using two mobile phases: A (0.1% formic acid in water) and B (0.1% formic acid in methanol) at a flow rate between 0.5-1 μL/min. For each 160 minute run, the gradient began with 5% B, rose to 25% B within 15 min, 90% B within 115 min, and then decreased back to 5% B within 145 min. Peptides were ionized using a nanospray source at 1.8 kV. Precursor ions were obtained via a full scan in the range of 300-2000 m/z. A data-dependent scan procedure was used for the tandem mass spectrometry (MS/MS) analysis, in which resultant ions from the full scan were selected and subjected to further fragmentation using collision-induced dissociation (CID). Analysis was completed in the positive ion mode with active dynamic exclusion, in which a precursor ion was excluded from the analysis for 180 seconds if it was selected for further fragmentation twice within 30 seconds.

2.3.9 Database Searches and Analysis

The MS/MS spectra were analyzed using software distributed by Thermo, specifically Finnigan Xcalibur Bioworks 3.2 and the Sequest search engine used for peptide/protein identification. Searches were performed against an NCBI RefSeq database for Homo sapiens, which was obtained on March 11, 2012 and contained 67,440 entries. Database sequences were
also reversed to enable decoy searches and false discovery rate approximations. No more than two missed cleavage sites were allowed. A fixed modification was set for carbamidomethylation (alkylated cysteines with mass shifts of +57.021464 Da) and a differential modification was set for oxidation of methionine (+15.9949 Da). Other differential modifications included: biotinylation at cysteine, histidine, and lysine for labels 1 (+298.4 Da), 2 (+411.5 Da), and 3 (+545.3 Da) and histidine and lysine for label 4 (+369.147075 Da). Trans-Proteomic Pipeline (TPP) (Versions 4.4 and 4.6.3; Institute for Systems Biology in Seattle, Washington) was used to refine Sequest search results. In order to validate peptide-matched MS/MS spectra, PeptideProphet was used and only peptides with a minimum probability of 0.9 were accepted. Semi-quantitation was achieved using spectral counting (SC), in which identified tandem mass spectra were counted for each HSA peptide.

2.3.10 FTC Assay

2.3.10.1 Preparation of Standards

The following methods were adapted from the work of Mohanty et al. Fluorescein-5-thiosemicarbazide (FTC) was initially dissolved in DMSO at 10 mM and stored at -20°C. Proteins from control plasma were isolated via TCA precipitation and reconstituted in 6 M GuHCl in 2-(N-morpholino)ethanesulfonic acid (MES) buffer. A solution of 5 µg/µL HSA in PBS was prepared and incubated with 0.015 M acrolein in DMSO (10:1 acrolein:protein) for 1 h on the ThermoMixer to carbonylate HSA. Excess acrolein was removed by TCA precipitation and proteins were reconstituted in 6 M GuHCl in MES. Both the plasma protein solution and oxidized HSA solution were diluted to 10 µg/µL. A new solution of 10 µg/µL HSA in PBS was prepared. To create HSA Standard Set 1, the carbonylated HSA was spiked into the plain 10
µg/µL HSA solution at 0, 5, 10, and 20% (v/v) for total volumes of 100 µL each. To create HSA Standard Set 2, the oxidized HSA was spiked into the plasma protein solution at 0, 5, 10, and 20% (v/v) for total volumes of 100 µL each. An equal volume of 304 µM FTC (10:1 FTC:HSA) was added and incubated overnight at room temperature, in the dark, and with gentle rotation. Proteins were isolated via TCA precipitation and reconstituted in 450 µL 3M GuHCl in PBS.

2.3.10.2 Fluorometry

Fluorescence measurements were made using a Varian Cary Eclipse Fluorescence Spectrophotometer and analyzed with Scan Software Version 1.1 (132). The instrument was zeroed with PBS and sample measurements were made one at a time with an excitation wavelength of 492 nm and an emission wavelength of 550 nm. The excitation and emission slit widths were 5 nm and measurements were made at medium scan speed (600 nm/min).

2.3.10.3 Fluorescence Anisotropy

Anisotropy measurements were made to estimate the degree of binding between the fluorescent label and protein. Measurements were made on the same instrument, but excitation and emitted light were polarized through the insertion of filters between the source and sample and the sample and detector. Sample solutions were excited with vertically polarized light and both vertically and horizontally polarized emissions were created via a filter oriented in both directions between the sample and detector. An instrumental correction factor known as the G-factor, which is simply a ratio comparing horizontal and vertical emissions, was determined prior to each day’s experiments. G-factors typically ranged between 1.5-1.6. A total of three anisotropy measurements were always made for each sample and resulting anisotropy (r) values were averaged.
2.4 RESULTS

2.4.1 Labeling Efficiency

The essential purpose of the following study was to develop analytical methods for identifying the sites of protein oxidative carbonylation by mass spectrometry. In this study, we allowed HSA to react with acrolein, an α,β-unsaturated aldehyde. As a strong electrophile, acrolein reacts readily with nucleophiles, namely the side chains of cysteine, histidine, and lysine, as depicted in Scheme 2-1. This produced aldehyde-containing Michael addition products similar to those formed from lipid peroxidation processes, providing a convenient method for reproducibly preparing carbonylated protein samples.

Scheme 2-1. Acrolein-based Michael addition forms adducts on cysteine, histidine, and lysine. (Redrawn from Ugur et al.)
In previous studies conducted by Dr. Ugur in our research lab, HSA was carbonylated, labeled, digested, and analyzed using a protocol similar to the one detailed above. Affinity tags, particularly biotin hydrazide reagents, were useful in our work in that they provided stability to the carbonyl and a means in which the samples could be enriched. A schematic showing the reaction between a hydrazide and aldehyde is pictured in **Scheme 2-2**. A total of five affinity tags were examined in terms of their labeling and ionization efficiency. For three of the tags, which were biotin hydrazide labels, monomeric avidin columns were utilized as a means of enrichment at the peptide level, affording an 8-9 fold enrichment factor when comparing the spectral counts for labeled peptides before and after enrichment (**Table 2-1**). There was little variation between the fraction of peptides detected after enrichment for the three labels, indicating none offered advantages during the enrichment process. Thus, enrichment was successful at the peptide level, but could labeled HSA be enriched at the protein level and in the presence of other proteins?

**Scheme 2-2.** Reaction between a hydrazide and an aldehyde to form a hydrazone. R represents the hydrazide reagent chain and P represents the protein.
Table 2-1. Fractions of biotinylated peptides detected with mass spectrometry before and after monomeric avidin column enrichment. Labels 1, 2, and 3 refer to biotin hydrazide, EZ-link hydrazide-LC-biotin, and EZ-link hydrazide-PEG4-biotin, respectively. (Recreated from Ugur et al.)

<table>
<thead>
<tr>
<th>LABEL</th>
<th>BEFORE</th>
<th>AFTER</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.041 ± 17%</td>
<td>0.39 ± 8%</td>
</tr>
<tr>
<td>2</td>
<td>0.044 ± 33%</td>
<td>0.34 ± 27%</td>
</tr>
<tr>
<td>3</td>
<td>0.037 ± 14%</td>
<td>0.33 ± 33%</td>
</tr>
</tbody>
</table>

Initial attempts to enrich biotin hydrazide-labeled HSA following TCA precipitation were unsuccessful. When eluted column fractions containing biotinylated HSA were analyzed using UV-Vis spectroscopy, no protein was detected at a wavelength of 280 nm. Upon stepwise alterations of the method, no improvements in enrichment were observed with this label. The biotin quantitation assay was adopted as a simpler and more direct means of assessing protein biotinylation prior to enrichment.

The noncovalent interaction between biotin and avidin is one of the strongest in nature, even surpassing the strength of interactions between ligands and their respective antibodies. The dissociation constant for the biotin-avidin interaction is $10^{-15}$ M. Due to such great affinity and specificity, the complex is stable in solutions at various pH values and can also withstand repeated washes without dissociating. The biotin quantitation assay utilizes this strong interaction in conjunction with the weaker interaction between HABA and avidin. The dissociation constant for the HABA-avidin interaction is $10^{-6}$ M, about 2.5 times less than the affinity between biotin and avidin. When a biotinylated sample is incubated with HABA-avidin complex, the stronger affinity between biotin and avidin compromises the HABA-avidin bonds,
and the HABA is displaced. HABA alone absorbs light at 356 nm, but following binding with avidin, tautomerizes and shifts to an absorption around 500 nm. A depiction of this shift was redrawn from Hofstetter et al. and can be seen in Scheme 2-3.\textsuperscript{157} The replacement of HABA with biotinylated material is stoichiometric,\textsuperscript{89} and thus the decrease in absorbance observed as the freed HABA tautomerizes can be used to calculate a relative biotin to protein ratio.

**Scheme 2-3.** Tautomerization of HABA upon addition of avidin with a subsequent red shift.

Test runs with the assay gave low biotin to protein ratios with Label 1, but higher ones with Labels 2 and 3. This indicated that either Label 1 was not the most efficient labeling reagent or was not as effective in the enrichment process. To gain more insight, collaboration with Dr. Ugur commenced. He prepared labeled HSA samples using the same three labeling reagents and then biotinylation was assessed using the quantitation assay. Inter- and intraday precision were considered: each labeled HSA sample was prepared once on three separate days and measured three times on each day. The nine measurements for each sample were averaged and the results are shown below in Figure 2-2.\textsuperscript{94}
These results suggested that Labels 2 and 3 were more efficient labeling reagents than Label 1. Interestingly, when these same samples were analyzed with mass spectrometry, this trend was not evident in terms of spectral counting (see Table 2-1). This implied that the label affected the ionization and detection of labeled peptides in the mass spectrometer, which apparently had a bias towards Label 1. The structures of all three labels are shown in Figure 2-7.

The labeling efficiencies of Labels 1, 2, and 4 were also examined via Western blotting. The resulting blot is pictured below in Figure 2-3. Label 4 is depicted in Figure 2-7. Minimal background was observed in the controls. Looking at the intensity of the main HSA band around 60,000 kDa, it appeared to be more intense for Label 2 (Lane 6 versus Lanes 3 and 9). However, no imaging software was used to calculate area intensities.
Figure 2-3. Labeling efficiencies visualized via Western blotting. The lane numbers correspond to: 1=Empty lane, 2=Label 1 no acrolein, 3=Label 1 + acrolein, 4=Empty lane, 5=Label 2 no acrolein, 6=Label 2 + acrolein, 7=Empty lane, 8=Label 4 no acrolein, 9=Label 4 + acrolein, Std. Ladder has corresponding molecular weights in red.
2.4.2 Enrichment at the Peptide Level

Next, we ventured to see if the enrichment trends presented previously would persist in samples containing labeled HSA spiked into protein mixtures composed of: alcohol dehydrogenase, cytochrome C, lysozyme, ribonuclease A, and ubiquitin. Mass spectrometric spectral counting results from one sample preparation are shown in Table 2-2. Tryptic digests of HSA produce about 75 peptides. If we use Ugur’s estimate of 2.7 carbonyls per HSA protein determined with the DNPH assay, we would predict about 3.6% of the peptides would carry a carbonyl modification (2.7/75). However, since the carbonylated HSA spikes are at 10% the concentration of the five additional proteins, we would actually expect only about 0.3-0.4% of the peptides to carry a modification. We would expect ratios higher than this though due to our use of enrichment with ratios closer to one suggesting more efficient enrichment. Varying ratios between the three labels would suggest differing enrichment capabilities based on the labels’ properties. Mass spectra for modified and unmodified peptides for each label can be found in Appendix 4.

<table>
<thead>
<tr>
<th>Label</th>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotin hydrazide</td>
<td>0.0065</td>
<td>0.23</td>
</tr>
<tr>
<td>EZ-link hydrazide-LC-biotin</td>
<td>0.015</td>
<td>0.39</td>
</tr>
<tr>
<td>EZ-link hydrazide-PEG4-biotin</td>
<td>0.0046</td>
<td>0.49</td>
</tr>
</tbody>
</table>

These results differed from Dr. Ugur’s results presented in Table 2-1. Even though HSA was used in both studies, it is key to remember that the protocols varied and these samples
contained a small amount of labeled HSA mixed into a solution of five proteins. These proteins competed for the spectral counts during the mass spectrometry analysis, which may explain the smaller fraction of labeled HSA detected prior to enrichment (Table 2-2). The variation in fractions of labeled peptides detected after enrichment also differed from Ugur’s study; this indicated that the labels offered varying enrichment efficiencies, possibly as a result of the more complex protein mixture. The ratios were much greater than the ratio predicted by the DNPH assay (0.036), showing that the enrichment was indeed successful. Theoretically, we would expect the ‘After’ ratio to equal one, but this was not the case due to incomplete release of biotinylated material from the columns. The small amount of material used in each MS run produced gaps in the analysis, ample time in which other non-biotinylated material could be identified and inadvertently incorporated into the enrichment ratio calculations.

We also examined thresholds of the monomeric avidin columns at the peptide level. Carbonylated HSA was spiked into plasma at 0, 10, 20, and 30 % (v/v) and derivatized with Label 1, followed by reduction and TCA precipitation steps. The rest of the procedure was performed according to sections 2.3.6, 2.3.7.1, 2.3.8 and 2.3.9 and included two mass spectrometry analyses: the first prior to enrichment and the second after enrichment. Results from one preparation are presented in Table 2-3. Percent spikes at 5% or below resulted in no identifiable modified peptides in either mass spectrometry analysis, suggesting high column thresholds. Following this protocol, the lowest detection of modified material was at a 10% spike, resulting in only 5% enrichment. Based on the DNPH assay, we presumed that 3.6% of HSA peptides would carry a carbonyl modification if the entire HSA sample was allowed to react with acrolein. In actuality, for the 0, 10, 20, and 30% spike samples, we expected 0, 0.36, 0.72, and 1.08% of HSA peptides to carry a carbonyl, respectively. Knowing this and comparing
the ‘Before’ and ‘After’ fractions, we observed about 50-60 fold improvement upon use of the columns, which was a significant enrichment. In terms of a biological sample though, carbonylated material would most often be below the column threshold and even enrichment would not provide enough modified material to elucidate carbonylation patterns.

**Table 2-3.** Fractions of carbonylated HSA spiked into plasma, labeled, and then detected with mass spectrometry before and after monomeric avidin column enrichment. Fractions were calculated by dividing the total number of spectra by the number of modified peptide spectra.

<table>
<thead>
<tr>
<th>% Carbonylated HSA</th>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>0.001</td>
<td>0.05</td>
</tr>
<tr>
<td>20</td>
<td>0.006</td>
<td>0.36</td>
</tr>
<tr>
<td>30</td>
<td>0.009</td>
<td>0.46</td>
</tr>
</tbody>
</table>

**2.4.3 Enrichment at the Protein Level**

Peptides were successfully enriched at the peptide level, but enriching at the protein level would allow us to isolate biotinylated proteins toward the beginning of the procedure, eliminating materials required to treat all samples and any procedural steps following the first mass spectrometry analysis. Regnier et al. have pioneered the incorporation of monomeric avidin columns at the protein level, successfully identifying carbonylated proteins in hydrogen peroxide-treated yeast, plasma from diabetic rats, human plasma from healthy patients, and human plasma from breast cancer patients. While they incorporated the columns into an HPLC format, we chose to use the columns provided with the commercially available kit. Initial experiments in which carbonylated HSA was spiked into serum and then derivatized with Label 1 and enriched were unsuccessful. However, improvements in enrichment and subsequent detection were seen when we used Labels 2 and 4. Results from experiments with these three
labels are shown in Table 2-4. Mass spectra for modified and unmodified peptides for Labels 2 and 4 can be found in Appendix 4.

**Table 2-4.** Enrichment results at the protein level – mass spectrometry spectral counting results for Labels 1, 2, and 4. Fractions were calculated by dividing the total number of spectra by the number of modified peptide spectra.

<table>
<thead>
<tr>
<th>Label</th>
<th># Spectra for Modified Peptides</th>
<th>Fraction of Modified Peptide Spectra</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotin hydrazide</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EZ-link hydrazide-LC-biotin</td>
<td>17</td>
<td>0.0026</td>
</tr>
<tr>
<td>Aldehyde Reactive Probe (ARP)</td>
<td>364</td>
<td>0.042</td>
</tr>
</tbody>
</table>

### 2.4.4 FTC Assay

Being that FTC contains a hydrazide group, the molecule reacts with protein carbonyls in a similar fashion as biotin hydrazide, as illustrated below in **Scheme 2-4**.

**Scheme 2-4.** The labeling of carbonylated proteins with FTC. Blue represents an aldehyde and orange represents a reactive hydrazide.
FTC was first synthesized and used to detect and quantitate protein carbonyls about 30 years ago. Like other colleagues in the same field, Stadtman et al. had worked with quantitative spectrophotometric methods for protein carbonyl detection, specifically the DNPH assay. However, more sensitivity was desired and thus, fluorescence techniques were explored. Labeling protein carbonyls with FTC provided the means for separation and visualization via SDS-PAGE and quantitation via fluorescence spectroscopy.\textsuperscript{159} Anisotropy (r) is a measure of the polarization of emitted light.\textsuperscript{160} Based on theories established by Perrin in the 1930s,\textsuperscript{161} anisotropy measurements have been used to investigate protein size, shape, fluidity, and interactions. In the following experiments, anisotropy measurements were used to assess protein-FTC interaction. Generally, polarized light is passed through a sample. If the fluorophores are bound to the proteins, there is minimal rotation and the emitted light remains polarized, giving higher anisotropy (r) values. If the fluorophores are not bound to the proteins, there is increased rotation and the emitted light is depolarized, giving lower r values. HSA bound to a fluorophore exhibits an anisotropy value around 0.2.\textsuperscript{160} In our experiments, free FTC (100 $\mu$M) exhibited an average r value of 0.026. The anisotropy principle is pictured below in Figure 2-4.

![Principle of fluorescence anisotropy](image)

**Figure 2-4.** Principle of fluorescence anisotropy.
The calibration curves depicted below in Figure 2-5 were constructed from the two types of carbonylated, FTC-labeled HSA standards. Samples in which carbonylated HSA was spiked into regular HSA are referred to as HSA Standard Set 1 and samples in which carbonylated HSA was spiked into control plasma are referred to as HSA Standard Set 2.

**Figure 2-5.** HSA Standard Set 1 includes (A) Emission spectrum and (B) Corresponding calibration curve with linear equation \( y = 34.08x + 74.731 \) and with a correlation coefficient \( (R^2) \) of 0.9926. HSA Standard Set 2 includes (C) Emission spectrum and (D) Corresponding calibration curve with linear equation \( y = 41.518x + 4.7003 \) and with correlation coefficient \( (R^2) \) of 0.9926.
Fluorescence intensity maxima at 518 nm were determined using the Scan Software and used to calculate the calibration curves. With $R^2$ values close to one, both experiments demonstrated the assay’s ability to detect various concentrations of carbonylated protein. Standard calibrations such as those in Figure 2-5 could be used to determine FTC to protein ratios in samples with unknown amounts of protein carbonylation. Applications involving this assay can be found in Chapters 4 and 5. These same sets of standards were stored at -20°C for about two weeks and then reanalyzed with fluorescence spectroscopy to assess protein-FTC stability over time. Results are shown in Figure 2-6. Little change was observed in the fluorescence intensities and calibration curves for both HSA Standard Sets over the two weeks.

Figure 2-6. HSA Standard Set 1 includes (A) Emission spectrum and (B) Corresponding calibration curve with linear equation $y=33.559x+36.031$ and with a correlation coefficient ($R^2$) of 0.9914. HSA Standard Set 2 includes (C) Emission spectrum and (D) Corresponding calibration curve with linear equation $y=40.072x-4.5568$ and with correlation coefficient ($R^2$) of 0.9656.
As a means of assessing the stability of the interaction between FTC and HSA, fluorescence anisotropy measurements were made. We ultimately wanted to confirm that the label remained bound to HSA proteins. Measurements were made for samples in both HSA Sample Sets. Average anisotropy r values calculated from three replicates per sample are displayed below in Table 2-5. Data corresponding to Figure 2-5 are in the column titled ‘First Analysis’ and data corresponding to Figure 2-6 are in the column titled ‘Second Analysis’ (measured 14 days after the First Analysis). Comparing r values for the first and second analyses, results indicated a decrease in value over time, which is consistent with release of the label and suggests that fluorescence measurements should be made immediately.

Table 2-5. Average anisotropy (r) values along with standard deviations for three replicate instrumental analyses for both Standard Sets. The Second Analysis was performed about 14 days after the First Analysis.

<table>
<thead>
<tr>
<th>Sample Set</th>
<th>% Carbonylated HSA</th>
<th>First Analysis</th>
<th>Second Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard Set 1 (HSA)</td>
<td>0%</td>
<td>0.153 ± 0.004</td>
<td>0.129 ± 0.003</td>
</tr>
<tr>
<td></td>
<td>5%</td>
<td>0.113 ± 0.006</td>
<td>0.080 ± 0.009</td>
</tr>
<tr>
<td></td>
<td>10%</td>
<td>0.106 ± 0.000</td>
<td>0.040 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>20%</td>
<td>0.118 ± 0.002</td>
<td>0.068 ± 0.008</td>
</tr>
<tr>
<td>Standard Set 2 (Plasma)</td>
<td>0%</td>
<td>0.064 ± 0.009</td>
<td>0.119 ± 0.010</td>
</tr>
<tr>
<td></td>
<td>5%</td>
<td>0.090 ± 0.011</td>
<td>0.055 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>10%</td>
<td>0.115 ± 0.001</td>
<td>0.060 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>20%</td>
<td>0.118 ± 0.003</td>
<td>0.063 ± 0.009</td>
</tr>
</tbody>
</table>
2.5 DISCUSSION

2.5.1 Labeling

In this work, four different biotin affinity labels and their capabilities in tagging protein carbonyls were investigated: Biotin hydrazide (Label 1), EZ-Link Biotin-LC-Hydrazide (Label 2), EZ-Link Biotin-PEG₄-Hydrazide (Label 3), and Aldehyde reactive probe (ARP) (Label 4). Detecting and quantifying carbonyls in vivo is problematic due to the variety of carbonyl modifications that exist. Thus, only a handful of broad studies, including our own, have been done to investigate labeling properties and efficiencies, while detailed and extensive studies are lacking in this area. A recent study published by Bollineni et al., one of the only of its kind, explored reactions between three different affinity labels (2,4-dinitrophenylhydrazdine, biotin hydrazide, and ARP) and three types of carbonyl species (aldehydes, ketones, and lactams). Using synthesized peptides, Bollineni et al. concluded that reagents were specific for reaction conditions and certain types of carbonyl modifications based on labeling efficiency analyses using HPLC, MALDI-TOF-MS, and ESI-LTQ-Orbitrap-MS. They also made the counterintuitive suggestion of labeling carbonyls at the peptide level, a finding in contradiction of Regnier et al. who proposed carbonyls should be labeled immediately.

We only focused on one type of carbonyl species (LPO-based aldehyde mimicks) and we discovered labeling efficiency trends for Labels 1-4, with less extensive work done with Label 4. Although varying interday biotin/BSA and biotin/HSA ratio values indicated that biotin quantitation assay results had significant uncertainties, general ratio trends suggested that Labels 2 and 3 were the most efficient, with corresponding ratios of about two and three biotin:protein, respectively. This was attributed to their varying spacer arm lengths, illustrated in Figure 2-7.
Longer chains could improve solubility and decrease steric hindrance and, in the case of Label 3, increase hydrophilicity. The concentrations of the affinity labels in solution were limited by their solubility. For instance, labels that failed to dissolve fully or crashed out of solution during use would affect the amount of label available to react with protein carbonyls. Longer chains decreased steric hindrance in two ways: 1. They allowed the hydrazide to react with protein carbonyls with less interference from the biotin moiety during labeling and 2. They allowed the biotin moiety to interact with avidin with less interference from the protein/peptide during enrichment. Lastly, hydrophilicity of the label determined at which sites on the protein it would react; hydrophilic labels are attracted to hydrophilic protein sites and vice versa.\textsuperscript{162}
Figure 2-7. Structures and molecular weights for Labels 1-4.
While longer chains may have contributed to monomeric avidin enrichment capabilities, mass spectrometry results indicated that this attribute hindered detection of the modification sites. Cumbersome tags can negatively affect ionization and fragmentation as demonstrated by Bollineni et al.\textsuperscript{92} Another contributing factor was the peptide structure, particularly the hydrophobicity/hydrophilicity of the amino acids comprising the peptides. An overview of the commonly modified residues detected with mass spectrometry before and after enrichment at the peptide level for Labels 1-3 is depicted in Figure 2-8. Interestingly, no modifications were observed at cysteine-34, the only free sulfur and most reactive site to acrolein modification.\textsuperscript{24} Lysine-545 was the most detectable modification site for the longer chain labels, 2 and 3, especially following enrichment. The greatest number of spectra identified for a modification site was histidine-146, derivatized with Label 2. Histidine-146 was found within the peptide sequence \texttt{HPYFYAPELLFFAK} and is one of the most hydrophobic peptides identified. Shockingly, this modified peptide, with the highest spectral counts, was only detected prior to enrichment. In contrast, lysine-12, from a hydrophilic peptide with the sequence \texttt{FKDLGEENFK}, was only detected after enrichment. This revealed that the columns discriminated against certain peptides, an undesirable trait in this work. The number of spectra varied for each of the three labels in regards to these two peptides, with Label 2 prevailing in both cases. This indicated that peptide properties played a role in the enrichment process and that some labels were versatile enough to allow detection of both hydrophobic and hydrophilic peptides. Location of the amino acids within the HSA protein structure was not a factor for enrichment and MS/MS analyses, since samples were at the peptide level. Overall, this study confirmed that label choice impacted which modification sites were derivatized, enriched, and
subsequently detected at the peptide level. We chose not to perform experimental repeats due to the peptide discriminating quality of the columns.

**Figure 2-8.** Peptide level: Plot depicting number of MS/MS spectra identified before and after enrichment of HSA derivatized with three different biotin labels (more extensive data related to Table 2-2).
Moving from the peptide to the protein level, we endeavored to apply enrichment to proteins reacted with Labels 1, 2, and 4. Upon obtaining poor results with Labels 1 and 2 as can be seen in Table 2-4 and Figure 2-9, we attempted to use Label 4, a biotin-containing alkoxyamine. Label 4, illustrated in Figure 2-7, was originally used to derivatize oxidation sites on DNA, but was utilized in the detection of protein carbonyls because of its oxime formation, which is more stable than the hydrazone formed with hydrazide reagents. Label 4 is relatively new, but an appealing option because it is water soluble, reactive at neutral pH and at room temperature, does not require reduction, and contains a biotin moiety for avidin enrichment. Several studies involving detection of Label 4-modified proteins in model systems and biological systems have been published. In our brief work with Label 4, we spiked carbonylated HSA into serum (20% v/v), derivatized with Label 4, and enriched with monomeric avidin columns, all at the protein level. While this approach saved time and materials, full-sized proteins could potentially cause steric hindrance during enrichment. Spectral counting results from one sample preparation are shown in Figure 2-9. Interestingly, similar and differing trends were distinguished between protein and peptide enrichment studies. It was expected that protein level enrichment would give patterns similar to those seen without enrichment. Again, the residue with the highest propensity for detection was the peptide containing histidine-146. This implied that since this peptide was not detected during peptide enrichment, protein enrichment was less discriminatory for this peptide. At protein level enrichment, Lysine-545 was hardly detected in contrast to peptide enrichment studies in which this residue was the most commonly detected site for Label 3. Histidine-367 was the second most commonly detected residue for Label 4 and, unlike the hydrophobic peptide containing histidine-146, this peptide (CCAAADPHECYAK) was composed mostly of hydrophilic amino acids.
These varying results confirmed that experimental factors such as choice of affinity label, reaction conditions, and experimental design were crucial to detecting certain modifications regardless of enrichment occurring at the peptide or protein level. Again, we chose to not do experimental repeats in order to exert our efforts in finding a simpler and more effective means of enrichment.

Figure 2-9. Protein Level: Plot depicting number of MS/MS spectra identified following protein enrichment for Label 4-derviatized HSA (more extensive data related to Table 2-4).
2.5.2 Spectral Counting

We chose to use spectral counting (SC) as a semi-quantitative technique because it is quicker, cheaper, and more user-friendly than label-based quantitation methods, such as iTRAQ, ICAT, SILAC, etc. It is also commonly used in bottom-up proteomics experiments such as our own. Our LTQ analysis included ten MS/MS events, which generated a large number of fragmentation spectra. This increased the probability of identifying more peptides and further facilitated the use of SC. Unlike labeling-based quantitation methods, SC does not require a unique label for each sample and can be applied to a variety of samples. Nonetheless, SC suffers from high variability and a lack of sensitivity, which is problematic when investigating low abundance proteins since the corresponding identified peptides would be limited. Issues persist with saturation of high abundance proteins as a result of ion trapping limitations as well. This results in a diminished dynamic range. Despite these downfalls, theoretically we would expect a linear relationship between protein abundance and spectra counts, and Liu et al. confirmed this assumption for dynamic ranges on the order of 2 or greater. Decreased linearity would be expected for more complex samples. Being that the majority of our samples, aside from the serum/plasma, were simple solutions with six or fewer proteins, we resorted to spectral counting. Our intent was not to use spectral counting as a measure of protein abundance, but simply to use it as a measure of labeling efficiency and instrument detectability. This proved to be successful in that we were able to quantitatively distinguish efficiency differences between affinity labels based on a comparison of spectra counts for the various samples.
2.5.3 Monomeric Avidin Column Enrichment

The studies in this chapter were centered on one mode of affinity purification, the monomeric avidin columns. Numerous successful studies involving monomeric avidin column enrichment of carbonylated proteins have been published, including one from our own lab and most notably from Regnier et al. For this reason, we proceeded with using the columns while also investigating their enrichment capabilities more fully. Tetrameric avidin and biotin have the strongest specificity in nature with a dissociation constant of $10^{-15}$ M. Such a strong affinity complicates elution; therefore, avidin variations were created that exhibited decreased specificity. One of these variations is monomeric avidin which, along with biotin, has a dissociation constant of $10^{-7}$ M, more than half that between tetrameric avidin and biotin. Other variations include chemically and genetically modified avidin. Biotin products that show less affinity for avidin, such as desthiobiotin and 2-iminobiotin, have been developed as well. The relatively easy elution of biotinylated material from the monomeric avidin columns allows for identification of the modification site, a quality lacking in affinity-based bead products. Identifying sites of carbonylation is crucial in helping us to understand carbonylation mechanisms and also provides more solid confirmation of the identified affinity-labeled peptide.

Spike experiments indicated that we had to reach a concentration of 10% carbonylated HSA (v/v) to achieve detection of modifications. This implied that the columns required large amounts of protein, which is not always feasible, or required highly carbonylated protein samples, again which is not common in biological samples. Although we only worked with one type of carbonyl species (LPO-based aldehyde mimicks), blood contains a variety of carbonyl species at lower concentrations potentially undetectable with the columns. Thus, their use with
true biological samples would not be amenable. Their incorporation into the procedure at either the peptide or protein level introduced large sample volumes that required extra measures to reduce and purify. Hence, they were neither the most economical nor time efficient enrichment technique. Results in Table 2-2 indicated that Label 3 was the most efficient label in terms of enrichment, yet even upon using this label in enrichment experiments at the protein level, little to no improvements in detection were made. Poor results from enrichment experiments at the protein level could be explained by the use of SDS. Being that proteins were precipitated and not all serum proteins are directly soluble in PBS, a small amount of SDS was used to reconstitute proteins. Monomeric avidin lacks the structural strength that natural avidin possesses and thus, would be vulnerable to structural change in the presence of denaturants. This could result in decreased binding capacity and ultimately a loss of biotinylated material.91

2.5.4 FTC Assay

HSA was carbonylated by allowing the protein to react with acrolein and then spiked into regular HSA or plasma at various spike amounts (v/v). FTC was added to label protein carbonyls and permit detection using fluorescence spectroscopy. Validation experiments were successful in that linear relationships ($R^2>0.99$) were determined between the percent of carbonylated HSA and the fluorescence intensity. The sample stability was also investigated – HSA Standard Sets 1 and 2 were stored at -20°C for two weeks and then reanalyzed. For both HSA Standard Sets, little change was observed in the fluorescence intensities and the calibration curves still offered a linear relationship between the percent of carbonylated HSA and the fluorescence intensity. Anisotropy experiments were performed to assess the stability of the interaction between FTC and HSA. For the first analysis, r values were roughly four to five times greater than that of free FTC, which indicated that the label was indeed bound to the HSA proteins. However, a decrease
in the r value from the First to the Second Analysis (measured 14 days after the First Analysis) indicated release of the label over time. Thus, fluorescence measurements should be made immediately. The one sample that exhibited a reverse trend was the 0% carbonylated HSA sample in HSA Standard Set 2, which showed a greater r value for the second analysis. This could have resulted from inefficient removal of excess label, which then reacted with proteins over time or a measurement error. Based on the validation results though, this assay shows promise for use in more complex systems.
2.6 CONCLUSION

Numerous analytical barriers still exist in proteomics experiments; however, our work has clarified some uncertainties concerning derivatization and enrichment techniques. Two new quantitative assays were also validated which can both be used as measures of labeling efficiency. Upon investigating labeling efficiency of various biotin labels, it was concluded that the labels affect enrichment and mass spectrometry analyses differently, depending on their chemical properties. In general, the longer chained, more hydrophilic labels offered better enrichment capabilities and were more readily detected via mass spectrometry. These results roughly correlated with research by Ugur et al. and Bollineni et al. Overall, it was confirmed that the choice of label influences the modification sites detected and extensive consideration should be given to this topic in future investigations of carbonyl modification sites. In terms of enrichment, the monomeric avidin columns discriminated against certain peptides at the peptide level and functioned poorly at the protein level, complicating experimental procedures and offering a high limit of detection. For these reasons, we began investigating the combination of a new label and a different form of enrichment. This new protocol allowed for detection of the protein carbonyl modification sites, like the monomeric avidin columns, but offered many advantages over the columns, as can be seen in the next chapter.
Chapter 3 – A CLEAVABLE BIOTIN TAG ENABLING ENRICHMENT AND IDENTIFICATION OF PROTEIN CARBONYLATION MODIFICATION SITES
3.1 ABSTRACT

Despite numerous variations in sample preparation, the use of monomeric avidin enrichment columns proved laborious and yielded unsatisfactory results in general. Thus, a different protocol was explored, one that still facilitated identification of modification sites just as the monomeric avidin columns had done, but was more user-friendly and time efficient. The combination of a cleavable biotin affinity tag and a streptavidin bead enrichment system allowed the best of both worlds: identification of modification sites and easy incorporation into existing protocols. General streptavidin bead enrichment protocols involve digestion off of the beads, resulting in various peptides assumed to have originated from the biotinylated protein, but without the modification sites still adhered to the beads. Our cleavable linker prevents this by allowing elution of the modification site from the beads. It contains a disulfide, which can be cleaved and alkylated with DTT and IAA, respectively, chemicals which are already incorporated into our procedure. We investigated the correlation between varying amounts of carbonylated protein and the percent of peptides carrying a modification site, along with various other procedural adjustments. The new cleavable biotin tag offered better labeling efficiency than previous labels, but also with preference for certain HSA sites, a trend alluded to in Chapter 2.
3.2 INTRODUCTION

A variety of derivatization methods for carbonyls have been used and include, but are not limited to: dinitrophenylhydrazine (DNPH),\textsuperscript{172} Girard P reagent (GRP),\textsuperscript{173} hydrazides,\textsuperscript{94} fluorescent probes,\textsuperscript{155,174} and alkoxyamines.\textsuperscript{164} DNPH-based techniques developed by Levine et al. hinge on UV-Vis Spectroscopy and Western blotting as detection methods and are primarily used for qualitative work. Two commonly used fluorescent probes are: fluorescein-5-thiosemicarbazide and Alexa 488 fluorescence hydroxylamine. Quantitation methods involve the use of fluorescence spectroscopy or SDS-PAGE, with gel work being the most advantageous since removal of excess fluorescent probe is difficult in solution.\textsuperscript{93} Hydrazides, and alkoxyamines, particularly biotin hydrazide and aldehyde reactive probe (ARP), respectively, and GRP are all commonly used in quantitative mass spectrometry analyses, with hydrazides and alkoxyamines prevailing.\textsuperscript{87,92} Hydrazides require a reduction step to stabilize the resultant hydrazone bond, whereas, this is unnecessary when using alkoxyamines since an oxime is formed.\textsuperscript{164} Both contain the biotin moiety, which can be utilized in conjugation with avidin products for enrichment and prior to mass spectrometry.\textsuperscript{93}

The noncovalent interaction between biotin and avidin is one of the strongest in nature, even surpassing the strength of interactions between ligands and their respective antibodies. Avidin is a carbohydrate-containing tetramer with a basic isoelectric point (pI=10).\textsuperscript{175} The dissociation constant for the biotin-avidin interaction is $10^{-15}$ M. Due to such great affinity and specificity, the complex is stable in solutions at various pH values and can also withstand repeated washes without dissociating.\textsuperscript{156} Conversely, the abundance of carbohydrates and basic pI encourage nonspecific binding and such a strong dissociation constant hinders release of
biotinylated material. Thus, avidin analogues with decreased affinity have been discovered. Popular products include monomeric avidin,\textsuperscript{176,177} neutrAvidin,\textsuperscript{178} and streptavidin,\textsuperscript{179,180} all of which have been used extensively as enrichment products for biotinylated carbonyls.\textsuperscript{175} One challenge when using streptavidin beads is the impossible feat of cleaving biotinylated material from the beads. This directs protocols to involve digestion on the beads, at the expense of leaving the modification site attached to the beads. Another challenge when working with biotin and avidin products is nonspecific binding, which can lead to peptides being falsely identified as part of a carbonylated protein.\textsuperscript{87} For this reason, our newly developed method is very useful because it allows for elution from the beads and identification of the modification site, providing steadfast confirmation of carbonylation.

In this work, we have developed an analytical method utilizing an alkoxyamine biotin-containing affinity tag and streptavidin beads. The EZ-Link Alkoxyamine-PEG\textsubscript{4}-SS-PEG\textsubscript{4}-biotin, pictured in \textbf{Scheme 3-1}, contains a carbonyl reactive site (alkoxyamine), a cleavable disulfide, and a biotin moiety permitting enrichment with avidin products and easy incorporation into existing protocols. Upon reaction between the protein carbonyl and alkoxyamine and subsequent enrichment, disulfide cleavage with DTT and alkylation with IAA can be achieved to yield two products, as depicted in \textbf{Scheme 3-1}. The most important product contains carbonylated protein conjugated to half of the alkylated label (alkoxyamine end), which provides a distinguishable mass when analyzing via mass spectrometry. The other product is the remainder of the label (biotin end) attached to the bead, which gets discarded. We’ve chosen to use human serum albumin (HSA) in our work because it is inexpensive and has been used previously in oxidative stress studies.\textsuperscript{181,182} We have developed a reproducible method of producing carbonylated protein in vitro in which HSA is treated with acrolein to carbonylate cysteines, histidines, and
lysines. These reactions mimic the nucleophilic addition of aldehyde-containing lipid peroxidation products to proteins in an environment under oxidative stress. To the best of our knowledge, this will be the first report of using this label to investigate protein carboxylation sites. Our validation work involved quantifying the labeling efficiency and investigating our instrumental limits of detection. Percent of modification and identification efficiencies were investigated via mass spectrometry and determined by spectral counting (SC). The results suggest that the alkoxyamine is superior to Labels 1, 2, and 3 in terms of labeling efficiency and shows preference for particular sites difficult to detect with other labels.
Scheme 3-1. Products resulting from reaction of the alkoxyamine label with a protein carbonyl and subsequent enrichment, reduction, and alkylation. $P=$protein.
3.3 EXPERIMENTAL DESIGN

3.3.1 Materials

HSA, IAA, and CaCl₂ were purchased from Sigma-Aldrich (St. Louis, MO). Formic Acid (LC/MS grade), urea, 10xPBS, water (HPLC), methanol (HPLC), ACN, DTT, DMSO, DTT, TFA, and biotinylated BSA were purchased from Fisher Scientific (Pittsburgh, PA). TCA was obtained from Acros Organics (Fair Lawn, NJ). The BCA Protein Assay, Biotin Quantitation kit, Streptavidin Ultralink Resin, and EZ-Link Alkoxyamine-PEG4-SS-PEG4-Biotin were purchased from Thermo Scientific (Rockford, IL). Oasis MCX extraction cartridges were purchased from Waters Corporation (Milford, MA). Sequencing grade modified trypsin was obtained from Promega (Madison, WI).

3.3.2 Carbonylating HSA

HSA (10 mg/mL) was reacted with acrolein at a 10:1 acrolein:protein ratio and incubated at room temperature with gentle shaking for 1 h. Initial experiments proceeded directly to labeling. For spike experiments, excess acrolein was removed via precipitation with 15% TCA (aq). Solutions were incubated on ice for 30 min and supernatant removed following centrifugation. Pellets were washed three times with ice cold acetone, air dried 5 min, and resuspended in 200 µL PBS. Following dissolution, carbonylated HSA concentrations were determined with the BCA Assay. A 10 mg/mL solution of carbonylated HSA was prepared and spiked into 10 mg/mL HSA in PBS at 0, 25, 50, and 75 % spikes (v/v). Other spikes prepared included: 5, 10, 12.5, 15, and 20%.
3.3.3 Protein Biotinylation and Enrichment

EZ-Link Alkoxyamine-PEG4-SS-PEG4-Biotin was prepared in DMSO at 250 mM and stored at -80°C. Alkoxyamine label was added to carbonylated HSA at a final concentration of 5 mM and incubated for 2 h at room temperature with gentle rotation. TCA precipitation was performed as detailed in the section above and pellets reconstituted in 500 µL PBS. Prior to enrichment, 500 µL aliquots of streptavidin bead slurry were washed four times with PBS. Labeled proteins (500 µg) were added and incubated overnight at room temperature with gentle rotation.

3.3.4 Biotin Quantitation Assay

The extent of biotinylation was assessed prior to enrichment by following the protocol provided by Thermo Scientific Pierce. Three experimental repetitions were performed. Each time, protein concentrations were measured using the BCA Assay prior to quantitating biotin. Briefly, 100 µL biotinylated HSA was added to a solution containing 100 µL HABA-avidin complex and 800 µL PBS. Biotin’s stronger affinity for avidin causes the displacement of HABA, invoking an absorbance change detectable with UV-visible spectroscopy at 500 nm. Using the extinction coefficient of 34,000 M⁻¹cm⁻¹, the known protein concentrations, and the change in absorbance before and after biotinylated protein addition, the biotin to protein ratios were calculated and the extent of biotinylation determined. Commercially available biotinylated BSA was used as a standard; the manufacturer reports nine equivalents of biotin per BSA molecule.
3.3.5 Trypsin Digestion

Following enrichment, nonbound protein fractions were reserved while excess reagents were removed by washing the beads four times with PBS. Both nonbound and bound proteins were denatured with 2 M urea and reduced with 10 mM DTT at 37°C, 300 rpm for 1 h. To alkylate cysteines, IAA was added to a final concentration of 15 mM and samples were again incubated as described above in the dark. A second 15 mM addition of DTT and 1 h incubation followed in order to quench excess IAA. Labeled proteins that were cleaved from the beads were transferred to new tubes and, along with nonbound samples, were diluted with PBS. CaCl₂ was added to a final concentration of 1 mM and overnight trypsin digestion was performed at 37°C, 300 rpm. Digestion was halted via acidification using 10% formic acid. The peptides were concentrated with Oasis MCX columns and dried with a Speed Vac concentrator. After evaporation, dried peptides were dissolved in 95/5 0.1% aqueous TFA/ACN and the solution concentrations were established using the BCA Assay.

3.3.6 LC-MS/MS

Peptide solutions were analyzed using liquid chromatography – tandem mass spectrometry (LC-MS/MS) on a Thermo (San Jose, CA) LTQ XL linear ion trap mass spectrometer with a nanospray ionization source. Utilizing a Perkin Elmer (Waltham, MA) Series 200 pump and an Agilent (Santa Clara, CA) 1100 series HPLC system equipped with an autosampler, peptides were separated on a C-18 reverse-phase column (150 µm × 10 cm, 5 µm particles, 300 Å pores) from Column Technology (Fremont, CA). Triplicate injections, each 20 µL, were made for each sample. Gradient elution from the column was accomplished using two mobile phases: A (0.1% formic acid in water) and B (0.1% formic acid in methanol) at a flow
rate between 0.5-1 μL/min. For each 160 minute run, the gradient began with 5% B, rose to 25% B within 15 min, 90% B within 115 min, and then decreased back to 5% B within 145 min. Peptides were ionized using a nanospray source at 1.8 kV and analysis was completed in the positive ion mode. Precursor ions were obtained via a full scan (300-2000 m/z) with a data-dependent scan procedure for MS/MS analyses. Active dynamic exclusion parameters excluded an ion from the analysis for 180 seconds if it was selected for further fragmentation twice within 30 seconds.

### 3.3.7 Data Analysis

The MS/MS spectra were analyzed using software distributed by Thermo, specifically Finnigan Xcalibur Bioworks 3.3.1 and the Sequest search engine used for peptide/protein identification. Searches were performed against an NCBI RefSeq database for Homo sapiens, which was obtained on January 15, 2013 and contained 71,806 entries. Database sequences were also randomized to enable decoy searches and false discovery rate approximations. No more than two missed cleavage sites were allowed. A fixed modification was set for carbamidomethylation (alkylated cysteines with mass shifts of +57.021464 Da). Differential modifications were set for three variations of cleaved alkoxyamine label products: reduced (+334.156 Da), alkylated (+391.177 Da), and full label (+882.38 Da), all at cysteine, histidine, and lysine. Identification of reduced or full label modified peptides would indicate inefficient alkylation or inefficient binding or washing, respectively. Trans-Proteomic Pipeline (TPP) (Version 4.6.3; Institute for Systems Biology in Seattle, Washington) was used to refine Sequest search results. In order to validate peptide-matched MS/MS spectra, PeptideProphet was used and only peptides with a minimum probability of 0.9 were accepted. Semi-quantitation was achieved using SC.
3.4 RESULTS

In this study, we reacted HSA with acrolein, an α,β-unsaturated aldehyde. As a strong electrophile, acrolein reacts readily with nucleophiles, namely side chains of cysteine, histidine, and lysine. This produces aldehyde-containing Michael addition products similar to those from lipid peroxidation processes\textsuperscript{24} and is a convenient method for reproducibly preparing carbonylated protein samples. Previous studies have indicated that a 10:1 acrolein:HSA ratio is optimum for this work and produces roughly 2.7 carbonyls per HSA molecule.\textsuperscript{94}

3.4.1 Labeling Efficiency

Knowing that each acrolein-modified HSA protein carries about 3 active carbonyls, we needed to determine how efficiently those carbonyls react with our alkoxyamine affinity label. In order to do this, we utilized a commercial biotin quantitation assay. Both a control sample (no acrolein) and a treated sample (+ acrolein) were derivatized with the alkoxyamine and analyzed. Results are plotted in Figure 3-1, along with assay results for three other biotin labels taken from a previous study in our lab (see Figure 2-2).\textsuperscript{94} A commercial biotinylated BSA standard, which contained about 9 biotin/protein, served as a positive control; whereas, a sample treated with the alkoxyamine and no acrolein was a negative control. Although varying interday biotin/BSA and biotin/HSA ratio values indicated that biotin quantitation assay results have significant uncertainties, general ratio trends were elucidated. The assay revealed that the alkoxyamine label offered the best labeling efficiency out of all of the biotin affinity tags with an estimated four biotin per molecule.
3.4.2 Identification of Modification Sites

Once labeling of the protein carbonyls was confirmed, samples were prepared containing varying amounts of acrolein-treated HSA (0, 25, 50, and 75 %) mixed with unmodified HSA (v/v). All samples were labeled, enriched, digested, and analyzed by LC-MS/MS. Nonbound protein fractions for each sample, which were collected following overnight incubation with the streptavidin beads and preceding PBS washes of the beads, were also digested, and analyzed by LC-MS/MS. Assuming all of the alkoxyamine-labeled HSA was captured by the beads, we would expect to find no modified material in the nonbound fractions. This protocol was repeated for a total of three separate sample preparations. The protocol was also repeated for other spiking levels, ranging from 5% to 20%. Little to no modifications were detected below 10% and the

Figure 3-1. Biotin quantitation assay results. BSA Standard refers to the commercially purchased biotinylated BSA which has approximately nine biotin/protein. Control Alkoxyamine was a sample of HSA prepared without acrolein and treated with the alkoxyamine. Error bars represent standard deviations.
most variation in the number of modified peptides detected was observed in quadruple repeat analyses of 10% carbonylated HSA. Thus, this was determined to be the point where our detection capabilities became less predictable. Spectral counting data for the 0, 10, 25, 50, and 75% carbonylated HSA samples is summarized in Figure 3-2. The percent of modified peptides were determined for each sample preparation by dividing the number of modified peptide spectra by the total number of HSA spectra and then multiplying by 100. Error bars represent standard deviations for the percent of modified peptide spectra identified between the three trials. Only modifications that were detected in two or more trials were included in the data set for each sample. One modified peptide in the 3+ charge state, YKAATFECQQAADK, was occasionally detected in the nonbound fractions (1-2 spectra) and in the 0% carbonylated HSA sample, which is visible in Figure 3-2. Upon manual inspection of the spectra for this peptide, the assignment was not accepted since the spectra contained few b+ and b2+ ions, poor runs of y+ and y2+ ions, and no coverage of the modified lysine.
Overall, a total of 12 modification sites were identified by mass spectrometry: five histidines, six lysines, and one cysteine. These sites are shown amidst the entire sequence for human serum albumin in Figure 3-3. There are 609 amino acids in the precursor form of HSA and 585 amino acids in its secreted form, sans the first 24 amino acids. Modification sites are labeled based on the secreted form of HSA; however, the first 24 amino acids are still visible in Figure 3-3 and are highlighted in yellow. Identified modification sites with a corresponding amino acid number are in red font. A value of 24 should be added to this number for the precursor form of HSA. The average sequence coverage in our mass spectrometry analyses was 87% based on the precursor form of HSA.

**Figure 3-2.** Percent of modified peptides for 0, 10, 25, 50, and 75 % carbonylated HSA spikes. Error bars represent standard deviation. The % carbonylated HSA represents the amount of carbonylated HSA spiked into regular HSA (v/v). Percent of modified peptides were determined by: \( \frac{\text{modified spectra}}{\text{total spectra}} \times 100 \).
MKWVTFISLL FLFSSAYSRG VFRRDAHKSE VAHRFKDLGE ENFKALVLIA
FAQYLQQC$_{34}$PF EDHVKLVNEV TEFA$_{51}$TCVAD ESAENCDKSL H$_{67}$TLFGDKLCT
VATLRETYGE MADCCAKQEP ERNECFLQHK DDNPNLPLRV RPEVDVMCTA
FH$_{128}$DNEETFLK KYLYEIARRH$_{146}$ PYFYAPELLL FAKRK$_{162}$AAFT ECCQAADKAA
CLLPKLDELR DEGKASSAKQ RLKCASLQKF GERAFAKAWAV ARLSQRFPKA
EFAEVSKLVT DLTKVHTHECC HGDLLECADD RADLA$_{262}$YICE NQDSISSKLK
ECCEKPLLEK SH$_{288}$CIAEVEND EMPADLPSLA ADFVESKDVC KNYAEAKDVF
LGMFLYEYAR RHPDYSVVLL LRLAKTYETT LEK$_{359}$CCAAADP H$_{367}$ECYAKVFDE
FK$_{378}$PLVEEPQN LIKQNCELFE QLGEYKFQNA LLVRYTK$_{4}$VP QVSTPTLVVE
SRNLGKVGSK CCKHPEAKRM PCAEDYLSSV LNQLCVLHEK TPVSDRVTKC
CTESLVNRRP CFSALEVDET YVPKEFNAET FTFHADICTL SEKERQIKKQ
TALVELVKHK PKATKEQLK$_{545}$A VMDDFAAFVE KCKADDKET CFAEGKKLV
AASQAALGL

**Figure 3-3.** HSA amino acid sequence. Amino acids only present in the precursor form are highlighted in yellow (first 24). Modification site numbers relate to the secreted form of HSA and are colored red.

Modifications at H-146 were most often detected (see **Figure 3-5**) and an example of one such MS/MS spectrum, in the +2 charge state, can be seen in **Figure 3-4.** The MS/MS spectrum of the corresponding non-modified version of this peptide, also in the +2 charge state, can be found in Appendix 4. A more complete set of modified peptide mass spectra and their non-modified counterparts can be viewed in the same appendix.
Figure 3-4. MS/MS spectrum of the peptide RH\textsubscript{528,24}PYFYAPELFFAK in the +2 charge state, m/z 1145.84, with the oxidative modification on the histidine (H) residue. Blue peaks represent b ions and red peaks represent y ions. Peaks containing b#* or y#* represent a loss of water and peaks containing y#^ represent a loss of ammonia. Gray peaks represent unlabeled doubly charged b ions or those other than b or y ions.
3.5 DISCUSSION

3.5.1 Labeling Efficiency

In the previous study with Labels 1, 2, and 3, increasing ratios were observed for labels with longer, more hydrophilic chains, which decreased steric hindrance and improved solubility, respectively. The alkoxyamine, which was the longest and most hydrophilic label we have studied, complied with this pattern and offered the greatest labeling capabilities we have seen thus far. Longer chains decreased steric hindrance in two ways: 1. They allowed the hydrazide to react with protein carbonyls with less interference from the biotin moiety during labeling and 2. They allowed the biotin moiety to interact with avidin with less interference from the protein/peptide during enrichment. One caveat was that the biotin:protein ratio for the alkoxyamine (~3.8) exceeded the amount of free carbonyls suggested by the DNPH assay (~2.7). However, the DNPH assay is known to give only approximate values. In addition, the control alkoxyamine sample, which gave about 1 biotin/protein, indicated that there was background reactivity in the samples. This has also been seen in work with other labels, including DNPH. Finally, it is possible that the alkoxyamine is actually a more efficient label than DNPH.

3.5.2 Spectral Counting

Spectral counting (SC) was used as a means of quantitation because it aligns with our goal of assessing the label’s ability to allow us to identify modification sites. Our LTQ analysis included ten MS/MS events, which generated a large number of fragmentation spectra and increased the probability of identifying more peptides, further facilitating the use of SC. Theoretically we would expect a linear relationship between protein abundance and spectral
counts, and Liu et al. confirmed this assumption for dynamic ranges on the order of 2 or greater. Decreased linearity would be expected for more complex samples. Being that all of our samples were simple solutions with only one protein (HSA), we decided to use spectral counting. Because we were focused on locating modification sites, our intent was not to use spectral counting as a measure of protein abundance, but simply to use it as a measure of detection efficiency of the labeled peptides. We were able to quantitatively distinguish differences in the amount of modified peptides between spike samples based on a comparison of spectral counts for each sample.

3.5.3 Identification of Modification Sites

As expected, very few modified peptides were detected in the 0% sample, which contained no acrolein-treated HSA. The few peptides that were identified could be attributed to false positive identifications or carry-over between instrumental runs. If complete separation was achieved using the streptavidin beads, we might expect the percent of modified peptide spectra (Figure 3-2) to be relatively constant across the samples. However, we observed increasing numbers of modified peptide spectra accompanying increasing amounts of carbonylated HSA in the spike, a phenomenon most likely caused by the high backgrounds in the samples. Each control sample produced several thousand spectra resulting from the cleavage, reduction, and digestion of non-biotinylated material stuck to the beads. The same should occur for the spike samples; thus, while the amount of carbonylated HSA varied across the spikes, the background remained roughly the same, causing the trend in Figure 3-2. In Appendix 2, Tables A2, A3, A4, and A5 display the number of modified peptide spectra detected for each site in the 0, 10, 25, 50, and 75% spike samples.
An overview of the commonly modified residues detected by mass spectrometry in each sample is depicted in Figure 3-5. The same trend persists in this plot as the spike size increases. The scale was decreased in order to better visualize peptides with fewer counts. The number of spectra for H-146 in the 75% samples totaled 284. Only modifications that were detected in two or more trials were included in the data set for each sample. We see that the most commonly identified peptide contained H-146 and was the most hydrophobic amino acid sequence in this set: RHPFYAPELLFFAK. As shown in Figure 2-8, this was also the most abundant peptide detected prior to enrichment using biotin hydrazide labels 1-3. The number of spectral counts for the alkoxyamine for this peptide (sum of three experiments) is considerably larger than the totals for Labels 1 and 3. It is similar to that of Label 2.

![Figure 3-5](image)

**Figure 3-5.** Spectral counting results for triplicate injections of 0, 10, 25, 50, and 75 % carbonylated HSA spikes. Error bars represent the corresponding standard deviations. The number of spectra for H-146 in the 75% samples totaled 284. All spike samples were prepared three times, except for the 10% spike sample which was prepared four times.
Compared to the previous study in our lab, the alkoxyamine affinity tag presented some different labeling patterns, with certain sites being more prone to modification and/or detection than others. This comparison is displayed in Figures 3-6 and 3-7. Using the alkoxyamine, we were able to identify five unique modification sites otherwise undetected with the biotin hydrazide reagents; whereas, only two sites were unique to the biotin hydrazide reagents. Seven sites were common to all four labels (Figure 3-6). Surprisingly, K-525, which gave many spectra with the biotin hydrazide labels, gave none with the alkoxyamine. Spectral counts for peptides derivatized with Labels 1-3 or the alkoxyamine at the seven common sites are plotted in Figure 3-7. A number of spectra were identified for carbonyl modification of C-34, the most reactive cysteine in HSA due to its nucleophilic sulfur atom and existence as the only free cysteine in HSA. This site has proven difficult to detect by mass spectrometry with some affinity tags, particularly Label 3. It has also been reported that some cysteinylation of C-34 occurs in commercial HSA samples, which would reduce the amount of free C-34 available for modification. The crystal structure of HSA reveals that C-34 is positioned in a crevice on the exterior of the protein and partially shielded by nearby amino acids. This justifies our findings; a longer-chained label would limit steric hindrance from the biotin moiety and promote reaction with the acrolein-modified cysteine. In our previous study, the longest-chained affinity tag, Label 3, yielded the least amount of spectra at this site, potentially as a result of poor ionization of the labeled peptide. Figure 3-7 suggests that Labels 1-3 offer more modified peptide spectra for the shared lysines, but the alkoxyamine performs equal to or better than Labels 1-3 in terms of modification and detection of derivatized histidines. This is especially true for modifications at H-146, in which the alkoxyamine offered five times more spectra, and H-288, in which the alkoxyamine surpassed Labels 1 and 2 and Label 3 offered no spectra at all. Using Labels 1-3,
five separate lysine sites were modified versus only three histidine sites, with spectral counts for lysine modifications also exceeding those for histidine modifications. Similarly, more lysine sites were modified with the alkoxyamine, but histidine modifications at H-146, H-288, and H-367 produced the highest number of modified peptide spectra by at least two fold. These same trends were also evident in Figure 2-8 (spectral counts before enrichment). Even though more lysine modification sites were identified, there were fewer overall spectra and larger uncertainties accompanying them. However, more rigorous quantitative experiments would be required to confirm the activities of these residues.

Figure 3-6. Comparison of modification sites identified between the alkoxyamine label versus the biotin hydrazide labels.
We decreased the percent spikes of carbonylated HSA in order to investigate regions of limited detection. Upon doing so, we found that the total number of modified peptide identifications fluctuated greatly once we reached 10% carbonylated HSA, with little to no material identified below that spike amount (i.e. 5% carbonylated HSA). As can be seen in Figure 3-2, the data offered an average percent modification of 1.07 ± 1.02 % at the 10% spike. It appears that the operational limit of detection of any modification is between 5 and 10% spike. However, the percent modification value of 1.07 falls in line with expectations from the trend for the 0, 25, 50, and 75 percent modification values presented in Figure 3-2. This pattern is not so clear in Figure 3-5, but it should be noted that the small numbers of spectra, sometimes less than 10 per peptide from triplicate preparations, offer large uncertainties. Thus, instances in which the
number of spectra for the 10% spike appear to be higher than those of the 25% spike are not significant.

We also compared the number of modified peptide spectra to the number of unmodified peptide spectra, but only for peptides detected in all four spike samples (10, 25, 50, and 75%). These fractions were calculated by dividing the number of modified peptide spectra by the total number of spectra (modified + unmodified) for each individual peptide and are presented in Figure 3-8. To be included in this plot, the modified peptide had to be detected in at least two trials for each of the 10, 25, 50, and 75% spike samples. Examining the plot, we see a similar trend as that in Figure 3-2. This is due to the fact that as the number of modified peptide spectra for a particular peptide increased, the number of unmodified peptide spectra remained roughly the same. In general, the peptides carrying lysine modifications had much higher fractions than the peptides carrying histidine modifications. Spectral counts for these unmodified peptides never totaled above 30, thus, small numbers of modified peptide spectra gave large fractions and uncertainties for each of these peptides. The one exception was K-378, in which over 500 unmodified peptide spectra were identified per sample per trial, but no more than 20 modified peptide spectra were identified in these same analyses. On the other hand, fractions for the cysteine and histidine-modified peptides were much lower, a result of moderate spectral counts for modified peptides and higher spectral counts (>100) for unmodified peptides. The fractions for the 10% spike sample were accompanied by large uncertainties, similar to those depicted in Figure 3-5. The C-34-containing peptide differences were more extreme, like those at K-378. While our label was able to offer better detection of this site compared to Label 3, the small fractions demonstrated this site is still difficult to label and detect.
In terms of MS/MS spectra, the modified peptide spectra for H-146 (Figure 3-4) demonstrated good runs of b, y, and b\textsuperscript{2+} ions, which confirmed the peptide identification. Its spectrum was also characterized by a more intense and shifted b\textsubscript{2} ion (corresponding to cleaved label mass) and intense y\textsubscript{8} ions, the latter being a result of the proline effect.\textsuperscript{187} Both modified and non-modified peptide spectra for this peptide presented strong b\textsubscript{7} ions and doubly charged b ions. The intense blue peaks were identified as doubly charged b ions, not ions derived from alkoxyamine label fragmentation. While these peak intensities dominated the spectra, the singly charged b and y ions still remained abundant and with good intensity. Few to none of the modified peptide spectra contained reduced or full label modified peptides. Identification of these products would indicate inefficient alkylation or inefficient binding or washing, respectively. Most importantly, significant fragmentation of the labeling group was not evident.

**Figure 3-8.** Comparison of modified peptide spectra to unmodified peptide spectra for selected peptides. Fractions were determined by dividing the number of modified peptide spectra by the total number of spectra (modified + unmodified) for a particular peptide and averaging over three trials. Error bars represent standard deviations of the ratios.
in the spectrum and intensity was not lost in this way. A more complete set of modified peptide spectra and their non-modified counterparts can be viewed in Appendix 4. Some modified peptide spectra showed a few unidentified moderate to high intensity peaks that could possibly have resulted from fragmentation of the label. Generally though, b or y ion intensities dominated the spectrum and overall, the majority of the modified peptide spectra offered good runs for at least three of the b, b²⁺, y, and y²⁺ series and coverage of the modification site.

### 3.5.4 Limitations

As mentioned previously, a major downfall of using streptavidin products is nonspecific binding of non-biotinylated material. Because elution of biotinylated material from streptavidin is difficult to achieve, many scientists resort to on-bead digestion. Despite repeated washes prior to digestion, nonspecific material commonly remains, which can result in high backgrounds and false positive identifications. The use of a cleavable label is thought to minimize these complications since carbonylated material is cleaved from the beads, which are then discarded and not involved in the digestion process. However, we still observed large backgrounds in our control samples. It should be noted that although we focused on the identification of modification sites, other non-modified HSA peptides were identified. These peptides could very well have been cleaved from a captured HSA peptide carrying a carbonyl, but we have no way to separate these from peptides resulting from non-specific binding.

Also, the structure of the label most likely played a crucial role in the ionization, fragmentation, and detection of modified peptides. Previous studies have suggested mass spectrometry analysis of the larger, more hydrophilic labels is less efficient than analysis of their smaller, hydrophobic counterparts. In our previous study, alkyl labels did not perform better
than hydrazide reagents overall, but did offer improved responses for certain modification sites, such as C-34, K-162, K-351, and H-288. This indicates that environmental conditions impacted the labeling process. For instance, K-351, a residue on albumin’s surface, was not identified in our studies as being modified. This suggests that other factors (hydrophilicity/hydrophobicity, etc.) besides accessibility play a role in residue modification or mass spectrometric detection. Using the alkoxyamine, about half as many peptide spectra were identified for modified lysines as were for the biotin hydrazide reagents, but nearly twice the amount of spectra were identified for modified histidines with the alkoxyamine. Thus, the long, pegylated chain on the alkoxyamine affinity tag could have caused a decrease in ionization efficiency for some labeled peptides, but still fared better than the long, pegylated biotin hydrazide label (Label 3) in terms of the number of modification sites identified. This makes sense because, although both labels are pegylated, the alkoxyamine is slightly smaller, offering better detectability in the mass spectrometer.

Spike experiments were modified in various fashions to determine if improvements in detection could be observed. Numerous experiments involving varying amounts of streptavidin beads and total protein (carbonylated HSA spikes in regular HSA) were performed and repeated. Thermo Scientific reports the binding capacity of the beads to be $\geq 2$ mg biotinylated material/mL of resin. (The resin is a slurry which contains roughly equal volumes of beads and storage solution.) Optimum experiments resulted from following those exact recommendations and using 500 $\mu$L slurry + 500 $\mu$g protein. Altered experiments included: 250 $\mu$L slurry + 500 $\mu$g protein, 250 $\mu$L slurry + 750 $\mu$g protein, 500 $\mu$L slurry + 1000 $\mu$g protein, 500 $\mu$L slurry + 1500 $\mu$g protein, and 1000 $\mu$L slurry + 1000 $\mu$g protein. In each of these experiments, either a decreased amount of modified spectra or absolutely no modified spectra were identified in
comparison to the 1 μL:1 μg sample. In their review, Dundas and coworkers made the claim that
the addition of more streptavidin beads does not correlate with more identifications.191 Multiple
streptavidin bead manufacturers advise adhering to bead capacity recommendations to obtain
capture of the target molecule; our experiments confirm this advice. Steric hindrance between
bead and label should not have been a problem considering the length of the alkoxyamine spacer
arm. However, the addition of excess protein could have promoted protein aggregation, which
can lead to limited label accessibilities. Too many streptavidin beads can lead to aggregation and
a concomitant decrease in binding capacity. Separate experiments involving increased
reducing/alkylating agents (DTT/IAA) and incorporation of urea washes prior to
reduction/alkylation were also unsuccessful. Excess DTT/IAA can lead to precipitation and
disulfides in the alkoxyamine label may not have been able to withstand the harsh urea washes,
leading to premature cleavage and loss of the modification site.

3.5.5 SpectraST

SpectraST was proposed as an alternative peptide identification tool in 2007. Instead of
performing a statistical validation of the comparison of sample MS/MS to theoretical peptide
spectra as in Peptide Prophet, this validation technique involves spectrum matching. Real sample
spectra are added to a library and then experimental MS/MS spectra are searched against this
library.192 Additional material was identified using this analysis technique as can be seen below
in Tables 3-1 and 3-2. This analysis technique is useful in a targeted study; however, false
positive identifications are more likely to occur. Thus, the use of this software was primarily
exploratory. We showed that upon identifying certain modified peptides in a global analysis, we
could target those particular peptides and identify more spectra based on spectral matching.
However, SpectraST would be better suited for strictly targeted analyses and thus no other samples were analyzed with this software.

Samples were prepared in which 100% carbonylated HSA was labeled and enriched; no spiking was performed. Two initial HSA samples were created: a control sample (no acrolein) and a treated sample (+ acrolein). Upon enrichment, bound (labeled, attached to beads) and nonbound (nonlabeled, not attached to beads) fractions were collected for each sample, giving a total of four samples prior to digestion. Samples were then analyzed via LC-MS/MS. Data was analyzed with Sequest software and simplified with Peptide Prophet. Experiments were repeated multiple times. Some sample sets were also simplified with SpectraST software to see if increased numbers of modified peptide spectra could be identified. A SpectraST library was created from the Peptide Prophet output for a treated sample and contained modified peptide spectra for C-34, C-487, H-67, H-146, H-288, H-367, K-162, K-359, and K-545. C-34 is the only free cysteine available for modification in HSA, thus the modified peptide spectrum for C-487 was most likely a false positive identification (only one spectrum was identified). The modification was included in the library though in order to observe how SpectraST would respond. Data from the preparation of a second treated sample was then compared to the spectra in this library and also analyzed using Peptide Prophet. The corresponding data sets for each of these analyses are presented in Tables 3-1 (green) and 3-2 (blue).
**Table 3-1.** Peptide Prophet Analysis: List of modified HSA peptides with mass shifts for modified cysteine (C), histidine (H), and lysine (K). *0.9 minimum probability for included spectra.

<table>
<thead>
<tr>
<th>SAMPLE NAME</th>
<th># TOTAL SPECTRA*</th>
<th>MODIFIED PEPTIDE</th>
<th># SPECTRA</th>
<th>MODIFIED RESIDUE</th>
<th>CHARGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Bound)</td>
<td>4556</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Treated (Bound)</td>
<td>7782</td>
<td>RH325,24PYFYAPEELLFFAK</td>
<td>69</td>
<td>H-146</td>
<td>+2/+3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SH325,2CIAEVENDEMPADLPSLAADFVESK</td>
<td>14</td>
<td>H-288</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCAAADPH325,2ECYAK</td>
<td>16</td>
<td>H-367</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>YK303,27AAFTECCQADK</td>
<td>18</td>
<td>K-162</td>
<td>+2/+3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TTYETTLEK303,2CCAAADPHECYAK</td>
<td>5</td>
<td>K-359</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EQLK303,2AVMDDFAAFFEK</td>
<td>7</td>
<td>K-545</td>
<td>+2/+3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ALVLIAFAQYLQQC503,1PFEDHVK</td>
<td>33</td>
<td>C-34</td>
<td>+2/+3</td>
</tr>
<tr>
<td>Control (Nonbound)</td>
<td>6676</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Treated (Nonbound)</td>
<td>3798</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

**Table 3-2.** SpectraST Analysis: List of modified HSA peptides with mass shifts for modified cysteine (C), histidine (H), and lysine (K). *0.9 minimum probability for included spectra.

<table>
<thead>
<tr>
<th>SAMPLE NAME</th>
<th># TOTAL SPECTRA*</th>
<th>MODIFIED PEPTIDE</th>
<th># SPECTRA</th>
<th>MODIFIED RESIDUE</th>
<th>CHARGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Bound)</td>
<td>6880</td>
<td>RPC494,10FSALEVDETYVPK</td>
<td>16</td>
<td>C-487</td>
<td>3</td>
</tr>
<tr>
<td>Treated (Bound)</td>
<td>11000</td>
<td>SLH325,2TLFGDK</td>
<td>8</td>
<td>H-67</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RH325,24PYFYAPEELLFFAK</td>
<td>175</td>
<td>H-146</td>
<td>+2/+3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SH325,2CIAEVENDEMPADLPSLAADFVESK</td>
<td>15</td>
<td>H-288</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CCAAADPH325,2ECYAK</td>
<td>29</td>
<td>H-367</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>YK303,27AAFTECCQADK</td>
<td>27</td>
<td>K-162</td>
<td>+2/+3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TTYETTLEK303,2CCAAADPHECYAK</td>
<td>7</td>
<td>K-359</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EQLK303,2AVMDDFAAFFEK</td>
<td>25</td>
<td>K-545</td>
<td>+2/+3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ALVLIAFAQYLQQC503,1PFEDHVK</td>
<td>100</td>
<td>C-34</td>
<td>+2/+3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RPC494,10FSALEVDETYVPK</td>
<td>23</td>
<td>C-487</td>
<td>3</td>
</tr>
<tr>
<td>Control (Nonbound)</td>
<td>9031</td>
<td>RPC494,10FSALEVDETYVPK</td>
<td>35</td>
<td>C-487</td>
<td>3</td>
</tr>
<tr>
<td>Treated (Nonbound)</td>
<td>5586</td>
<td>RPC494,10FSALEVDETYVPK</td>
<td>12</td>
<td>C-487</td>
<td>3</td>
</tr>
</tbody>
</table>
Based on this analysis, we can see that more total spectra and modified spectra were identified using SpectraST. Roughly 2000-3000 more spectra were identified for each sample and over double the amount of modified peptide spectra were identified versus Peptide Prophet. The percent of modified peptide spectra increased from 2.08% (Peptide Prophet) to 3.51% (SpectraST). One peptide, SLHTLFGDK, was identified by spectrum matching using SpectraST, but not by theoretical spectrum comparison using Peptide Prophet. The peptide RPCFSALEVDETYVPK, carrying a modification on C-487, was identified in all control and treated samples during the SpectraST analysis, but was not identified in the Peptide Prophet analysis. As predicted, false positive identifications occurred using SpectraST.

An example spectrum for the peptide RHPYFYAPELLFFAK carrying a modification on the histidine (H-146) can be seen in Figures 3-4. Comparing the library and query spectra in Figure 3-4, we can see that the query spectrum was deemed a match based on the large number of matched peaks (in red). Black peaks in the query spectrum do not match assigned peaks in the library spectrum. Blue peaks in the library spectrum represent unassigned peaks. The red labels for the b and y ions signify that those particular ions were also found in the query spectrum. In summation, SpectraST software behaved as expected, providing more hits for modified peptide spectra, but also more false positive identifications. This was simply an exploratory study, and thus we did not analyze other sample data with this software.
Figure 3-9. SpectraST spectrum for RH$_{528.24}$PYFYAPELFFAK in the $+2$ charge state. The x-axis represents mass to charge (m/z) and the y-axis represents Relative Abundance. In the Query spectrum, red lines signify a peak match to those in the library spectrum.
3.6 CONCLUSION

A new method involving the use of a cleavable biotin tag and streptavidin bead enrichment was validated for HSA samples. On-bead digestion was avoided while still maintaining the ability to identify modification sites. Experiments aimed at correlating the percent of carbonylated HSA with the number of modified spectra highlighted issues with nonspecific binding and limited ranges of detection. However, it was discovered that the alkoxyamine offered the highest labeling efficiency of all labels tested in the past and greater coverage of modification sites than biotin hydrazide reagents, particularly Label 3. This work coincided with previous research indicating that many affinity labels prefer certain peptides in terms of labeling and detection. Future work should focus on protocol improvements concerning non-specific binding and detection limits and also validation of the method in a more complex sample matrix, with the hopes of one day applying the method to real biological samples.
Chapter 4 – APPLICATION: INVESTIGATION OF PROTEIN CARBONYLATION IN HUMAN PLASMA COLLECTED FROM CHRONIC KIDNEY DISEASE (CKD) PATIENTS ON DIALYSIS
Studies suggest a correlation between CKD progression and increasing oxidative stress, particularly at the final stage of the disease when patients are placed on dialysis and the highest oxidation levels have been observed. Being that we are focused on identifying sites and mechanisms of protein oxidative carbonylation with various tools, here we are applying our analytical methods and investigating protein carbonylation in the plasma of CKD patients. The overall goal of the study is to prepare and analyze CKD samples using our own methods in order to help us better understand the nature of oxidative damage to plasma proteins. This work was preliminary and mainly served as a means of method application. Although various protocols utilizing UV-Vis spectroscopy, fluorescence techniques, and mass spectrometry were used to investigate oxidative carbonylation in these samples, the data was not consistent enough to determine overall oxidation patterns.
4.2 INTRODUCTION

Chronic kidney disease (CKD) has gained status as a worldwide “public health problem” over the last ten years. The disease has become so prevalent across the globe that in 2006, March was deemed National Kidney Month and the second Thursday of March as World Kidney Day. The Centers for Disease Control and Prevention (CDC) deemed CKD as the 13th leading cause of death in the United States in 2011. An estimated 25 million people in the US, which is equivalent to about 12% of the population, have been diagnosed with CKD. The disease is most common in older populations; nearly 50% of the elderly over the age of 70 have CKD.

In the simplest of terms, CKD is defined as a condition affecting the performance of the kidneys. According to the National Kidney Foundation’s Kidney Disease Outcomes Quality Initiative (KDOQI), CKD is diagnosed if a patient has kidney damage or a decreased glomerular filtration rate (GFR) for more than 3 months, neither of which stems from a particular cause. If an individual’s GFR is measured below 60 mL/min per 1.73 m², this indicates about 50% loss of kidney function and the risk for CKD complications increases. One’s GFR naturally decreases with age due to the loss of muscle mass over time. This factor contributes to higher incidence of CKD amongst the elderly. As can be seen in Table 4-1, which was recreated from Anderson and Glynn, there are five main stages of CKD as determined by GFR:

<table>
<thead>
<tr>
<th>STAGE</th>
<th>CLINICAL FEATURES</th>
<th>GFR (mL/min/1.73m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Kidney damage with normal or increased GFR</td>
<td>≥ 90</td>
</tr>
<tr>
<td>II</td>
<td>Kidney damage with a mild decrease in GFR</td>
<td>60-89</td>
</tr>
<tr>
<td>III</td>
<td>Moderate decrease in GFR</td>
<td>30-59</td>
</tr>
<tr>
<td>IV</td>
<td>Severe decrease in GFR</td>
<td>15-29</td>
</tr>
<tr>
<td>V</td>
<td>Kidney failure</td>
<td>&lt;15 or dialysis</td>
</tr>
</tbody>
</table>
Sadly, there is no cure for CKD. The disease progresses until the patient’s kidneys can no longer function independently and the patient must receive dialysis or a transplant. As observed in Table 4-1, this constitutes Stage V of CKD. Upon receiving such assistance, the patient is diagnosed with End Stage Renal Disease (ESRD). In terms of dialysis, the two most common types are hemodialysis (HD) and peritoneal dialysis (PD). The first instance of continuous HD performed in the United States occurred in 1960 in Seattle, Washington. A Teflon tubing shunt was inserted into the patient’s arm vein and connected to Skeggs-Leonards dialyzers. These machines passed blood and dialysate, contained in a household freezer, in opposite directions across a membrane, allowing for the removal of excess waste from the blood via diffusion and are similar to present-day dialysis machines. This patient successfully received dialysis for 11 years before succumbing to a heart attack.

The first instance of PD in the United States also occurred around this time in San Francisco, California. However, survival rates were low and the process was not fully automated until 1976. At this time, continuous ambulatory peritoneal dialysis (CAPD) was developed in Austin, Texas. A catheter is inserted into the peritoneal cavity. Dialysate is introduced into the cavity and prompts diffusion, resulting in waste removal across the peritoneum membrane. The waste is extracted and dialysate replaced multiple times a day. CAPD allows for more freedom in that the patient is not attached to a machine for prolonged amounts of time; although, patients are at higher risks for infection.

Kidney transplantation is the closest option to a cure, but waiting lists can be long and risk of organ rejections high. However, survival rates of transplant patients are higher than those of dialysis patients. While both types of treatment incur yearly costs around $22 billion,
establishment of the Medicare ESRD Program in 1973 offers monetary assistance for ESRD patients receiving dialysis and/or a transplant.  

Although our work was merely application based and no biological conclusions were made, connections between oxidation and CKD have been elucidated previously. Oxidative stress plays a role not only in CKD, but also in an assortment of other associated conditions, such as aging, inflammation, diabetes, cardiovascular disease (CVD), atherosclerosis, etc. This complicates the assignment of biomarkers to particular conditions. Regardless, studies indicate an increase in oxidative stress in both CKD and ESRD patients and generally even higher oxidative stress when compound conditions are present.  

In terms of CKD, research shows an increase in protein carbonyl content and a decrease in protein thiol content in stages 3-5 CKD patients, signifying increased oxidation and decreased antioxidant defenses, respectively. In terms of ESRD, an increase in oxidative stress has been observed and postulated to be a result from various factors depending on the type of dialysis.  

This project was in collaboration with Dr. Donald Brophy (VCU School of Pharmacy/Department of Pharmacotherapy and Outcomes Science) and Erika Martin (VCU School of Pharmacy/Coagulation Advancement Lab). We received 50 plasma samples: 20 CKD patients on HD, 20 CKD patients on PD, and 10 healthy controls. This was the first study in which we were applying our proteomic methods to true biological samples and also our first attempts at working with blood samples. The overall goal of the study was to detect and relatively quantify oxidative damage to plasma proteins and develop an assay for monitoring oxidative damage during disease progression. Also, since the method development work in
Chapters 2 and 3 was occurring concomitantly with this application, the methods utilized for this study do not always reflect the optimized methods from these chapters.
4.3 EXPERIMENTAL DESIGN

4.3.1 Materials

Urea, 10xPBS, water (HPLC), methanol (HPLC), DMSO, DTT, TFA, Tween® 20, Brilliant blue, BSA (Fraction V), and Biotinylated BSA were purchased from Fisher Scientific (Pittsburgh, PA). HSA, (+)-Biotin hydrazide, NaBH$_3$CN, SDS, IAA, and CaCl$_2$ were purchased from Sigma-Aldrich (St. Louis, MO). TCA and GuHCl were obtained from Acros Organics (Fair Lawn, NJ). The BCA Protein Assay, Biotin Quantitation kits, EZ-Link Biotin-LC-Hydrazide, and EZ-Link Biotin-PEG$_4$-Hydrazide were purchased from Thermo Scientific (Rockford, IL). Oasis MCX extraction cartridges were purchased from Waters Corporation (Milford, MA). Sequencing grade modified trypsin was obtained from Promega (Madison, WI). Mini PROTEAN® TGX™ gels, 10x TGS Buffer, 10xTBS, 10x TG Buffer, Laemmli sample buffer, Mini Trans-Blot filter paper, Immun-Blot PVDF Membranes for Protein Blotting, and Blotting Grade Blocker were obtained from Bio-Rad (Hercules, CA). The OxyBlot Protein Oxidation Detection kit was purchased from Millipore (Billerica, MA). The Enhanced Chemiluminescence (ECL) Plus Western Blotting Detection System and PD midiTrap™ G-25 columns were obtained from GE Healthcare (Pittsburgh, PA). FTC, HCl salt was purchased from Marker Gene Technologies, Inc. (Eugene, OR).

4.3.2 Plasma Preparation

The Coagulation Advancement Lab provided the CKD plasma samples. Prior to our involvement in the study, blood was drawn from volunteers/patients via arm vein or dialysis access into 4.5 mL Vacutainer tubes containing 3.2% sodium citrate (Becton Dickinson, Franklin
Lakes, NJ). The blood was centrifuged for 10 min at 3500 rpm and plasma drawn off for analysis and long term storage.

4.3.3 Protein Labeling

The plasma was diluted in PBS at a 1:100 ratio. Biotin hydrazide (Label 1) was prepared in DMSO at 50 mM and added to a final concentration of 5 mM. The samples were incubated at room temperature for 2 h using an Eppendorf ThermoMixer. To reduce and stabilize hydrazone bonds formed during labeling, NaBH₃CN was prepared in PBS at 30 mM and added to a final concentration of 15 mM. The samples were incubated at room temperature for 1 h. To precipitate the proteins, 100% TCA was added at 20% of the total solution. Following a 15 min slushy ice incubation, protein pellets were washed three times with ice cold acetone. The supernatant was removed and the pellets resuspended in PBS at a concentration of 1-2 mg/mL.

4.3.4 Protein Digestion

Following labeling, 8 M urea was added to the protein solutions as a denaturant. To reduce disulfide bonds, DTT was added to a final concentration of 10 mM and samples were incubated on the ThermoMixer for 1 h at 37°C. To alkylate cysteines, IAA was added to a final concentration of 20 mM and samples were again incubated as described above. Following dilution with PBS, CaCl₂ was added to a final concentration of 1 mM and overnight trypsin digestion was performed at 37°C and 300 rpm on the ThermoMixer. Formic acid was added to halt digestion and the acidified peptide samples were concentrated with individual Oasis MCX columns using the protocol supplied by Waters Corporation. Three 1-mL fractions were eluted from each column and then all fractions were dried with a Speed Vac concentrator. After evaporation, dried peptides were dissolved in 95/5 0.1% aqueous TFA/ACN. The solution
concentrations were established using a BCA assay kit following the protocol provided by Thermo Scientific Pierce.

### 4.3.5 Mass Spectrometry

Peptide solutions were analyzed using LC-MS/MS on a Thermo (San Jose, CA) LTQ XL linear ion trap mass spectrometer with a nanospray ionization source. Utilizing a Thermo (San Jose, CA) Finnigan Micro ASII autosampler and Surveyor Plus HPLC System, peptides were separated on a C18 reverse-phase column (150 µm × 10 cm, 5 µm particles, 300 Å pores) from Column Technology (Fremont, CA). Triplicate injections, each 20 µL, were made for each sample. Gradient elution from the column was accomplished using two mobile phases: A (0.1% formic acid in water) and B (0.1% formic acid in methanol) at a flow rate between 0.5-1 µL/min. For each 160 minute run, the gradient began with 5% B, rose to 25% B within 15 min, 90% B within 115 min, and then decreased back to 5% B within 145 min. Peptides were ionized using a nanospray source at 1.8 kV. Precursor ions were obtained via a full scan in the range of 300-2000 m/z. A data-dependent scan procedure was used for the MS/MS analysis, in which ten resultant ions from the full scan were selected and subjected to further fragmentation using CID. Analysis was completed in the positive ion mode with active dynamic exclusion, in which a precursor ion was excluded from the analysis for 180 seconds if it was selected for further fragmentation twice within 30 seconds.

### 4.3.6 Database Searches and Analysis

The MS/MS spectra were analyzed using software distributed by Thermo, specifically Finnigan Xcalibur Bioworks 3.2 and the Sequest search engine used for peptide/protein identification. Searches were performed against an NCBI RefSeq database for Homo sapiens,
which was obtained on January 15, 2013 and contained 71,806 entries. Database sequences were also reversed to enable decoy searches and false discovery rate approximations. No more than two missed cleavage sites were allowed. A fixed modification was set for carboxamidomethylation (alkylated cysteines with mass shifts of +57.021464 Da) and differential modifications were set for: biotinylation at arginine (+199.066655), lysine (+241.088455), and proline (+257.107175) and oxidation at methionine (+15.994915), histidine (+15.994915), and cysteine (+15.994915, +31.98983, +47.984745). Trans-Proteomic Pipeline (TPP) (Version 4.6.3; Institute for Systems Biology in Seattle, Washington) was used to refine Sequest search results. In order to validate peptide-matched MS/MS spectra, PeptideProphet was used and only peptides with a minimum probability of 0.9 were accepted. Semi-quantitation was achieved using spectral counting (SC).

4.3.7 Western Blotting

Western blotting was performed according to the manual provided with the commercial OxyBlot Protein Oxidation Detection Kit. Plasma proteins were isolated via TCA precipitation and reconstituted in 5% SDS in PBS. Equal amounts of protein (4 µg) were labeled with 7.5 µL DNPH in 2M hydrochloric acid and incubated at room temperature for 15 min. Proteins were neutralized and reduced with 5 µL neutralization solution and 1 µL 5% 2-mercaptoethanol, respectively. The samples, along with an oxidized protein standard and protein standard ladder, were loaded onto a 4-20% gradient polyacrylamide SDS-PAGE gel and run in TGS buffer at 100 V and 90 mA for 1 h and 30 min. Proteins were then transferred to a PVDF membrane and run in TG buffer at 100 V and 300 mA for 1 h and 15 min. Following immunoblot, the membrane was blocked with 5% non-fat dry milk in 1X TBS-T for 1 h and then incubated with rabbit anti-DNP antibody at a 1:1200 dilution in 5% BSA and TBS-T overnight at 4°C. The membrane was
washed with TBS-T and incubated with goat anti-rabbit IgG antibody (HRP conjugated) at a 1:2400 dilution in 3% non-fat dry milk and TBS-T for 1 h at room temperature. The membrane was washed with TBS-T and then developed using the ECL Plus Western Blotting Detection System according to the manufacturer’s protocol.

**4.3.8 Biotin Quantitation Assay**

Plasma proteins were precipitated with TCA and reconstituted in PBS and 8 M urea. Each sample was incubated with an excess (5 mM) of one of three biotin hydrazide reagents: standard biotin hydrazide (Label 1), EZ-link hydrazide-LC-biotin (Label 2), or EZ-link hydrazide-PEG4-biotin (Label 3), and then reduced with NaBH$_3$CN. Excess materials were removed using PD midiTrap™ G-25 columns and proteins were eluted in PBS. The protein concentrations were determined using the BCA assay.

The extent of biotinylation was assessed following the protocol provided by Thermo Scientific Pierce. Biotinylated protein was added to a solution containing HABA-avidin complex. Biotin’s stronger affinity for avidin causes the displacement of HABA, invoking an absorbance change detectable with UV-visible spectroscopy. Using the extinction coefficient of 34,000 M$^{-1}$ cm$^{-1}$, the known protein concentrations, and the change in absorbance before and after biotinylated protein addition, the biotin to protein ratios were calculated and the extent of biotinylation determined. Commercially available biotinylated BSA was used as a standard; the manufacturer reports nine equivalents of biotin per BSA molecule.
4.3.9 FTC Assay

The following procedure was adapted from the methods of Mohanty et al. Standards were prepared according to the protocol in section 2.3.10.1. CKD samples were prepared accordingly. Plasma proteins were isolated via TCA precipitation and reconstituted in 6M GuHCl in MES. Protein concentrations were determined using the BCA Assay kit. Protein solutions were diluted to 10 µg/µL with MES and incubated with 304 µM FTC (10:1 FTC:HSA) overnight at room temperature with gentle rotation. Proteins were then isolated via TCA precipitation and reconstituted in 450 µL 3M GuHCl in PBS. Fluorescence measurements were made using a Varian Cary Eclipse Fluorescence Spectrophotometer. The instrument was zeroed with PBS and sample measurements were made one at a time with an excitation wavelength of 492 nm and an emission wavelength of 550 nm. The slit widths were 5 nm and measurements were made at medium scan speed. Fluorescence anisotropy measurements were made according to the procedure described in Section 2.3.10.3. Plasma diluted in PBS was used as a control.
4.4 RESULTS

4.4.1 Western Blotting

Western blotting is a commonly used technique to separate the components of a mixture for visualization; proteins are separated by molecular weight on SDS-PAGE gels, transferred to a membrane via immunoblot, and then often visualized using a combination of an HRP-conjugated antibody and a chemiluminescent reagent. Prior to Western blotting, DNPH was used to derivatize plasma protein carbonyls in various CKD patient samples. The general reaction was redrawn from Hensley and Floyd and is presented in Scheme 4-1.210 DNPH is the most commonly used reagent for oxidative modification labeling, detection, and quantification. The two most popular methods involving DNPH are spectrophotometric and Western blotting methods developed by Levine et al.211

Scheme 4-1. Reaction between a protein carbonyl and DNPH to form the derivatized product.

Not only is chemiluminescence a highly sensitive technique, but it can be achieved with a variety of substances. In our system, we used hydrogen peroxide (H$_2$O$_2$) as the oxidant, horseradish peroxidase (HRP) as the catalyst, and luminol as the light-emitting molecule. The general reaction was redrawn from Marquette & Blum and is presented below in Scheme 4-2.212
HRP, which has specificity for H$_2$O$_2$, will complex with the oxidant. This complex oxidizes luminol to form a dianion in the excited state, 3-aminophthalate. As this molecule decays from the excited state to the ground state, light is emitted, which is detected and visualized.$^{212,213}$

**Scheme 4-2.** Chemiluminescence reaction. *Catalyst/cooxidant* represents HRP/H$_2$O$_2$ in our experiments and hv denotes emitted light. The asterisk signifies an excited state.

Observable changes in Western blot band intensities were used as a measure of carbonylation. Hence, lighter bands indicated less carbonylation while darker bands indicated more carbonylation. Patterns in band intensities were observed for the various CKD samples. **Figures 4-1** and **4-2** demonstrate these patterns for two sets of patient samples. Each set of samples were prepared twice and analyzed on separate days, creating four total analyses. The first set of samples included sample numbers: 907 (control), 935 (HD), and 967 (PD). The second set of samples included sample numbers: 910 (control), 930 (HD), and 952 (PD).
Figure 4-1. SAMPLE SET #1: Duplicate Western blot analyses of patient samples 907, 935, and 967. The lane numbers correspond to: 2=907 no DNPH, 3=907 + DNPH, 4=Empty lane, 5=935 no DNPH, 6=935 + DNPH, 7=Empty lane, 8=967 no DNPH, 9=967 + DNPH, 10=Oxidized protein standard with corresponding molecular weights in red.
Figure 4-2. SAMPLE SET #2: Duplicate Western blot analyses of patient samples 910, 930, and 952. The lane numbers correspond to: 2=910 no DNPH, 3=910 + DNPH, 4=Empty lane, 5=930 no DNPH, 6=930 + DNPH, 7=Empty lane, 8=952 no DNPH, 9=952 + DNPH, 10=Oxidized protein standard with corresponding molecular weights in red.
While bands for the PD patient sample proteins were consistently darker than the other bands indicating increased carbonylation in these samples, the bands for the HD patient samples were not as consistent, although did appear darker than the control bands in most instances. Background-level intensities for the control bands were expected due to inherent oxidation present in human blood. The method provides a qualitative measure of carbonylation, but lacks specificity and is not amenable to screening large sample sets.

### 4.4.2 Biotin Quantitation Assay

The following biotin to protein ratios were determined for two subsets of patient samples and can be seen in Table 4-2. Carbonyls were derivatized with one of three biotin hydrazide reagents: standard biotin hydrazide (Label 1), EZ-link hydrazide-LC-biotin (Label 2), or EZ-link hydrazide-PEG4-biotin (Label 3). Biotin:protein ratios separated by commas indicate repeat experiments were performed.

**Table 4-2.** Calculated biotin to protein ratios for two subsets of CKD patient samples.

<table>
<thead>
<tr>
<th>Subset #</th>
<th>Patient #</th>
<th>Label 1</th>
<th>Label 2</th>
<th>Label 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control 905</td>
<td>0.94, 0.20, 0.24</td>
<td>0.29</td>
<td>5.70</td>
</tr>
<tr>
<td></td>
<td>HD 928</td>
<td>0.57, 0.46, 0.22</td>
<td>-</td>
<td>4.04</td>
</tr>
<tr>
<td></td>
<td>PD 954</td>
<td>1.11, 0.71, 0.28</td>
<td>-</td>
<td>3.45</td>
</tr>
<tr>
<td>2</td>
<td>Control 909</td>
<td>&lt;0</td>
<td>0.27</td>
<td>9.28, 7.15</td>
</tr>
<tr>
<td></td>
<td>HD 922</td>
<td>&lt;0</td>
<td>0.33</td>
<td>5.57, 4.13</td>
</tr>
<tr>
<td></td>
<td>PD 951</td>
<td>&lt;0</td>
<td>0.16</td>
<td>11.10, 11.86</td>
</tr>
</tbody>
</table>
For patient sample numbers 905, 928, and 954 in Subset #1, the biotin:protein ratios calculated when using Label 1 varied greatly across three interday analyses. The %RSD values for patient sample numbers 905, 928, and 954 were 90.5%, 43.0%, and 59.3%, respectively. However, the assay effectively relies on measuring the change in absorbance of avidin-bound HABA and there are high uncertainties when only a small amount is displaced by biotin. The majority of the measurements for Labels 1 and 2-derivatized samples from both Subsets #1 and #2 were at or close to 0 indicating very limited derivatization by these tagging reagents. Overall, ratios for Label 3-derivatized samples were much higher than those for Labels 1 and 2-derivatized samples. This is a result we would expect since Label 3 offers the highest labeling efficiency when compared to Labels 1 and 2 when using the biotin quantitation assay as a measurement tool (Figure 2-2).

### 4.4.3 FTC Assay

As described in Chapter 2, the FTC assay was validated using carbonylated HSA spiked into regular HSA and plasma, but was not applied to biological samples. Upon receiving the CKD samples, we took this as our first opportunity to test our assay. Two sets of patient samples were analyzed with the assay and results are presented below. The first set, referred to as CKD Sample Set 1, included: Control 909, HD 922, and PD 951. The second set, referred to as CKD Sample Set 2, included: Control 906, HD 938, and PD 956. Carbonylated proteins in all six plasma samples were labeled with FTC and analyzed with fluorescence spectroscopy. To assess interday precision, CKD Sample Set 1 was prepared three times, each on a different day. The emission spectra from each of these three analyses are depicted in Figure 4-3. Because CKD Sample Set 1 produced variable results across the days, CKD Sample Set 2 was prepared to test preparation and instrument reproducibility. These results are shown in Figure 4-4.
Figure 4-3. CKD Sample Set 1: emission spectra from three interday FTC assay analyses of patient samples 909, 922, and 951.
Figure 4-4. CKD Sample Set 2 emission spectra from: A. First sample preparation on Day 1. B. Second sample preparation on Day 2. C. First sample preparation solutions reanalyzed on Day 1. D. Second sample preparation solutions reanalyzed on Day 2.
To ensure that our fluorescent label was indeed reacting with carbonyl sites on the protein and that there was no label detachment occurring between the protein reconstitution and fluorescence measurements, we investigated binding between protein and FTC label by measuring anisotropy. Triplicate anisotropy measurements were made for each of the patient samples in CKD Sample Set 1: Control 909, HD 922, and PD 951. Results are depicted below in Figure 4-5. R values are averages of nine measurements with error bars representing the standard deviations. Free FTC (100 µM) exhibited an average r value of 0.026. Patient sample r values were much higher than that of free FTC, suggesting that the FTC label was indeed bound to the protein.

![Figure 4-5](image)

**Figure 4-5.** Average anisotropy (r) values and standard deviations for triplicate analyses of patient samples control 909, HD 922, and PD 951. Free FTC represents 100 µM FTC in PBS.
4.4.4 Mass Spectrometry

Of the 50 patient samples, five have been analyzed with our protocol: Sample numbers 907 (healthy control), 924 and 935 (HD patients), and 962 and 967 (PD patients). It was not possible to definitively identify any peptides in these samples that carried an expected modification along with the biotin labeling group. No enrichment was used in these studies and past experience in the research group suggests that it was unlikely to find carbonylation modifications under these circumstances. As an alternative approach that would not require labeling, methionine and histidine oxidation levels were characterized for various proteins. These could also offer insight as to the level of oxidative stress in the patient. Figure 4-6 illustrates the percent oxidation values calculated using spectral counts for non-labeled samples (no biotin hydrazide). This was done to minimize sample preparation time and to potentially reduce adventitious oxidation. These samples were also analyzed a second time with LC-MS/MS, hence the error bars representing the deviations. The second LC-MS/MS analysis served to assess instrument reproducibility. Varying oxidation levels were detected amongst the five patient samples; however, due to the fact that methionine oxidation can be induced during sample preparation and/or reversed by certain enzymes, these experiments present some risks. The CKD project was terminated so additional samples were not processed by this approach.
Figure 4-6. Average percentage of methionine/histidine oxidation for three LC-MS/MS analyses of sample numbers 907, 924, 935, 962, and 967. Percents were determined by: \[
\frac{\# \text{oxidized spectra}}{\# \text{total spectra}} \times 100.
\]
4.5 DISCUSSION

4.5.1 Analytical Assays

Going into this project, we had no idea as to how much carbonylation would be detected in the control and patient samples aside from what we had gleaned from the literature. Based on correlations between final stage CKD patients and increased carbonylation in the literature, we expected to encounter this correlation in our research. Pertaining to distinguishing carbonylation patterns between HD and PD patients, we were unsure what to expect being that oxidation had been observed and reported in both HD and PD patient samples.\textsuperscript{5,8,84,86,207}

Initial attempts at distinguishing carbonylation patterns proved successful in that we were able to reproducibly visualize what appeared to be increased carbonylation in PD patients using Western blotting. However, labeling of the carbonylation sites with DNPH using the OxyBlot kit was often interrupted by precipitation observed during incubation of the plasma with the acidified DNPH solution. No precipitation was encountered in experiments presented in the Section 4.4.1, but it continued to be a reoccurring problem throughout derivatization procedures. Before other options could be explored, such as labeling with biotin hydrazide and detecting with streptavidin Poly-HRP, it was decided that Western blotting was too costly and time consuming for analyzing 50 or more patient samples with a minimum of three replicates each.

As discovered in Chapter 2, considerable uncertainties accompany the biotin quantitation assay (\textbf{Figure 2-2}). Inherent uncertainty resides in the assay because measurements are made against a non-zero background. Bound HABA absorbs around 500 nm and as biotinylated material replaces the HABA, a decrease in absorbance is observed. Thus, resulting biotin to protein ratios are based on a change in absorbance and small changes will always offer large
standard deviations, even with numerous experimental repeats. Since we didn’t know what to expect for the CKD patient samples in terms of biotin to protein ratios, we can only conclude by stating that Label 3 functioned as the most efficient affinity label. Label 3 showed higher preference for carbonylated material in the CKD plasma samples, similar to experiments in Chapter 2 in which Label 3 showed a higher preference for carbonylated HSA. High biotin to protein ratios could also have resulted from reaction of Label 3 with a variety of carbonylated species, which would require mass spectrometry for full elucidation. We did not proceed with these experiments since we were focused on establishing a quick assay which would give us relative quantitation values in a clinical setting.

We had hoped that since the FTC assay was validated with carbonylated HSA that we would be able to use the assay to establish general carbonylation patterns, but large variations in fluorescence intensities across interday experimental and instrumental analyses prevented us from doing so. We also questioned the binding stability of the label. Anisotropy experiments allowed us to confirm binding between FTC label and protein. As shown in Figure 2-4, FTC label bound to protein causes a decrease in rotation of the fluorophore and, upon interaction with polarized light, does not disrupt the polarization. Free FTC label would rotate more freely and would disrupt the polarized light, leading to emission in the form of depolarized light. In accordance with the literature,160 we expected high anisotropy values for non-labeled plasma, medium anisotropy values for FTC labeled-proteins, and low anisotropy values for free FTC label, and we achieved just that. Non-labeled plasma exhibited an anisotropy around 0.4, CKD patient samples exhibited an anisotropy around 0.9-1.2, and free FTC label exhibited an anisotropy around 0.026. Due to the mid-range values obtained for the patient samples, we were able to infer that the majority of the FTC label was indeed bound to plasma protein. Nonetheless,
the high variability in the FTC measurements in plasma made it an unsuitable assay for the samples.

The sensitivities of the various assays also most likely played a role in the experimental results. UV-Visible spectroscopy is the fastest technique, but is known for offering less sensitivity than other analytical techniques. Thus, it is mainly used for turbidity, concentration, and enzymatic activity measurements. One of the oldest techniques in analytical chemistry dating back to the 1850s, fluorescence spectroscopy, offers greater sensitivity than UV-Visible spectroscopy.\textsuperscript{214} According to the manufacturer, the Varian Cary Eclipse Fluorometer offers detection limits of the fluorophore in the picomolar range. In terms of electrophoresis and Western blotting, SDS-PAGE techniques combined with coomassie blue staining as detection offer visualization of proteins at the low nanogram scale.\textsuperscript{215} In our experiments, the use of the OxyBlot Protein Oxidation Detection Kit and ECL reagents also contributed to the sensitivity and selectivity of the experiments, hence the varying band intensities and the minimal backgrounds, respectively. According to the manufacturer, the OxyBlot kit offers a limit of detection of five femtomoles and ECL techniques are known to be 10-100 times more sensitive than other luminescence detection methods.\textsuperscript{216} Although UV-Visible and fluorescence spectroscopy are not destructive to samples as in Western blotting, there are potential solution interferences in the spectroscopic techniques (in Western blotting, excess material is separated out). The lower sensitivity and lack of matrix effects was most likely the cause for success with Western blotting. Unfortunately, analyzing 150 samples (a minimum of three repeats per sample) with Western blotting would not be time efficient or cheap.

Pertaining to mass spectrometry experiments, no biotinylated peptides were identified during these analyses, most likely because our protocol did not include an enrichment step.
Previous experiments in our lab have shown that it is more difficult to detect biotinylated material without enriching first. Regardless, varying levels of methionine and histidine oxidation were detected, but the reversibility of methionine oxidation was a concern. As can be seen in Scheme 4-3, which was recreated from Schöneich’s work, methionine is involved in multiple redox processes. Methionine oxidation can be irreversible depending on the mechanism following radical formation, but is most often in a reversible state in which enzymes like MsrA and MsrB can reverse the oxidation. Both MsrA and MsrB proteins are expressed in the liver, which serves as the main source for blood proteins. MsrA and MsrB activities have been detected in neutrophils, a key component in plasma, which means these enzymes could very well have played a role in methionine sulfoxide detoxification in these experiments. Another factor in reversibility is the methionine location in the protein; buried methionines would not be as accessible as those on the protein surface. No form of cysteine oxidation (sulfenic, sulfinic, or sulfonic acid) was identified, thus reversibility of cysteine oxidation was not considered.
4.5.2 Other Factors to Consider

Protein carbonyls are chemically stable species, especially once reacted with an affinity label such as biotin hydrazide. Without a label though, there is a limit to their survival upon storage, even at -80 degrees Celsius. While one study claims that protein carbonyls are stable for three months upon storage at -80 degrees Celsius, another study claims that non-labeled protein carbonyls can degrade over time due to reaction with lysines on other proteins to form Schiff bases. Thus, carbonyl sites must be derivatized immediately. Ideally, we strived to derivatize carbonyls as soon as possible; however, being that we were not present for the CKD blood sample collection, we do not have full record of the samples’ history prior to receiving them. Our collaborators received the blood, isolated the plasma, analyzed the samples, and then froze them while also documenting any samples that were thawed and refrozen. The time from which the blood was drawn to the time when the samples were in our collaborators’ possession is the gray area – it was unknown how long the samples were in transit and how the samples were handled.

This study ultimately confirmed that the application of proteomics-based analytical methods to biological samples has numerous challenges, as discussed in Chapter 2. These were the initial studies in the lab with blood samples and a whole array of new challenges arose in regards to biological sample preparation. Limitations included: initial sample handling, sample availability, protein solubility, instrumental sensitivity, reproducibility, etc. Time was another factor; experiments took anywhere from 2 days for simpler assays to 7-9 days for mass spectrometry-based analyses. Finally, the CKD study ended before we were able to work with fresh samples and employ more orthogonal techniques to establish the oxidation levels in the
samples. However, this work was a useful stepping stone in terms of analytical method development relating to blood samples.
4.6 CONCLUSION

In conclusion, we applied our analytical methods to plasma samples for the first time in this study. Disappointingly, no oxidation patterns were apparent in the samples that we had from the CKD studies. While the Western blotting results consistently suggested higher oxidation levels in the PD patient samples, other means of analysis failed to confirm this. Causes for this outcome may include but are not limited to the fact that: methionine oxidation is reversible, oxidation can be induced during sample preparation, and/or the CKD samples were over two years old. Despite this outcome, this project marked the beginning of blood analyses in which existing protocols were adapted to the plasma samples. Although this project was eventually terminated, it proved our methods were adaptable to blood samples and could potentially provide useful data with a fresh set of biological samples. For a new set of patient samples, our top priorities would be incorporating an enrichment step into the biotinylation protocol and acquiring quantitative mass spectrometry data through the use of stable isotope labeling.
Chapter 5 - APPLICATION: INVESTIGATION OF PROTEIN CARBONYLATION IN HUMAN PLASMA/SERUM COLLECTED FROM TRAUMA PATIENTS
5.1 ABSTRACT

Trauma-induced oxidative changes and patterns in blood proteins have been investigated over the years. Most notably are patterns that have been elucidated amongst the acute phase proteins, proteins which are key responders to inflammation. In this study, we investigated protein carbonylation in plasma/serum collected from trauma patients over the course of many days using our own methods we had optimized. The overall goal was to apply these existing analytical methods in order to characterize oxidation levels in patient blood samples, similar to our aim in the CKD study. Carbonylated proteins were labeled with biotin hydrazide and a portion analyzed via Western blotting. The remaining sample was affinity enriched, digested, and then quantitated via iTRAQ labeling and mass spectrometry. Due to the abundance of certain proteins in blood, such as human serum albumin, techniques are necessary to decrease the concentration of said proteins in order to observe the potential changes in low abundance proteins. Not only were we able to increase the number of modified protein identifications using the ProteoMiner depletion techniques, but we were also able to visualize and quantitate temporal changes in particular oxidized proteins. The study demonstrated that our methods were applicable to this sample set and that the oxidation profiles of a large number of proteins could be simultaneously tracked via quantitative mass spectrometry.
5.2 INTRODUCTION

In the United States, trauma is the leading cause of death for Americans aged 1 to 44 years old and the third ranked cause of death for all ages. Every year, emergency room visits total about 41 million, with about 2 million of those resulting in hospital admissions.\cite{219} This leads to trauma-related costs totaling roughly $406 billion.\cite{220} Each year, about 180,000 deaths result from trauma-related injuries, which amounts to about 30% of all deaths in the United States followed by cancer at 16% and heart disease at 12%.\cite{219} Severe trauma or infection often result in a condition known as sepsis, which is one of the leading causes of death in intensive care units. In the United States, about 700,000 sepsis cases are treated each year.\cite{79}

Trauma and sepsis patients experience moderate to severe inflammation, which has been correlated with oxidative stress in a tremendous number of literature reports throughout the years. Cytokines, released in response to inflammation, produce ROS, along with the leukocytes that consume oxygen as they travel toward the inflammation site.\cite{221} Sepsis and subsequent organ failure have been linked to mitochondrial damage, in which the release of superoxide, nitric oxide, and cytokines/leukocytes can hinder mitochondrial function.\cite{77} A decrease in antioxidant levels has also been observed in the plasma of trauma patients\cite{80} and in damaged mitochondrial systems,\cite{77} which further complicates the body’s ability to combat oxidative stress. With such imbalances between ROS production and antioxidant protection, oxidative stress would undeniably be present in such systems.

Since the mid-1950s, research has been published linking various inflammation-related proteins to trauma and injury.\cite{222} These changes are most often observed by examining blood protein concentrations. The fact that large amounts of blood serum and plasma can be easily and
rapidly collected, each of which contain equally large amounts of protein (on the mg scale), makes blood the most commonly biological fluid used in protein analyses. Blood proteins are linked to various tissues throughout the human body and thus, provide real time information about a person’s health and/or disease state. The blood proteins of most interest in trauma patients are called acute phase proteins (APPs). There are two types: positive APPs experience concentration increases and negative APPs experience concentration decreases, both by a minimum of 25% during trauma-related events. Changes in these proteins have been observed not only during trauma, but also during conditions such as: infection, burns, surgery, inflammation, and cancer. These proteins would be of most interest to us in terms of investigating carbonylated protein levels in trauma patients. Inflammation, regardless of the source, triggers activation of cytokines, most notably the interleukins, which ultimately induce hepatic production or reduction of the acute phase proteins. This process is called the acute phase response (APR). An overview of the process is illustrated below in Figure 5-1. Examples of these particular proteins include, but are not limited to: C-reactive protein, fibrinogen, α1-antitrypsin, haptoglobin, serum amyloid A, and ceruloplasmin (positive APPs) along with transthyretin, albumin, transferrin, and retinol-binding protein (negative APPs). For instance, C-reactive protein functions to identify foreign cells, activate the complement system, prohibit superoxide generation from neutrophils, etc. These are pro-inflammatory effects and justify the protein’s increase in concentration, just as the negative APPs are decreased due to their anti-inflammatory effects or simple lack of inflammation-related functions.
Many studies have been published in which blood protein carbonylation has been investigated in trauma patient samples. Early studies, such as those performed by Winterbourn et al., focused on quantifying total protein carbonyl content via ELISA. For trauma and sepsis patients, initial protein carbonyl concentrations were ten times those of carbonyl concentrations in normal patients and levels remained elevated in injury patients ten days following the traumatic event/diagnosis of sepsis. These results were corroborated by similar experiments and outcomes achieved by Abu-Zidan et al. More recently, researchers have examined blood protein levels in patients with burn injuries, which are classified as one of the severest types of trauma. These studies incorporated immunoaffinity chromatography and quantitative mass spectrometric proteomics techniques and resulted in confirmation of changes in important APP
concentrations along with possible identification of proteins specific to burn injury.\textsuperscript{76,78} Such studies show promise for the application of our analytical techniques to trauma patient samples.

This project was in collaboration with Dr. Dayanjan Shanaka Wijesinghe (VCU Medical Center/Department of Biochemistry & Molecular Biology) and Dr. Suresh Narayanasamy, a former graduate student in Dr. Gronert’s Research Group (VCU Department of Chemistry). Over the course of five months, one set of trauma patient samples and one set of elective surgery patients were received through Dr. Wijesinghe. Each set contained 4-10 blood samples per patient. Dual studies of the samples were completed: lipidomics by Dr. Wijesinghe and proteomics on our end. The first set of samples was subjected to Western blot analysis, affinity-enrichment techniques, iTRAQ labeling, and subsequent mass spectrometry analysis. The second set of samples was also subjected to the same techniques as the first set, but only after low-abundance protein enrichment in order to equalize protein concentrations. Dr. Narayanasamy and I worked together on labeling blood proteins, but then I focused on Western blotting and he on iTRAQ labeling/mass spectrometry. The main goal of this project was to apply our analytical methods to plasma/serum samples in order to characterize oxidation in these patient blood samples. A broader, discovery-based approach was taken being that we were not targeting specific proteins and ultimately testing our recently developed analytical methods. To the best of our knowledge, we had not seen a method such as ours published in the literature. Although quantitative mass spectrometry had been applied to trauma patients previously,\textsuperscript{76} we were unaware of an applicable method combining biotin/avidin affinity enrichment along with iTRAQ-based mass spectrometry for these types of samples. Finally, since the method development work in Chapters 2 and 3 was occurring concomitantly with this application, the methods utilized for this study were not those optimized in that method development work.
5.3 EXPERIMENTAL DESIGN

5.3.1 Materials

Urea, 10xPBS, water (HPLC), methanol (HPLC), DMSO, DTT, TFA, Tween® 20, Brilliant blue, BSA (Fraction V), and Biotinylated BSA were purchased from Fisher Scientific (Pittsburgh, PA). (+)-Biotin hydrazide, NaBH₃CN, SDS, IAA, and CaCl₂ were purchased from Sigma-Aldrich (St. Louis, MO). TCA was supplied by Acros Organics (Fair Lawn, NJ). Ammonium bicarbonate was obtained from J.T. Baker (Phillipsburg, NJ). The BCA Protein Assay, Streptavidin Poly-HRP, and Streptavidin UltraLink Resin were purchased from Thermo Scientific (Rockford, IL). Sep-Pak C18 cartridges were purchased from Waters Corporation (Milford, MA). Sequencing grade modified trypsin and TMB stabilized substrate were obtained from Promega (Madison, WI). The iTRAQ reagent kits were obtained from AB SCIEX (Framingham, MA). ProteoMiner low-abundance protein enrichment kits, Mini PROTEAN® TGX™ gels, 10x TGS Buffer, 10xTBS, 10x TG Buffer, Laemmli sample buffer, Mini Trans-Blot filter paper, Immun-Blot PVDF Membranes for Protein Blotting, and Blotting Grade Blocker were obtained from Bio-Rad (Hercules, CA). FTC, HCl salt was purchased from Marker Gene Technologies, Inc. (Eugene, OR).

5.3.2 Preparation of Plasma/Serum

The plasma and serum samples were provided by Dr. Martin Mangino (VCU Medical Center/Department of Surgery). We received four samples: trauma Patients W and X in Sample Set 1 and elective surgery Patients Y and Z in Sample Set 2. Following the traumatic event, blood was collected in 4.5 mL Vacutainer tubes containing 3.2% sodium citrate (Becton
Dickinson, Franklin Lakes, NJ) from Patients W and X. For Patient W, blood was collected for eight consecutive days (Day 1 – Day 8). For Patient X, blood was collected for six consecutive days (Day 0 – Day 5). The tubes were mixed by inversion and then incubated for 30 min at room temperature in order to allow the blood to clot. Samples were centrifuged at 1,000-2,000 x g for 10 min at 4°C. Serum was drawn off and stored in polypropylene tubes at -80°C. Following elective surgery, blood was collected in 4.5 mL Vacutainer tubes containing 3.2% sodium citrate (Becton Dickinson, Franklin Lakes, NJ) from Patients Y and Z. For Patient Y, blood was collected for seven consecutive days (Day 1 – Day 7). For Patient Z, blood was collected for four consecutive days (Day 1 – Day 4) and then on Day 7 and Day 11. The blood was not allowed time to clot in these cases and samples were immediately centrifuged at 1,000-2,000 x g for 10 min at 4°C. Plasma was drawn off and stored in polypropylene tubes at -80°C.

5.3.3 Protein Biotinylation

Within a week of sample collection, the serum and plasma proteins were biotinylated. Serum and plasma samples were thawed on ice for 30 min and then diluted in PBS at a 1:4 ratio. Initial protein amounts in each diluted sample ranged from approximately 3-4 mg. Biotin hydrazide (Label 1) was prepared in DMSO at 50 mM and added to a final concentration of 5 mM. The samples were incubated at room temperature for 1 h using an Eppendorf ThermoMixer. SDS was added at a final concentration of 1% followed by a second addition of biotin hydrazide and sample incubation continued for 1 h. To reduce and stabilize hydrazone bonds formed during labeling, NaBH₃CN was prepared in PBS at 30 mM and added to a final concentration of 15 mM. The samples were incubated at room temperature for 1 h. To precipitate the proteins, 100% TCA was added at 20% of the total solution. Following a 30-minute slushy ice incubation, protein pellets were washed three times with ice cold acetone. The supernatant was removed and
the pellets resuspended in PBS and 2.5% SDS at protein concentrations ranging from 2-6 mg/mL, as determined by the BCA Assay.

5.3.4 ProteoMiner Depletion

Following biotinylation and reduction for patient samples Y and Z, the samples were divided into two aliquots: one aliquot was subjected to Western blotting and the second was subjected to high abundance protein depletion using the ProteoMiner ligand-based affinity columns. Plasma samples were prepared according to the manufacturers’ protocol. The spin columns were washed with PBS and then incubated with 1 mg plasma proteins with gentle rotation, at room temperature for 2 h. The columns were washed three times with PBS in order to remove nonbound material. Bound proteins were eluted with three-20 μL aliquots of elution reagent (8M Urea, 2% CHAPS) making for a total of 60 μL per sample. Sample concentrations were determined using the BCA Assay.

5.3.5 Western Blotting

Directly following biotinylation and reduction, an aliquot of the samples were analyzed using Western blotting (these samples were not affinity-depleted). Equal amounts of biotinylated protein (10 μg) were incubated with 50% Laemmlli buffer and 5% 2-mercaptoethanol for 5 min at 95°C. The samples, along with a protein standard ladder, were loaded onto a 4-20% gradient polyacrylamide SDS-PAGE gel and run in TGS buffer at 100 V and 90 mA for 1 h and 30 min. Proteins were then transferred to a PVDF membrane and run in TG buffer at 100 V and 300 mA for 1 h and 15 min. Following immunoblot, the membrane was blocked with 5% non-fat dry milk in 1X TBS-T for 1 h and then incubated with Streptavidin Poly-HRP at a 1:5000 dilution in 5% BSA and TBS-T overnight at 4°C. The membrane was washed with TBS-T and incubated with
TMB substrate for 5 min for visualization of protein bands. For patient samples Y and Z, Western Blotting was performed prior to ProteoMiner depletion.

### 5.3.6 Affinity Enrichment and Tryptic Digestion

Streptavidin UltraLink resin (200 μL/sample) was washed three times with PBS. Biotinylated/reduced serum and ProteoMiner depleted plasma samples were added to the resin (200 μg/sample) and incubated with gentle rotation, at room temperature overnight. The resin was first washed three times with PBS in order to remove nonbound protein and then washed twice with 8 M urea and 10 mM DTT in PBS. Proteins were then incubated with 8 M urea and 10 mM in DTT in PBS for 1 h at 500 rpm, 37°C in order to promote denaturation and reduction. To alkylate reduced cysteines, IAA was added to 55 mM final concentration and incubation continued for 1 h at 500 rpm, 37°C, in the dark. Following was a second addition of DTT in order to saturate unreacted IAA during a 1 h, 500 rpm, 37°C incubation. The resin was washed four times with 50 mM aqueous ammonium bicarbonate in order to remove reagents. Digestion commenced through the addition of calcium chloride to a final concentration of 1 mM and trypsin at a 1:50 trypsin:protein ratio and continued for 16 h at 500 rpm, 37°C. The beads were washed four times with 95/5 0.1% TFA/ACN and washes were pooled together in order to retain as many peptides as possible. The peptide samples were desalted by passing samples through C₁₈, reversed-phase SPE cartridges and dried using Speed Vac concentration.

### 5.3.7 iTRAQ Labeling

Peptides were dissolved in 20 μL iTRAQ dissolution buffer (0.5 M triethylammonium bicarbonate, pH 8.5). iTRAQ 8-plex reagents were used to label peptides. The labels were
dissolved in 80 μL of isopropyl alcohol, added to peptide solutions, and incubated at 300 rpm, room temperature, for two h. All respective samples were combined for each patient and dried using Speed Vac concentration. Each peptide sample was resuspended in 20 μL of 0.1% aqueous formic acid.

5.3.8 Mass Spectrometry

Peptide solutions were analyzed using LC-MS/MS on a Thermo (Waltham, MA) LTQ Orbitrap Velos mass spectrometer with a Waters nanoACQUITY UPLC system. Total sample injection volumes were 2 μL. Loading times were minimized using a nanoACQUITY trapping column. Peptides were separated on a C-18, reverse-phase nanoACQUITY column (100 μm × 100 mm, 1.7 μm particles). Gradient elution from the column was accomplished using two mobile phases: A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) at a flow rate set to 0.4 μL/min. For each 120 minute run, the gradient began with 1% B, rose to 15% B within 25 min, 25% B within 35 min, 35% B within 40 min, and ended with 85% B within 20 min. Peptides were ionized using a nanospray source at 3.5 kV. Precursor ions were obtained via a full scan in the range of 400-2000 m/z at 60,000 resolution. A ten-event data-dependent scan procedure was used for the MS/MS analysis, in which higher energy C-trap dissociation (HCD) was used to fragment iTRAQ-labeled ions at a collision energy of 40 and activation time of 0.1 ms. Analysis was completed in the positive ion mode with active dynamic exclusion, in which a precursor ion was excluded from the analysis for 180 seconds if it was selected for further fragmentation twice within 30 seconds.
5.3.9 Database Searches and Analysis

Peptide identifications were made using an X! Tandem variation in the Trans-Proteomic Pipeline (TPP) software (Version 4.6.3; Institute for Systems Biology in Seattle, Washington). Searches were performed against an NCBI RefSeq database for Homo sapiens, which was obtained on January 15, 2013 and contained 71,806 entries. Database sequences were also reversed to enable decoy searches and false discovery rate approximations. No more than one missed cleavage site was allowed. Fixed modifications were set for carbamidomethylation (alkylated cysteines with mass shifts of +57.021464 Da) and 8-plex iTRAQ label additions on lysine and peptide N-terminus (+304.205360 Da). A differential modification was set for methionine sulfoxide (+15.994915 Da). In order to validate peptide-matched MS/MS spectra suggested by X! Tandem, PeptideProphet was used and only peptides with a minimum peptide-spectrum matches (PSMs) score of 0.9 were accepted. The false positive rates (FPs) were approximated by calculating: 2 x accepted decoy identifications. Quantitative mass spectrometry data was prepared in the following manner to make it presentable via histogram plots. The data was corrected via trypsin ratios and normalized to the first day for each sample (i.e. 0 or 1). Data was then averaged and displayed on histograms, with standard deviations representing errors between the three injections for each sample. For each patient sample, the ten proteins that exhibited the most distinct changes in carbonylation across the collection days were reported.

5.3.10 FTC Assay

The following procedure was adapted from the methods of Mohanty et al. Standards were prepared according to the protocol in section 2.3.10.1. Trauma patient serum, which was
stored at -80°C, was thawed and diluted in PBS. Protein concentration was determined using the BCA Assay according to the manufacturers’ protocol (10-13 mg/mL). The serum was further diluted to create 10 mg/mL solutions and incubated with 304 µM FTC (10:1 FTC:HSA) overnight at room temperature with gentle rotation. Proteins were then isolated via TCA precipitation and reconstituted in 450 µL 3M GuHCl in PBS. Fluorescence measurements were made using a Varian Cary Eclipse Fluorescence Spectrophotometer. The instrument was zeroed with PBS and sample measurements were made one at a time with an excitation wavelength of 492 nm and an emission wavelength of 550 nm. The slit widths were 5 nm and measurements were made at medium scan speed.
5.4 RESULTS

5.4.1 Western Blotting: Patients W, X, Y, and Z

For these experiments, we again took advantage of the strong noncovalent interaction between biotin and avidin.\cite{156} Being that our serum and plasma proteins were labeled with biotin hydrazide, streptavidin poly-HRP was used to detect these modified proteins and TMB substrate was used for visualization. For trauma patients W and X, we analyzed 8 and 6 serum samples, respectively via Western blotting. This provided us with a visual estimation of protein oxidation in the patients over the course of 8 or 6 consecutive days following the traumatic event and can be seen below in Figure 5-2. Most notable in these two analyses would be Lanes 2 and 5 for Patient W and Lane 0 for Patient X, which show many intense bands across the entire lane. It is unclear why these samples behaved differently than the others and this may have been an artifact of the sample collection procedure. For patients Y and Z, we analyzed 7 and 6 plasma samples, respectively via Western blotting. Results are below in Figure 5-3. Patient Y samples were collected over 7 consecutive days, but Patient Z samples were only collected over 4 consecutive days with the last two samples being collected a couple days apart from one another. Some variations in band intensities across the time points can be seen in these analyses, but they weren’t as pronounced as those in Figure 5-2. For all four patients, samples were analyzed twice via Western Blotting, with the two blots correlating for each patient (only one blot/patient is pictured below in Figures 5-2 and 5-3). This confirmed Western blot technique reproducibility. A second set of Patient X samples were labeled and analyzed via Western Blotting. These results also correlated with those from the initial labeling experiment, confirming reproducibility of biotinylation.
Figure 5-2. Western blot analyses of trauma patient samples W and X. Std. represents a protein standard ladder with corresponding molecular weights in red. The lane numbers represent the days following the traumatic injury.
**Patient Y:**

![Western blot analysis of trauma patient samples Y and Z. Std. represents a protein standard ladder with corresponding molecular weights in red. The lane numbers represent the days following the traumatic injury.](image)

**Patient Z:**

![Western blot analysis of trauma patient samples Y and Z. Std. represents a protein standard ladder with corresponding molecular weights in red. The lane numbers represent the days following the traumatic injury.](image)

**Figure 5-3.** Western blot analyses of trauma patient samples Y and Z. Std. represents a protein standard ladder with corresponding molecular weights in red. The lane numbers represent the days following the traumatic injury.
5.4.2 FTC Assay: Patient X

As described in Chapter 2, the FTC assay was validated using carbonylated HSA spiked into regular HSA and plasma. The assay was applied to CKD patient samples in Chapter 4, but results varied. This left us unsure of the assay’s capabilities. Upon receiving the trauma samples, we took this as second opportunity to test our assay. Patients W and X showed the most interesting oxidation trends based on Western blotting results, but due to limited serum for Patient W, we could only apply replicates of our FTC assay to serum from Patient X. Carbonylated proteins in all six serum samples for Patient X were labeled with FTC and analyzed with fluorescence spectroscopy three times, each on a different day. The emission spectra from each of these three analyses are depicted in Figure 5-4. The patient samples from Day 0 demonstrated the highest carbonylation levels in all three analyses, albeit varying fluorescence intensities across the three analysis days. The relative values for other collection days showed greater variability.
Figure 5-4. Emission spectra from triplicate FTC assay analyses of Patient X.
5.4 iTRAQ Quantitation: Patients W and X

When performing a discovery-based proteomics experiment, a researcher not only desires to determine what is present, but also how much is present, with the latter always proving to be the most difficult to accomplish. Quantitative mass spectrometry dates back to the early 1980s in which internal standards were used as a means of quantitation. The use of chemical labels in quantitative proteomics was first demonstrated by Gygi et al. in 1999. Five years later, isobaric tags for relative and absolute quantitation (iTRAQ) were introduced by Ross et al. In this technique, peptides are reacted with the moiety pictured in Figure 5A. The reporter group is composed of N-methylpiperazine, which contains various nitrogen isotopes that exhibit different low mass reporter ions in fragmentation spectra. The intensities of these ions are used to quantitate peptides and proteins in a given sample. The carbonyl is a mass balance group that is neutral during fragmentation. The N-Hydroxysuccinimide (NHS) ester is protein reactive and forms an amide upon reaction with N-termini or lysines, which is demonstrated in Figure 5B. Figure 5 was recreated from the work by Ross and coworkers.

**Figure 5-5.** A. Components of an iTRAQ label. B. Product following reaction between iTRAQ label and a peptide (indicated by amide bond). Below the product are the various isotopic components for each different label.
Prior to Orbitrap analysis, all samples for each respective patient were combined to create two total samples, one for Patient W and one for Patient X. Data was collected for three injections for each sample. With a minimum PSM score of 0.9, 832 PSMs were identified for Patient W. With two of those being identified as decoy proteins, this suggests a FP of 0.5% for Patient W. This is based on the theory by Elias and Gygi in which it is assumed that false positive identifications will occur in targeted matches as often they will in decoy matches, and thus the amount of decoy matches is doubled, divided by the total PSMs, and multiplied by two.\textsuperscript{226,231} Upon rejection of the decoy matches, 23 carbonylated proteins were identified for Patient W. Again, with a minimum score of 0.9, 2244 PSMs were identified for Patient X. Three of those were identified as decoy proteins, which implies a FP of 0.3% for Patient X.\textsuperscript{231} Upon rejection of the decoy matches, 37 carbonylated proteins were identified for Patient X. Figures 5-6 and 5-7\textsuperscript{231} display the changes in selected carbonylated proteins over the various time points for both Patient W and X, respectively. The entire data sets are located in Appendix 3, Tables A6 and A7. All figures were reprinted with permission from Dr. Narayanasamy.
Figure 5-6. Carbonylated protein levels for ten proteins that exhibited the most change over the course of eight days for Patient W. All data was normalized to Day 1.
Figure 5-7. Carbonylated protein levels for ten proteins that exhibited the most change over the course of six days for Patient X. All data was normalized to Day 0.
5.4.4 ProteoMiner Depletion and iTRAQ Quantitation: Patients Y and Z

Blood is a prime matrix for proteomics work. It is one of the most abundant and attainable biological fluids in the human body. And in terms of proteomics, there are about 7500 identifiable proteins in human plasma (Human Plasma Proteome Project).\textsuperscript{232} While this provides an array of opportunities for discovery proteomics, the fact that about 50 proteins dominate 99% of the entire proteomic composition of plasma complicates detection techniques. Various techniques have been used to equalize protein concentrations such as: precipitation methods, fractionation (HPLC), 2-D gel electrophoresis, or immunoaffinity devices.\textsuperscript{233,234} However, a new technique has been developed within the last ten years that utilizes specificity between ligands and proteins. The ligands are developed based on solid-phase peptide synthesis methods pioneered by Merrifield.\textsuperscript{235} Generating a library of hexapeptides from the twenty natural amino acids results in nearly 64 million ligands, which is generally enough for reaction with most proteins present in a biological sample. The ligands are synthesized onto beads (one ligand per bead). When a mixture of proteins is added, the high concentration proteins rapidly saturate their respective ligands and excess are washed away. Meanwhile, the low concentration proteins bind entirely with their respective ligands, with no excess to be washed away. In this sense, high concentration proteins are depleted and low concentration proteins are enriched to decrease the dynamic range of all protein concentrations.\textsuperscript{233,234} A schematic of the process can be seen below in Figure 5-8.
Prior to Orbitrap analysis, all samples for each respective patient were combined to create two total samples, one for Patient Y and one for Patient Z. Data was collected for three injections for each sample. With a minimum PSM score of 0.9, 4051 PSMs were identified for Patient Y with a false positive rate of 0.1%. Upon rejection of the decoy matches, 59 carbonylated proteins were identified for Patient Y. Again, with a minimum score of 0.9, 3443 PSMs were identified for Patient Z with a false positive rate of 0.2%. Upon rejection of the decoy matches, 47 carbonylated proteins were identified for Patient Z. Figures 5-9 and 5-10\textsuperscript{231} show carbonylation levels for selected proteins over the various time points for both Patient Y and Z, respectively. The entire data sets are located in Appendix 3, Tables A8 and A9. All figures were reprinted with permission from Dr. Narayanasamy.

\textbf{Figure 5-8.} ProteoMiner concept depicting equalization of proteins. (Redrawn from a poster titled “Enriching Low-Abundance Proteins Using ProteoMiner\textsuperscript{TM} Protein Enrichment Technology,” by S. Freeby et al.)
**Figure 5-9.** Carbonylated protein levels for ten proteins that exhibited the most change over the course of four days for Patient Y. All data was normalized to Day 1.
**Figure 5-10.** Carbonylated protein levels for ten proteins that exhibited the most change over the course of four days for Patient Z. All data was normalized to Day 1.
5.5 Discussion

5.5.1 Western Blotting

Unusual carbonylation patterns were visualized in Western blots for Patients W and X. Protein oxidation appeared to increase, then decrease, and repeat across the timepoints for Patient W; whereas, for Patient X, protein oxidation was initially very high, decreased, and then slowly increased over the course of four days. Three of the lanes in these Western blots suggested intense carbonylation across a wide range of proteins – lanes 2 and 5 for Patient W and lane 0 for Patient X. However, quantitative mass spectrometry results did not correlate with these patterns. Thus, we attributed these results to artifacts of initial sample processing and instead, focused our attention on changes to particular bands, such as the fluctuating intensity of the HSA band around 60 kDa. Due to the fact that we were not present for the initial sample collection and handling, we believe that oxidation could have been induced by exposure to air and/or long delays before freezing. On the other hand, carbonylation patterns were indistinguishable in Western blots for Patients Y and Z – only minimal variations in band intensities were observed. Patients Y and Z were elective surgery patients, thus, samples were collected under a better controlled environment and outcomes were more predictable for these patients.

5.5.2 FTC Assay: Patient X

The carbonylation trends exhibited in the emission spectra roughly correlated with one another across the three days, with Day 0 showing the highest intensity and Day 1 showing low intensity. However, fluorescence intensity ranges fluctuated across the three analyses with
unusually high intensities in the third analysis. This is similar to the issues that arose in the CKD samples. While differences in protein carbonylation in the samples could be distinguished, resulting fluorescence intensities were not as consistent across multiple analyses as we had hoped. This variability could be a result of excess fluorescent label remaining in the samples during analysis. Unreacted tagging reagents, particularly fluorescent labels, can be difficult to completely remove from solution.\textsuperscript{93} Due to limited sample, more experimental repeats could not be performed. Initial validation experiments with HSA produced expected results; however, the assay has performed poorly in our studies with biological samples.

**5.5.3 iTRAQ: Patients W and X**

Temporal protein carbonylation trends were observed for a handful of APPs, which correlated with results from many previously published studies involving the analysis of trauma patient blood with mass spectrometry techniques.\textsuperscript{76,78,79} In Patient W, we observed general carbonylation increases in alpha-1-antitrypsin and complement C3 proteins (positive APPs) and a decrease in serum albumin protein (negative APP). Interestingly, significant drops in fibrinogen carbonylation levels were observed for all three fibrinogen chains (alpha, beta, and gamma) on Days 2 and 5. In contrast, samples from these two days exhibited the highest carbonylation levels via Western blotting (Figure 5-2). These observations further our claim that results for these two days were artifacts of sample handling. Finally, fibrinogen is an interesting protein to study in trauma patients since it is involved in the clotting process;\textsuperscript{10} however, we cannot draw conclusions concerning this particular protein for multiple reasons. First, serum is clotted and this would affect overall fibrinogen concentrations. Second, we did not measure overall protein concentrations – only carbonylation levels. This creates ambiguity as to whether
increasing carbonylation levels are a result of increasing protein concentrations or more protein is actually being carbonylated.

In Patient X, we observed strong carbonylation increases in many of the positive APPs such as: alpha, beta, and gamma fibrinogen chains, alpha-1-antichymotrypsin, alpha-1-antitrypsin, haptoglobin, alpha-2-macroglobulin, and ceruloplasmin. As can be seen in Figures 5-6 and 5-7, the carbonylated protein abundances for Patient X were nearly 5 fold higher than those for Patient W in some instances, which could be explained by the fact that Patient X had a complicated recovery period and did not survive. Day 0 data did not correlate between Western blotting and mass spectrometry experiments because the mass spectrometry data was normalized to Day 0. We did not give as much concern to Day 0 anyways since the Western blotting results for this day were attributed to artifacts of sample handling.

5.5.4 ProteoMiner and iTRAQ: Patients Y and Z

Again, we observed temporal carbonylation trends in APPs, particularly serum amyloid and C-reactive proteins. Both are positive APPs and the carbonylation levels increased for these proteins in each patient sample, as expected. Interestingly, these two proteins have been reported to exhibit the most rapid change during inflammation, generally increasing anywhere between 5-fold and 1000-fold. Increases in these two proteins were more pronounced for Patient Z, suggesting a more difficult recovery period. This is merely postulation though being that we were not privy to details concerning the patients’ conditions or recoveries. Moderate oxidation changes were observed in this set of patients versus the first possibly due to the fact that Patients Y and Z were elective surgery patients rather than trauma patients, in which the injuries were less predictable. However, the ProteoMiner depletion was effective and led to a greater number
of protein quantifications. Nearly twice as many carbonylated proteins were identified in Patients Y and Z plasma versus Patients W and X serum. This implies that lower abundance proteins are being carbonylated in inflammation-associated samples, but we are limited by our detection capabilities in terms of identifying these particular proteins. Despite this limitation, we confirmed that techniques such as ProteoMiner depletion offer an opportunity to detect and investigate some of these lower abundance species. Investigating carbonylation in these lower abundance species might lead us to identifying a more prominent marker of inflammation.
5.6 Conclusion

Upon successfully applying some of our analytical methods to trauma patient samples, we were able to visualize and quantitate temporal protein carbonylation patterns. Western blotting results illustrated interesting carbonylation changes over time and quantitative mass spectrometry confirmed these results, along with identifying carbonylation changes in important inflammation-associated proteins. ProteoMiner experiments proved successful in that we were able to identify a larger and more diverse amount of carbonylated proteins via mass spectrometry. These analytical protocols were not only unique to this field of study, but also hold promise for application to future blood samples. We anticipate receiving a larger sample set and continuing our applications in the hopes of confirming the utility of our methods and working toward protein carbonylation biomarker identification. With more samples, we also hope to incorporate Chapter 3 methods involving the cleavable biotin affinity tag and streptavidin bead enrichment into our workflow as an alternative novel method application. The ultimate goal for this study is the development of therapeutic interventions which would one day assist in emergency room preventative care.
REFERENCES


APPENDICES

Appendix 1

Table A1

The table below depicts elution profiles for the monomeric avidin columns. Two commercially purchased biotinylated proteins were used: HRP and BSA, reported to have 1 and 9 biotin/protein, respectively. Both were dissolved in 500 µL PBS giving BSA a concentration of 2 mg/mL and HRP a concentration of 1 mg/mL. Samples were enriched following the procedure detailed in Section 2.3.7. Six two-mL aliquots were collected for both nonbound proteins (NB) and bound proteins (B). Protein concentrations in the first four NB fractions and all six B fractions were determined with the BCA assay. No bound protein was eluted for HRP, but 73% of biotinylated BSA was recovered. Total protein recovery (in NB fractions) for HRP was 40%.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Absorbance</th>
<th>Concentration</th>
<th>µg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA NB1</td>
<td>-0.0025811</td>
<td>-0.0274</td>
<td>-54.03</td>
</tr>
<tr>
<td>BSA NB2</td>
<td>0.0099173</td>
<td>-0.0192</td>
<td>-37.63</td>
</tr>
<tr>
<td>BSA NB3</td>
<td>-0.00091076</td>
<td>-0.0263</td>
<td>-51.87</td>
</tr>
<tr>
<td>BSA NB4</td>
<td>-0.0027676</td>
<td>-0.0275</td>
<td>-54.28</td>
</tr>
<tr>
<td>BSA B1</td>
<td>0.023527</td>
<td>-0.0102</td>
<td>-19.93</td>
</tr>
<tr>
<td>BSA B2</td>
<td>0.58521</td>
<td>0.345</td>
<td>673.4</td>
</tr>
<tr>
<td>BSA B3</td>
<td>0.081578</td>
<td>0.0279</td>
<td>54.37</td>
</tr>
<tr>
<td>BSA B4</td>
<td>0.018648</td>
<td>-0.0147</td>
<td>-28.74</td>
</tr>
<tr>
<td>BSA B5</td>
<td>0.014004</td>
<td>-0.0165</td>
<td>-32.12</td>
</tr>
<tr>
<td>BSA B6</td>
<td>0.0030594</td>
<td>-0.0237</td>
<td>-46.13</td>
</tr>
<tr>
<td>HRP NB1</td>
<td>0.18623</td>
<td>0.0966</td>
<td>190.7</td>
</tr>
<tr>
<td>HRP NB2</td>
<td>0.042794</td>
<td>0.00242</td>
<td>4.789</td>
</tr>
<tr>
<td>HRP NB3</td>
<td>0.019162</td>
<td>-0.0131</td>
<td>-25.85</td>
</tr>
<tr>
<td>HRP NB4</td>
<td>0.041647</td>
<td>0.00167</td>
<td>3.302</td>
</tr>
<tr>
<td>HRP B1</td>
<td>0.015952</td>
<td>-0.0152</td>
<td>-29.63</td>
</tr>
<tr>
<td>HRP B2</td>
<td>0.033066</td>
<td>-0.00396</td>
<td>-7.723</td>
</tr>
<tr>
<td>HRP B3</td>
<td>0.015194</td>
<td>-0.0157</td>
<td>-30.60</td>
</tr>
<tr>
<td>HRP B4</td>
<td>0.0017300</td>
<td>-0.0245</td>
<td>-47.83</td>
</tr>
<tr>
<td>HRP B5</td>
<td>0.015542</td>
<td>-0.0155</td>
<td>-30.15</td>
</tr>
<tr>
<td>HRP B6</td>
<td>0.015450</td>
<td>-0.0155</td>
<td>-30.27</td>
</tr>
</tbody>
</table>
Appendix 2

Table A2

The table below depicts the number of modifications for each particular peptide in the first analysis of the 0, 25, 50, and 75% spike samples. Nonbound samples produced no modified peptides.

**TRIAL 1**

<table>
<thead>
<tr>
<th>RESIDUE</th>
<th>MODIFIED PEPTIDE</th>
<th>SAMPLE NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cleaved and Alkylated:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-34</td>
<td>ALVLIAFAQYLQQC&lt;sub&gt;494.19&lt;/sub&gt;PFEDHK (+2)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>ALVLIAFAQYLQQC&lt;sub&gt;494.19&lt;/sub&gt;PFEDHK (+3)</td>
<td></td>
</tr>
<tr>
<td>H-67</td>
<td>SLH&lt;sub&gt;328.24&lt;/sub&gt;TLFGDK (+2)</td>
<td></td>
</tr>
<tr>
<td>H-128</td>
<td>LVRPEVDVMCTAFH&lt;sub&gt;528.24&lt;/sub&gt;DNEETFLKK (+3)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>H&lt;sub&gt;528.24&lt;/sub&gt;PFYAPELFFFAK (+2)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>H&lt;sub&gt;528.24&lt;/sub&gt;PFYAPELFFFAK (+3)</td>
<td>2</td>
</tr>
<tr>
<td>H-146</td>
<td>RH&lt;sub&gt;528.24&lt;/sub&gt;PFYAPELFFFAK (+2)</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>RH&lt;sub&gt;528.24&lt;/sub&gt;PFYAPELFFFAK (+3)</td>
<td>14</td>
</tr>
<tr>
<td>H-242</td>
<td>VEH&lt;sub&gt;528.24&lt;/sub&gt;TECHGDLDLEACDAR (+3)</td>
<td></td>
</tr>
<tr>
<td>H-288</td>
<td>SH&lt;sub&gt;528.24&lt;/sub&gt;CIAEVENDEMPADLPSLAADFVESK (+3)</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>CCAADPH&lt;sub&gt;528.24&lt;/sub&gt;ECYA (+2)</td>
<td>16</td>
</tr>
<tr>
<td>K-51</td>
<td>LVNEVTEFAK&lt;sub&gt;519.27&lt;/sub&gt;TCVDESAENCDAK (+3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>YK&lt;sub&gt;519.27&lt;/sub&gt;AAFTECCQAADK (+2)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>YK&lt;sub&gt;519.27&lt;/sub&gt;AAFTECCQAADK (+3)</td>
<td>8</td>
</tr>
<tr>
<td>K-162</td>
<td>ADLAK&lt;sub&gt;519.27&lt;/sub&gt;YICENQDSSK (+3)</td>
<td></td>
</tr>
<tr>
<td>K-359</td>
<td>TYETTLEK&lt;sub&gt;519.27&lt;/sub&gt;CIAADPHCEYAK (+3)</td>
<td>5</td>
</tr>
<tr>
<td>K-378</td>
<td>VFDEFK&lt;sub&gt;519.27&lt;/sub&gt;PLVEEPQNLIK (+3)</td>
<td>2</td>
</tr>
<tr>
<td>K-414</td>
<td>K&lt;sub&gt;519.27&lt;/sub&gt;PFVSPSTPLVEVR (+3)</td>
<td></td>
</tr>
<tr>
<td>K-545</td>
<td>EQLK&lt;sub&gt;519.27&lt;/sub&gt;AVMDDFAFAFEK (+2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EQLK&lt;sub&gt;519.27&lt;/sub&gt;AVMDDFAFAFEK (+3)</td>
<td>1</td>
</tr>
<tr>
<td>Cleaved and Reduced:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-289</td>
<td>SHC&lt;sub&gt;437.14&lt;/sub&gt;IAEVENDEMPADLPSLAADFVESK (+3)</td>
<td></td>
</tr>
<tr>
<td>H-288</td>
<td>SH&lt;sub&gt;437.14&lt;/sub&gt;CIAEVENDEMPADLPSLAADFVESK (+3)</td>
<td>1</td>
</tr>
</tbody>
</table>

| TOTAL SPECTRA | 4791 | 6170 | 5965 | 5137 |
| TOTAL HSA SPECTRA | 4738 | 6127 | 5927 | 5116 |

| TOTAL MODIFIED SPECTRA | 8 | 76 | 107 | 204 |
| % MODIFIED | 0.17 | 1.24 | 1.81 | 3.99 |
Table A3
The table below depicts the number of modifications for each particular peptide in the second analysis of the 0, 25, 50, and 75% spike samples. Nonbound samples produced no modified peptides.

<table>
<thead>
<tr>
<th>TRIAL 2</th>
<th>SAMPLE NAME</th>
<th>RESIDUE</th>
<th>MODIFIED PEPTIDE</th>
<th>0%</th>
<th>25%</th>
<th>50%</th>
<th>75%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cleaved and Alkylated:</td>
<td></td>
<td>C-34</td>
<td>ALVLIAFAQYLQQC_{494,19}PFEDHVK (+2)</td>
<td>7</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ALVLIAFAQYLQQC_{494,19}PFEDHVK (+3)</td>
<td>3</td>
<td>13</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>H-67</td>
<td>SLH_{528,24}TLFGDK (+2)</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>H_{528,24}PYFYAPELFFAK (+2)</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>H_{528,24}PYFYAPELFFAK (+3)</td>
<td></td>
<td></td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H-146</td>
<td>RH_{528,24}PYFYAPELFFAK (+2)</td>
<td></td>
<td></td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RH_{528,24}PYFYAPELFFAK (+3)</td>
<td></td>
<td></td>
<td>22</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H-288</td>
<td>SH_{528,24}CIAEVENDEMPADLPSLAADFVESK (+3)</td>
<td>24</td>
<td>19</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>H-367</td>
<td>CCAADPH_{528,24}ECYAK (+2)</td>
<td></td>
<td>19</td>
<td>18</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>K-51</td>
<td>LVNEVTEFAK_{519,27}TCVADESAENCDK (+3)</td>
<td></td>
<td></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>K-162</td>
<td>YK_{519,27}AAFTECCQAADK (+2)</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>YK_{519,27}AAFTECCQAADK (+3)</td>
<td></td>
<td>4</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>K-262</td>
<td>ADLAK_{519,27}YICENQDSISSK (+3)</td>
<td></td>
<td></td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>K-359</td>
<td>TYETTLEK_{519,27}CCAAADPHECYAK (+3)</td>
<td></td>
<td>1</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>K-378</td>
<td>VFDEFK_{519,27}PLVEEPQNLIK (+3)</td>
<td>8</td>
<td>7</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>K-545</td>
<td>EQLK_{519,27}AVMDDFAAFVEK (+2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EQLK_{519,27}AVMDDFAAFVEK (+3)</td>
<td>1</td>
<td>2</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Cleaved and Reduced:</td>
<td></td>
<td>C-289</td>
<td>SHC_{437,16}CIAEVENDEMPADLPSLAADFVESK (+3)</td>
<td>2</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>H-288</td>
<td>SHL_{471,21}CIAEVENDEMPADLPSLAADFVESK (+3)</td>
<td></td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOTAL SPECTRA</td>
<td></td>
<td></td>
<td></td>
<td>4053</td>
<td>7110</td>
<td>6041</td>
<td>9015</td>
</tr>
<tr>
<td>TOTAL HSA SPECTRA</td>
<td></td>
<td></td>
<td></td>
<td>3996</td>
<td>7076</td>
<td>6006</td>
<td>8981</td>
</tr>
<tr>
<td>TOTAL MODIFIED SPECTRA</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>95</td>
<td>112</td>
<td>217</td>
</tr>
<tr>
<td>% MODIFIED</td>
<td></td>
<td></td>
<td></td>
<td>0.00</td>
<td>1.34</td>
<td>1.86</td>
<td>2.42</td>
</tr>
</tbody>
</table>
Table A4

The table below depicts the number of modifications for each particular peptide in the third analysis of the 0, 25, 50, and 75% spike samples. Nonbound samples produced no modified peptides.

**TRIAL 3**

<table>
<thead>
<tr>
<th>RESIDUE</th>
<th>MODIFIED PEPTIDE</th>
<th>0%</th>
<th>25%</th>
<th>50%</th>
<th>75%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cleaved and Alkylated:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-34</td>
<td>ALVLIAFAQYLQQC&lt;sub&gt;494.15&lt;/sub&gt;PFEDHKV (+2)</td>
<td>3</td>
<td>3</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ALVLIAFAQYLQQC&lt;sub&gt;494.15&lt;/sub&gt;PFEDHKV (+3)</td>
<td></td>
<td></td>
<td></td>
<td>11</td>
</tr>
<tr>
<td>H-67</td>
<td>SLH&lt;sub&gt;328.24&lt;/sub&gt;TLFGDKLCTVATLR (+3)</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-128</td>
<td>LVRPEVDMCTAFH&lt;sub&gt;328.24&lt;/sub&gt;DNEEFLK (+3)</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H&lt;sub&gt;328.24&lt;/sub&gt;PYYAPELFFFAK (+2)</td>
<td></td>
<td>4</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H&lt;sub&gt;328.24&lt;/sub&gt;PYYAPELFFFAK (+3)</td>
<td></td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-146</td>
<td>RH&lt;sub&gt;328.24&lt;/sub&gt;PYYAPELFFFAK (+2)</td>
<td>12</td>
<td>19</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RH&lt;sub&gt;328.24&lt;/sub&gt;PYYAPELFFFAK (+3)</td>
<td>12</td>
<td>22</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>H-288</td>
<td>SH&lt;sub&gt;328.24&lt;/sub&gt;IAEVENDEMPADLPSLAADFVESK (+3)</td>
<td>10</td>
<td>19</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>H-367</td>
<td>CAAADPH&lt;sub&gt;328.24&lt;/sub&gt;EICYAK (+2)</td>
<td>11</td>
<td>16</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>K-162</td>
<td>YK&lt;sub&gt;519.27&lt;/sub&gt;AAFTECCQAADK (+2)</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>YK&lt;sub&gt;519.27&lt;/sub&gt;AAFTECCQAADK (+3)</td>
<td>1</td>
<td>4</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>K-359</td>
<td>TYETTLEK&lt;sub&gt;519.27&lt;/sub&gt;CAAADPHECYAK (+3)</td>
<td></td>
<td></td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>K-378</td>
<td>VFDEFK&lt;sub&gt;519.27&lt;/sub&gt;PLVEEPQLIK (+3)</td>
<td></td>
<td></td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>K-545</td>
<td>EQLK&lt;sub&gt;519.27&lt;/sub&gt;AVMDDFAAFVEK (+2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EQLK&lt;sub&gt;519.27&lt;/sub&gt;AVMDDFAAFVEK (+3)</td>
<td></td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Cleaved and Reduced:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-289</td>
<td>SHC&lt;sub&gt;437.16&lt;/sub&gt;IAEVENDEMPADLPSLAADFVESK (+3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-288</td>
<td>SH&lt;sub&gt;471.23&lt;/sub&gt;IAEVENDEMPADLPSLAADFVESK (+3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**TOTAL SPECTRA** 6868 5154 6140 6642

**TOTAL HSA SPECTRA** 6824 5101 6111 6614

| TOTAL MODIFIED SPECTRA | 1 | 52 | 95 | 194 |
| % MODIFIED | 0.01 | 1.02 | 1.55 | 2.93 |
**Table A5**

The table below depicts the number of modifications for each particular peptide in all four analyses of the 10% spike sample.

<table>
<thead>
<tr>
<th>RESIDUE</th>
<th>MODIFIED PEPTIDE (CHARGE)</th>
<th>10% LABELED</th>
<th>10% NB</th>
<th>10% LABELED</th>
<th>10% NB</th>
<th>10% LABELED</th>
<th>10% NB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cleaved and Alkylated:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-34</td>
<td>ALVLIAFAQYLQQC&lt;sub&gt;494.19&lt;/sub&gt;PFEDHVK (+2)</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ALVLIAFAQYLQQC&lt;sub&gt;494.19&lt;/sub&gt;PFEDHVK (+3)</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-146</td>
<td>H&lt;sub&gt;528.24&lt;/sub&gt;PFFYAPELFFAK (+2)</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>H&lt;sub&gt;528.24&lt;/sub&gt;PFFYAPELFFAK (+3)</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RH&lt;sub&gt;528.24&lt;/sub&gt;PFFYAPELFFAK (+2)</td>
<td>5</td>
<td></td>
<td>14</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RH&lt;sub&gt;528.24&lt;/sub&gt;PFFYAPELFFAK (+3)</td>
<td></td>
<td></td>
<td>36</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-288</td>
<td>SH&lt;sub&gt;528.24&lt;/sub&gt;CIAEVENDEMPADLPSLAADFVESK (+3)</td>
<td>5</td>
<td>18</td>
<td>18</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-367</td>
<td>CCAAADPH&lt;sub&gt;528.24&lt;/sub&gt;ECYAK (+2)</td>
<td>2</td>
<td>25</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K-51</td>
<td>LVNEVT&lt;sub&gt;501.27&lt;/sub&gt;TCAVESAE&lt;sub&gt;501.27&lt;/sub&gt;CDK (+3)</td>
<td>1</td>
<td></td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>YK&lt;sub&gt;501.27&lt;/sub&gt;AAFT&lt;sub&gt;501.27&lt;/sub&gt;CCQADK (+2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K-162</td>
<td>YK&lt;sub&gt;501.27&lt;/sub&gt;AAFT&lt;sub&gt;501.27&lt;/sub&gt;CCQADK (+3)</td>
<td>1</td>
<td>7</td>
<td>9</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K-359</td>
<td>TYETT&lt;sub&gt;501.27&lt;/sub&gt;LEK&lt;sub&gt;501.27&lt;/sub&gt;CCAAADPHECYAK (+3)</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K-378</td>
<td>VFDEFK&lt;sub&gt;532.27&lt;/sub&gt;PLVE&lt;sub&gt;532.27&lt;/sub&gt;EPPQLIK (+3)</td>
<td>10</td>
<td></td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K-545</td>
<td>EQLK&lt;sub&gt;501.27&lt;/sub&gt;AVMD&lt;sub&gt;501.27&lt;/sub&gt;DAFVEK (+2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EQLK&lt;sub&gt;501.27&lt;/sub&gt;AVMD&lt;sub&gt;501.27&lt;/sub&gt;DAFVEK (+3)</td>
<td>3</td>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cleaved and Reduced:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-289</td>
<td>SH&lt;sub&gt;537.16&lt;/sub&gt;CIAEVENDEMPADLPSLAADFVESK (+3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H-288</td>
<td>SH&lt;sub&gt;547.27&lt;/sub&gt;CIAEVENDEMPADLPSLAADFVESK (+3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOTAL SPECTRA</td>
<td>4220</td>
<td>3330</td>
<td>6406</td>
<td>2851</td>
<td>4929</td>
<td>4367</td>
<td>6696</td>
</tr>
<tr>
<td>TOTAL HSA SPECTRA</td>
<td>4191</td>
<td>3317</td>
<td>6377</td>
<td>2664</td>
<td>4886</td>
<td>4329</td>
<td>6640</td>
</tr>
<tr>
<td>TOTAL SPECTRA FOR MODIFIED PEPTIDES</td>
<td>13</td>
<td>0</td>
<td>119</td>
<td>0</td>
<td>99</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>% MODIFIED PEPTIDES</td>
<td>0.31</td>
<td>0.00</td>
<td>1.87</td>
<td>0.00</td>
<td>2.03</td>
<td>0.00</td>
<td>0.06</td>
</tr>
</tbody>
</table>
Appendix 3

Table A6

The following are changes in carbonylation protein relative abundances in blood serum samples that were collected from Patient W over the course of eight days. The changes were not normalized to Day 1. No PeptideProphet scores below 0.9 were accepted. The proteins were grouped using ProteinProphet; however, the alpha-2-macroglobulin and pregnancy zone protein group did not contain related proteins like the other groups. The relative abundances were calculated by dividing the reporter ion intensity by the total intensity for the eight reporter ions for each PSM and then averaging all PSMs composing the various protein groups.

<table>
<thead>
<tr>
<th>Protein Group Description</th>
<th>Associated Gene</th>
<th>Supporting PSM Count</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptavidin</td>
<td>not available</td>
<td>940</td>
<td>21.3%</td>
<td>14.6%</td>
<td>9.5%</td>
<td>9.9%</td>
<td>13.6%</td>
<td>10.3%</td>
<td>10.1%</td>
<td>10.7%</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>ALB</td>
<td>461</td>
<td>21.5%</td>
<td>20.5%</td>
<td>8.9%</td>
<td>12.0%</td>
<td>12.2%</td>
<td>9.6%</td>
<td>7.5%</td>
<td>7.8%</td>
</tr>
<tr>
<td>Trypsin (Pig)</td>
<td>not available</td>
<td>84</td>
<td>15.8%</td>
<td>14.5%</td>
<td>8.0%</td>
<td>13.0%</td>
<td>13.3%</td>
<td>12.4%</td>
<td>11.2%</td>
<td>11.8%</td>
</tr>
<tr>
<td>Ig gamma chain C region</td>
<td>IGHG</td>
<td>61</td>
<td>18.7%</td>
<td>16.6%</td>
<td>6.6%</td>
<td>9.0%</td>
<td>12.9%</td>
<td>11.9%</td>
<td>11.2%</td>
<td>13.0%</td>
</tr>
<tr>
<td>Fibrinogen alpha chain</td>
<td>FGA</td>
<td>57</td>
<td>12.4%</td>
<td>4.2%</td>
<td>12.6%</td>
<td>20.8%</td>
<td>3.3%</td>
<td>20.6%</td>
<td>12.8%</td>
<td>13.4%</td>
</tr>
<tr>
<td>Complement C3</td>
<td>C3</td>
<td>33</td>
<td>12.9%</td>
<td>13.0%</td>
<td>7.4%</td>
<td>9.0%</td>
<td>14.9%</td>
<td>17.6%</td>
<td>12.1%</td>
<td>13.1%</td>
</tr>
<tr>
<td>Alpha-1-antitrypsin</td>
<td>SERPINA1</td>
<td>31</td>
<td>7.7%</td>
<td>11.7%</td>
<td>8.7%</td>
<td>13.5%</td>
<td>18.5%</td>
<td>15.1%</td>
<td>12.0%</td>
<td>12.8%</td>
</tr>
<tr>
<td>Ig kappa chain C region</td>
<td>IGKC</td>
<td>28</td>
<td>18.4%</td>
<td>12.8%</td>
<td>9.0%</td>
<td>11.0%</td>
<td>13.0%</td>
<td>11.3%</td>
<td>11.3%</td>
<td>13.2%</td>
</tr>
<tr>
<td>Decoy</td>
<td>not available</td>
<td>16</td>
<td>10.7%</td>
<td>8.6%</td>
<td>9.9%</td>
<td>15.1%</td>
<td>16.9%</td>
<td>11.3%</td>
<td>13.2%</td>
<td>14.4%</td>
</tr>
<tr>
<td>Vitronectin</td>
<td>VTN</td>
<td>12</td>
<td>14.5%</td>
<td>15.8%</td>
<td>9.9%</td>
<td>12.5%</td>
<td>16.4%</td>
<td>13.1%</td>
<td>8.2%</td>
<td>9.6%</td>
</tr>
<tr>
<td>Fibrinogen beta chain</td>
<td>FGB</td>
<td>11</td>
<td>10.6%</td>
<td>3.1%</td>
<td>13.8%</td>
<td>21.4%</td>
<td>3.0%</td>
<td>21.1%</td>
<td>13.6%</td>
<td>13.5%</td>
</tr>
<tr>
<td>Complement C4-A &amp; B</td>
<td>C4A &amp; C4B</td>
<td>10</td>
<td>16.0%</td>
<td>12.0%</td>
<td>9.7%</td>
<td>13.0%</td>
<td>17.2%</td>
<td>11.0%</td>
<td>9.4%</td>
<td>11.6%</td>
</tr>
<tr>
<td>Ig alpha chain C region</td>
<td>IGH A</td>
<td>10</td>
<td>13.9%</td>
<td>14.6%</td>
<td>9.8%</td>
<td>9.5%</td>
<td>12.6%</td>
<td>13.1%</td>
<td>12.6%</td>
<td>13.9%</td>
</tr>
<tr>
<td>Fibrinogen gamma chain</td>
<td>FGG</td>
<td>7</td>
<td>19.9%</td>
<td>8.8%</td>
<td>10.3%</td>
<td>14.2%</td>
<td>9.0%</td>
<td>14.0%</td>
<td>11.4%</td>
<td>12.5%</td>
</tr>
<tr>
<td>Serotransferrin</td>
<td>TF</td>
<td>6</td>
<td>30.0%</td>
<td>26.1%</td>
<td>5.5%</td>
<td>7.6%</td>
<td>8.1%</td>
<td>7.7%</td>
<td>7.6%</td>
<td>7.3%</td>
</tr>
<tr>
<td>Hemoglobin subunit beta</td>
<td>HBB</td>
<td>5</td>
<td>18.4%</td>
<td>26.7%</td>
<td>5.6%</td>
<td>14.1%</td>
<td>7.7%</td>
<td>13.1%</td>
<td>6.3%</td>
<td>8.1%</td>
</tr>
<tr>
<td>Keratin, type II cytoskeletal 1</td>
<td>KRT1</td>
<td>4</td>
<td>22.5%</td>
<td>16.7%</td>
<td>6.7%</td>
<td>9.5%</td>
<td>12.4%</td>
<td>10.6%</td>
<td>8.3%</td>
<td>13.4%</td>
</tr>
<tr>
<td>Trypsin-1 &amp; 2</td>
<td>PRSS1 &amp; PRSS2</td>
<td>4</td>
<td>19.2%</td>
<td>17.2%</td>
<td>7.9%</td>
<td>11.3%</td>
<td>11.3%</td>
<td>11.5%</td>
<td>10.0%</td>
<td>11.7%</td>
</tr>
<tr>
<td>Alpha-2-macroglobulin &amp; Pregnancy zone protein</td>
<td>A2M &amp; PZP</td>
<td>2</td>
<td>15.9%</td>
<td>14.2%</td>
<td>9.3%</td>
<td>10.9%</td>
<td>11.8%</td>
<td>15.1%</td>
<td>10.4%</td>
<td>12.4%</td>
</tr>
<tr>
<td>Apolipoprotein A-1</td>
<td>APOA1</td>
<td>2</td>
<td>24.6%</td>
<td>17.6%</td>
<td>8.3%</td>
<td>11.0%</td>
<td>9.2%</td>
<td>11.8%</td>
<td>7.6%</td>
<td>9.8%</td>
</tr>
</tbody>
</table>
Table A7

The following are changes in carbonylation protein relative abundances in blood serum samples that were collected from Patient X over the course of six days. The changes were not normalized to Day 1. No PeptideProphet scores below 0.9 were accepted. The proteins were grouped using ProteinProphet; however, the alpha-2-macroglobulin and pregnancy zone protein group did not contain related proteins like the other groups. The relative abundances were calculated by dividing the reporter ion intensity by the total intensity for the six reporter ions for each PSM and then averaging all PSMs composing the various protein groups.

<table>
<thead>
<tr>
<th>Protein Group Description</th>
<th>Associated Gene</th>
<th>Supporting PSM Count</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptavidin</td>
<td>not available</td>
<td>1661</td>
<td>15.7%</td>
<td>13.3%</td>
<td>16.4%</td>
<td>10.3%</td>
<td>18.8%</td>
<td>18.9%</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>ALB</td>
<td>908</td>
<td>18.3%</td>
<td>12.0%</td>
<td>16.4%</td>
<td>15.6%</td>
<td>21.0%</td>
<td>17.9%</td>
</tr>
<tr>
<td>Ig gamma chain C region</td>
<td>IGHG</td>
<td>214</td>
<td>17.1%</td>
<td>16.7%</td>
<td>20.4%</td>
<td>20.6%</td>
<td>28.0%</td>
<td>20.9%</td>
</tr>
<tr>
<td>Fibrinogen alpha chain</td>
<td>FGA</td>
<td>199</td>
<td>8.0%</td>
<td>5.7%</td>
<td>12.8%</td>
<td>12.8%</td>
<td>22.2%</td>
<td>26.1%</td>
</tr>
<tr>
<td>Alpha-1-antitrypsin</td>
<td>SERPINA1</td>
<td>143</td>
<td>8.0%</td>
<td>5.7%</td>
<td>12.8%</td>
<td>22.2%</td>
<td>26.1%</td>
<td>25.2%</td>
</tr>
<tr>
<td>Trypsin (Pig)</td>
<td>not available</td>
<td>106</td>
<td>18.3%</td>
<td>15.2%</td>
<td>12.7%</td>
<td>17.7%</td>
<td>18.1%</td>
<td>17.9%</td>
</tr>
<tr>
<td>Complement C3</td>
<td>C3</td>
<td>100</td>
<td>8.4%</td>
<td>7.3%</td>
<td>14.2%</td>
<td>14.2%</td>
<td>20.9%</td>
<td>24.6%</td>
</tr>
<tr>
<td>Fibrinogen beta chain</td>
<td>FGB</td>
<td>95</td>
<td>5.0%</td>
<td>4.5%</td>
<td>7.3%</td>
<td>14.2%</td>
<td>20.9%</td>
<td>24.6%</td>
</tr>
<tr>
<td>Serotransferrin</td>
<td>TF</td>
<td>83</td>
<td>12.2%</td>
<td>10.3%</td>
<td>25.4%</td>
<td>18.3%</td>
<td>17.0%</td>
<td>16.9%</td>
</tr>
<tr>
<td>Protein</td>
<td>Symbol</td>
<td>Value</td>
<td>Value</td>
<td>Value</td>
<td>Value</td>
<td>Value</td>
<td>Value</td>
<td>Value</td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>--------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>Fibrinogen gamma chain</td>
<td>FGG</td>
<td>78</td>
<td>6.9%</td>
<td>8.6%</td>
<td>14.9%</td>
<td>18.2%</td>
<td>24.6%</td>
<td>26.9%</td>
</tr>
<tr>
<td>Ig kappa chain C region</td>
<td>IGKC</td>
<td>67</td>
<td>17.4%</td>
<td>10.9%</td>
<td>19.6%</td>
<td>19.3%</td>
<td>16.4%</td>
<td>16.4%</td>
</tr>
<tr>
<td>Alpha-2-macroglobulin &amp; Pregnancy zone protein</td>
<td>A2M &amp; PZP</td>
<td>46</td>
<td>8.4%</td>
<td>6.3%</td>
<td>22.2%</td>
<td>23.8%</td>
<td>20.3%</td>
<td>19.1%</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>HP</td>
<td>40</td>
<td>5.1%</td>
<td>4.6%</td>
<td>14.1%</td>
<td>7.9%</td>
<td>27.2%</td>
<td>41.1%</td>
</tr>
<tr>
<td>Complement C4-A &amp; B</td>
<td>C4A &amp; C4B</td>
<td>39</td>
<td>12.6%</td>
<td>6.2%</td>
<td>17.6%</td>
<td>22.5%</td>
<td>20.2%</td>
<td>20.9%</td>
</tr>
<tr>
<td>Alpha-1-antichymotrypsin</td>
<td>SERPINA3</td>
<td>37</td>
<td>3.9%</td>
<td>2.9%</td>
<td>14.3%</td>
<td>26.7%</td>
<td>27.4%</td>
<td>24.7%</td>
</tr>
<tr>
<td>Apolipoprotein A-I</td>
<td>APOA1</td>
<td>35</td>
<td>10.5%</td>
<td>8.2%</td>
<td>26.9%</td>
<td>29.7%</td>
<td>12.0%</td>
<td>12.7%</td>
</tr>
<tr>
<td>Vitronectin</td>
<td>VTN</td>
<td>29</td>
<td>13.0%</td>
<td>11.2%</td>
<td>16.9%</td>
<td>17.1%</td>
<td>20.9%</td>
<td>20.9%</td>
</tr>
<tr>
<td>Decoy</td>
<td>not available</td>
<td>27</td>
<td>17.2%</td>
<td>17.2%</td>
<td>13.7%</td>
<td>18.6%</td>
<td>16.9%</td>
<td>16.3%</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>CP</td>
<td>25</td>
<td>9.3%</td>
<td>6.4%</td>
<td>15.6%</td>
<td>19.8%</td>
<td>23.6%</td>
<td>25.3%</td>
</tr>
<tr>
<td>Ig alpha chain C region</td>
<td>IGHA</td>
<td>24</td>
<td>14.2%</td>
<td>10.6%</td>
<td>22.8%</td>
<td>19.6%</td>
<td>16.0%</td>
<td>16.8%</td>
</tr>
<tr>
<td>Serum amyloid A-1 &amp; 2 protein</td>
<td>SAA1 &amp; SAA2</td>
<td>21</td>
<td>3.9%</td>
<td>3.3%</td>
<td>18.9%</td>
<td>25.9%</td>
<td>18.5%</td>
<td>29.5%</td>
</tr>
<tr>
<td>Hemopexin</td>
<td>HPX</td>
<td>16</td>
<td>11.7%</td>
<td>9.8%</td>
<td>43.2%</td>
<td>12.0%</td>
<td>10.6%</td>
<td>12.6%</td>
</tr>
<tr>
<td>Ig heavy chain V-III regions</td>
<td>not available</td>
<td>12</td>
<td>13.8%</td>
<td>10.9%</td>
<td>23.7%</td>
<td>21.9%</td>
<td>15.1%</td>
<td>14.5%</td>
</tr>
<tr>
<td>Trypsin-1 &amp; 2</td>
<td>PRSS1 &amp; PRSS2</td>
<td>10</td>
<td>19.5%</td>
<td>16.1%</td>
<td>10.3%</td>
<td>19.4%</td>
<td>17.9%</td>
<td>16.8%</td>
</tr>
<tr>
<td>Plasma protease C1 inhibitor</td>
<td>SERPING1</td>
<td>5</td>
<td>7.5%</td>
<td>6.0%</td>
<td>15.1%</td>
<td>28.5%</td>
<td>20.8%</td>
<td>22.1%</td>
</tr>
<tr>
<td>Apolipoprotein B-100</td>
<td>APOB</td>
<td>4</td>
<td>13.6%</td>
<td>9.5%</td>
<td>14.5%</td>
<td>24.9%</td>
<td>19.4%</td>
<td>18.1%</td>
</tr>
<tr>
<td>Ig lambda chain C region</td>
<td>IGLC</td>
<td>4</td>
<td>15.9%</td>
<td>13.8%</td>
<td>15.8%</td>
<td>21.4%</td>
<td>17.0%</td>
<td>16.0%</td>
</tr>
<tr>
<td>Alkaline ceramidase I</td>
<td>ACER1</td>
<td>3</td>
<td>12.5%</td>
<td>11.6%</td>
<td>22.2%</td>
<td>24.4%</td>
<td>15.6%</td>
<td>13.6%</td>
</tr>
<tr>
<td>Keratin, type I cytoskeletal (9, 10, 12, 14-17, 19)</td>
<td>KRT9, 10, 12, 14-17, 19</td>
<td>3</td>
<td>25.4%</td>
<td>12.2%</td>
<td>13.7%</td>
<td>15.5%</td>
<td>18.8%</td>
<td>14.5%</td>
</tr>
<tr>
<td>Keratin, type II cytoskeletal 1</td>
<td>KRT1</td>
<td>3</td>
<td>21.0%</td>
<td>11.4%</td>
<td>14.2%</td>
<td>15.3%</td>
<td>19.4%</td>
<td>18.7%</td>
</tr>
<tr>
<td>Protein AMBP</td>
<td>AMBP</td>
<td>2</td>
<td>19.8%</td>
<td>7.0%</td>
<td>11.6%</td>
<td>19.1%</td>
<td>20.2%</td>
<td>22.2%</td>
</tr>
<tr>
<td>Angiotensinogen</td>
<td>AGT</td>
<td>1</td>
<td>10.3%</td>
<td>6.3%</td>
<td>14.0%</td>
<td>15.5%</td>
<td>28.9%</td>
<td>25.0%</td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>CRP</td>
<td>1</td>
<td>5.1%</td>
<td>5.6%</td>
<td>16.2%</td>
<td>30.5%</td>
<td>24.6%</td>
<td>18.0%</td>
</tr>
<tr>
<td>Decoy</td>
<td>not available</td>
<td>1</td>
<td>16.2%</td>
<td>10.6%</td>
<td>16.0%</td>
<td>24.6%</td>
<td>15.9%</td>
<td>16.7%</td>
</tr>
<tr>
<td>Decoy</td>
<td>not available</td>
<td>1</td>
<td>5.1%</td>
<td>5.4%</td>
<td>41.6%</td>
<td>14.8%</td>
<td>20.6%</td>
<td>20.6%</td>
</tr>
</tbody>
</table>
### Table A8

The following are changes in carbonylation protein relative abundances in ProteoMiner-depleted blood serum samples that were collected from Patient Y over the course of four days. The changes were not normalized to Day 1. No PeptideProphet scores below 0.9 were accepted. The proteins were grouped using ProteinProphet; however, the alpha-2-macroglobulin and pregnancy zone protein group did not contain related proteins like the other groups. The relative abundances were calculated by dividing the reporter ion intensity by the total intensity for the six reporter ions for each PSM and then averaging all PSMs composing the various protein groups.

<table>
<thead>
<tr>
<th>Protein Group Description</th>
<th>Associated Gene</th>
<th>Supporting PSM Count</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complement C4-A &amp; B</td>
<td>C4A &amp; C4B</td>
<td>589</td>
<td>36.8%</td>
<td>24.8%</td>
<td>15.6%</td>
<td>22.8%</td>
</tr>
<tr>
<td>Fibrinogen alpha chain</td>
<td>FGA</td>
<td>402</td>
<td>32.3%</td>
<td>21.9%</td>
<td>19.1%</td>
<td>26.7%</td>
</tr>
<tr>
<td>Complement C3</td>
<td>C3</td>
<td>279</td>
<td>36.5%</td>
<td>23.8%</td>
<td>17.1%</td>
<td>22.6%</td>
</tr>
<tr>
<td>Fibrinogen beta chain</td>
<td>FGB</td>
<td>249</td>
<td>31.9%</td>
<td>22.3%</td>
<td>19.8%</td>
<td>26.0%</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>ALB</td>
<td>226</td>
<td>31.0%</td>
<td>25.5%</td>
<td>18.1%</td>
<td>25.3%</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>CP</td>
<td>209</td>
<td>29.1%</td>
<td>28.9%</td>
<td>16.9%</td>
<td>25.1%</td>
</tr>
<tr>
<td>Vitronectin</td>
<td>VTN</td>
<td>199</td>
<td>32.8%</td>
<td>19.5%</td>
<td>16.9%</td>
<td>30.7%</td>
</tr>
<tr>
<td>Fibrinogen gamma chain</td>
<td>FGG</td>
<td>192</td>
<td>33.4%</td>
<td>21.4%</td>
<td>18.7%</td>
<td>26.4%</td>
</tr>
<tr>
<td>Thrombin</td>
<td>F2</td>
<td>186</td>
<td>31.4%</td>
<td>25.0%</td>
<td>18.2%</td>
<td>25.5%</td>
</tr>
<tr>
<td>Apolipoprotein A-I</td>
<td>APOA1</td>
<td>164</td>
<td>34.3%</td>
<td>27.7%</td>
<td>17.6%</td>
<td>20.4%</td>
</tr>
<tr>
<td>Serum amyloid A-1 &amp; 2 protein</td>
<td>SAA1 &amp; SAA2</td>
<td>144</td>
<td>15.9%</td>
<td>11.8%</td>
<td>18.9%</td>
<td>53.4%</td>
</tr>
<tr>
<td>Apolipoprotein B-100</td>
<td>APOB</td>
<td>114</td>
<td>33.1%</td>
<td>20.8%</td>
<td>17.9%</td>
<td>28.2%</td>
</tr>
<tr>
<td>Ig gamma chain C region</td>
<td>IGHG</td>
<td>110</td>
<td>36.0%</td>
<td>27.5%</td>
<td>14.8%</td>
<td>21.7%</td>
</tr>
<tr>
<td>Clusterin</td>
<td>CLU</td>
<td>100</td>
<td>28.8%</td>
<td>24.8%</td>
<td>19.6%</td>
<td>26.8%</td>
</tr>
<tr>
<td>Apolipoprotein E</td>
<td>APOE</td>
<td>97</td>
<td>28.4%</td>
<td>19.7%</td>
<td>20.4%</td>
<td>31.5%</td>
</tr>
<tr>
<td>Alpha-1-antitrypsin</td>
<td>SERPINA1</td>
<td>86</td>
<td>27.9%</td>
<td>27.0%</td>
<td>15.3%</td>
<td>29.8%</td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>CRP</td>
<td>77</td>
<td>15.4%</td>
<td>11.5%</td>
<td>15.2%</td>
<td>57.9%</td>
</tr>
<tr>
<td>Protein Name</td>
<td>Abbreviation</td>
<td>Sample Count</td>
<td>20.2%</td>
<td>17.2%</td>
<td>17.2%</td>
<td>45.4%</td>
</tr>
<tr>
<td>-------------------------------------------------</td>
<td>--------------</td>
<td>--------------</td>
<td>-------</td>
<td>--------</td>
<td>--------</td>
<td>-------</td>
</tr>
<tr>
<td>Lipopolysaccharide-binding protein</td>
<td>LBP</td>
<td>71</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ig mu chain C region</td>
<td>IGHM</td>
<td>65</td>
<td>34.1%</td>
<td>25.7%</td>
<td>17.0%</td>
<td>23.1%</td>
</tr>
<tr>
<td>Serum paraoxonase/ arylesterase I</td>
<td>PON1</td>
<td>62</td>
<td>36.2%</td>
<td>25.8%</td>
<td>14.8%</td>
<td>23.3%</td>
</tr>
<tr>
<td>Apolipoprotein A-IV</td>
<td>APOA4</td>
<td>59</td>
<td>28.2%</td>
<td>19.9%</td>
<td>22.5%</td>
<td>29.5%</td>
</tr>
<tr>
<td>Ig kappa chain C region</td>
<td>IGKC</td>
<td>59</td>
<td>34.2%</td>
<td>23.6%</td>
<td>20.8%</td>
<td>21.4%</td>
</tr>
<tr>
<td>Alpha-1-antichymotrypsin</td>
<td>SERPINA3</td>
<td>43</td>
<td>28.4%</td>
<td>20.4%</td>
<td>17.9%</td>
<td>33.3%</td>
</tr>
<tr>
<td>Alpha-2-macroglobulin &amp; Pregnancy zone protein</td>
<td>A2M &amp; PZP</td>
<td>32</td>
<td>34.2%</td>
<td>27.1%</td>
<td>16.4%</td>
<td>22.4%</td>
</tr>
<tr>
<td>Complement C1q subcomponent subunit C</td>
<td>C1QC</td>
<td>31</td>
<td>38.7%</td>
<td>21.7%</td>
<td>18.2%</td>
<td>21.3%</td>
</tr>
<tr>
<td>C4b-binding protein alpha chain</td>
<td>C4BPA</td>
<td>24</td>
<td>28.1%</td>
<td>33.6%</td>
<td>15.7%</td>
<td>22.6%</td>
</tr>
<tr>
<td>Galectin-3-binding protein</td>
<td>LGALS3BP</td>
<td>24</td>
<td>28.7%</td>
<td>23.1%</td>
<td>25.0%</td>
<td>23.2%</td>
</tr>
<tr>
<td>Inter-alpha-trypsin inhibitor heavy chain H4</td>
<td>ITIH4</td>
<td>24</td>
<td>27.2%</td>
<td>27.7%</td>
<td>19.7%</td>
<td>25.4%</td>
</tr>
<tr>
<td>Streptavidin</td>
<td>not available</td>
<td>23</td>
<td>34.2%</td>
<td>24.9%</td>
<td>18.5%</td>
<td>22.4%</td>
</tr>
<tr>
<td>Complement C1s subcomponent</td>
<td>C1S</td>
<td>20</td>
<td>31.7%</td>
<td>22.9%</td>
<td>16.1%</td>
<td>29.3%</td>
</tr>
<tr>
<td>Trypsin (Pig)</td>
<td>not available</td>
<td>16</td>
<td>29.5%</td>
<td>27.0%</td>
<td>17.3%</td>
<td>26.2%</td>
</tr>
<tr>
<td>Angiotensinogen</td>
<td>AGT</td>
<td>15</td>
<td>25.9%</td>
<td>18.7%</td>
<td>23.0%</td>
<td>32.5%</td>
</tr>
<tr>
<td>Decoy</td>
<td>not available</td>
<td>15</td>
<td>29.3%</td>
<td>19.6%</td>
<td>24.0%</td>
<td>27.1%</td>
</tr>
<tr>
<td>Complement C1q subcomponent subunit B</td>
<td>C1QB</td>
<td>14</td>
<td>33.3%</td>
<td>28.3%</td>
<td>14.3%</td>
<td>24.1%</td>
</tr>
<tr>
<td>Decoy</td>
<td>not available</td>
<td>11</td>
<td>29.6%</td>
<td>25.6%</td>
<td>19.3%</td>
<td>25.6%</td>
</tr>
<tr>
<td>Ig alpha chain C region</td>
<td>IGHA</td>
<td>9</td>
<td>32.0%</td>
<td>26.4%</td>
<td>17.4%</td>
<td>24.2%</td>
</tr>
<tr>
<td>Carboxypeptidase N catalytic chain</td>
<td>CPN1</td>
<td>8</td>
<td>24.0%</td>
<td>18.3%</td>
<td>26.1%</td>
<td>31.6%</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>HP</td>
<td>6</td>
<td>37.3%</td>
<td>33.8%</td>
<td>13.7%</td>
<td>15.1%</td>
</tr>
<tr>
<td>Vitamin K-dependent protein S</td>
<td>PROS1</td>
<td>6</td>
<td>37.3%</td>
<td>33.8%</td>
<td>13.7%</td>
<td>15.1%</td>
</tr>
<tr>
<td>C4b-binding protein beta chain</td>
<td>C4BPB</td>
<td>5</td>
<td>32.8%</td>
<td>32.0%</td>
<td>15.1%</td>
<td>20.1%</td>
</tr>
<tr>
<td>Complement component C9</td>
<td>C9</td>
<td>4</td>
<td>30.2%</td>
<td>27.4%</td>
<td>15.1%</td>
<td>27.3%</td>
</tr>
<tr>
<td>Ig lambda chain C region</td>
<td>IGLC</td>
<td>4</td>
<td>39.9%</td>
<td>27.9%</td>
<td>13.8%</td>
<td>18.4%</td>
</tr>
<tr>
<td>Protein Name</td>
<td>Gene Symbol</td>
<td>Exon</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------------------------------------------------</td>
<td>-------------</td>
<td>------</td>
<td>------------</td>
<td>------------</td>
<td>------------</td>
<td>------------</td>
</tr>
<tr>
<td>Phospholipid transfer protein</td>
<td>PLTP</td>
<td>4</td>
<td>25.3%</td>
<td>23.1%</td>
<td>23.4%</td>
<td>28.2%</td>
</tr>
<tr>
<td>Serum amyloid A-4 protein</td>
<td>SAA4</td>
<td>4</td>
<td>30.1%</td>
<td>26.7%</td>
<td>17.2%</td>
<td>26.0%</td>
</tr>
<tr>
<td>Apolipoprotein A-II</td>
<td>APOA2</td>
<td>3</td>
<td>32.8%</td>
<td>17.4%</td>
<td>17.9%</td>
<td>31.9%</td>
</tr>
<tr>
<td>Apolipoprotein C-III</td>
<td>APOC3</td>
<td>3</td>
<td>33.7%</td>
<td>29.7%</td>
<td>17.1%</td>
<td>19.4%</td>
</tr>
<tr>
<td>Apolipoprotein M</td>
<td>APOM</td>
<td>3</td>
<td>29.1%</td>
<td>18.0%</td>
<td>24.0%</td>
<td>28.9%</td>
</tr>
<tr>
<td>Coiled-coil domain-containing protein 40</td>
<td>CCDC40</td>
<td>3</td>
<td>29.1%</td>
<td>18.0%</td>
<td>24.0%</td>
<td>28.9%</td>
</tr>
<tr>
<td>Complement C1q subcomponent subunit A</td>
<td>C1QA</td>
<td>3</td>
<td>36.4%</td>
<td>23.4%</td>
<td>13.7%</td>
<td>26.5%</td>
</tr>
<tr>
<td>Decoy</td>
<td>not available</td>
<td>3</td>
<td>32.5%</td>
<td>23.1%</td>
<td>20.2%</td>
<td>24.2%</td>
</tr>
<tr>
<td>Serum amyloid P-component</td>
<td>APCS</td>
<td>3</td>
<td>31.7%</td>
<td>26.9%</td>
<td>14.5%</td>
<td>26.9%</td>
</tr>
<tr>
<td>Collectin-10</td>
<td>COLEC10</td>
<td>2</td>
<td>31.5%</td>
<td>20.7%</td>
<td>20.8%</td>
<td>27.0%</td>
</tr>
<tr>
<td>Decoy</td>
<td>not available</td>
<td>2</td>
<td>29.9%</td>
<td>31.9%</td>
<td>14.4%</td>
<td>23.8%</td>
</tr>
<tr>
<td>Fibulin-1</td>
<td>FBLN1</td>
<td>2</td>
<td>30.6%</td>
<td>24.2%</td>
<td>19.1%</td>
<td>26.1%</td>
</tr>
<tr>
<td>Hemoglobin subunit beta</td>
<td>HBB</td>
<td>2</td>
<td>28.7%</td>
<td>32.9%</td>
<td>15.8%</td>
<td>22.6%</td>
</tr>
<tr>
<td>Ig kappa chain V-IV regions</td>
<td>not available</td>
<td>2</td>
<td>33.0%</td>
<td>25.0%</td>
<td>19.6%</td>
<td>22.3%</td>
</tr>
<tr>
<td>Complement C1r subcomponent</td>
<td>C1R</td>
<td>1</td>
<td>29.8%</td>
<td>21.2%</td>
<td>23.5%</td>
<td>25.6%</td>
</tr>
<tr>
<td>Complement C5</td>
<td>C5</td>
<td>1</td>
<td>32.2%</td>
<td>29.1%</td>
<td>18.6%</td>
<td>20.0%</td>
</tr>
<tr>
<td>Complement factor H</td>
<td>CFH</td>
<td>1</td>
<td>32.2%</td>
<td>28.4%</td>
<td>19.0%</td>
<td>20.3%</td>
</tr>
<tr>
<td>Decoy</td>
<td>not available</td>
<td>1</td>
<td>28.7%</td>
<td>29.4%</td>
<td>17.2%</td>
<td>24.7%</td>
</tr>
<tr>
<td>Hemopexin</td>
<td>HPX</td>
<td>1</td>
<td>20.2%</td>
<td>25.5%</td>
<td>26.9%</td>
<td>27.4%</td>
</tr>
<tr>
<td>Inter-alpha-trypsin inhibitor heavy chain H3</td>
<td>ITIH3</td>
<td>1</td>
<td>26.3%</td>
<td>9.0%</td>
<td>21.8%</td>
<td>42.9%</td>
</tr>
<tr>
<td>Leucine-rich alpha-2-glycoprotein</td>
<td>LRG1</td>
<td>1</td>
<td>23.9%</td>
<td>14.8%</td>
<td>17.8%</td>
<td>43.6%</td>
</tr>
<tr>
<td>Microtubule cross-linking factor 1</td>
<td>MTCL1</td>
<td>1</td>
<td>31.5%</td>
<td>20.1%</td>
<td>17.1%</td>
<td>31.3%</td>
</tr>
<tr>
<td>Steryl-sulfatase</td>
<td>STS</td>
<td>1</td>
<td>36.6%</td>
<td>24.4%</td>
<td>15.2%</td>
<td>23.8%</td>
</tr>
<tr>
<td>WD repeat domain phosphoinositide-interacting protein 1</td>
<td>WIP11</td>
<td>1</td>
<td>28.2%</td>
<td>23.4%</td>
<td>24.9%</td>
<td>23.5%</td>
</tr>
</tbody>
</table>
Table A9

The following are changes in carbonylation protein relative abundances in ProteoMiner-depleted blood serum samples that were collected from Patient Z over the course of four days. The changes were not normalized to Day 1. No PeptideProphet scores below 0.9 were accepted. The proteins were grouped using ProteinProphet; however, the alpha-2-macroglobulin and pregnancy zone protein group did not contain related proteins like the other groups. The relative abundances were calculated by dividing the reporter ion intensity by the total intensity for the six reporter ions for each PSM and then averaging all PSMs composing the various protein groups.

<table>
<thead>
<tr>
<th>Protein Group Description</th>
<th>Associated Gene</th>
<th>Supporting PSM Count</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complement C4-A &amp; B</td>
<td>C4A &amp; C4B</td>
<td>634</td>
<td>25.8%</td>
<td>24.1%</td>
<td>24.8%</td>
<td>25.3%</td>
</tr>
<tr>
<td>Fibrinogen alpha chain</td>
<td>FGA</td>
<td>293</td>
<td>26.8%</td>
<td>24.6%</td>
<td>25.4%</td>
<td>23.3%</td>
</tr>
<tr>
<td>Fibrinogen beta chain</td>
<td>FGB</td>
<td>279</td>
<td>27.2%</td>
<td>26.0%</td>
<td>23.9%</td>
<td>23.0%</td>
</tr>
<tr>
<td>Complement C3</td>
<td>C3</td>
<td>278</td>
<td>26.2%</td>
<td>25.9%</td>
<td>24.1%</td>
<td>23.8%</td>
</tr>
<tr>
<td>Fibrinogen gamma chain</td>
<td>FGG</td>
<td>193</td>
<td>26.1%</td>
<td>25.3%</td>
<td>24.2%</td>
<td>24.4%</td>
</tr>
<tr>
<td>Vitronectin</td>
<td>VTN</td>
<td>179</td>
<td>26.7%</td>
<td>21.9%</td>
<td>20.4%</td>
<td>31.0%</td>
</tr>
<tr>
<td>Thrombin</td>
<td>F2</td>
<td>165</td>
<td>26.0%</td>
<td>24.3%</td>
<td>23.4%</td>
<td>26.2%</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>CP</td>
<td>162</td>
<td>26.7%</td>
<td>25.5%</td>
<td>23.8%</td>
<td>23.9%</td>
</tr>
<tr>
<td>Apolipoprotein A-1</td>
<td>APOA1</td>
<td>143</td>
<td>29.7%</td>
<td>25.6%</td>
<td>24.2%</td>
<td>20.5%</td>
</tr>
<tr>
<td>Serum amyloid A-1 &amp; 2 protein</td>
<td>SAA1 &amp; SAA2</td>
<td>139</td>
<td>5.0%</td>
<td>3.7%</td>
<td>30.5%</td>
<td>60.8%</td>
</tr>
<tr>
<td>Serum albumin</td>
<td>ALB</td>
<td>124</td>
<td>25.8%</td>
<td>18.7%</td>
<td>34.9%</td>
<td>20.6%</td>
</tr>
<tr>
<td>Apolipoprotein B-100</td>
<td>APOB</td>
<td>110</td>
<td>31.1%</td>
<td>22.9%</td>
<td>21.4%</td>
<td>24.6%</td>
</tr>
<tr>
<td>Serum paraoxonase/ arylesterase 1</td>
<td>PON1</td>
<td>95</td>
<td>27.7%</td>
<td>26.1%</td>
<td>25.0%</td>
<td>21.2%</td>
</tr>
<tr>
<td>Ig gamma chain C region</td>
<td>IGHG</td>
<td>81</td>
<td>33.1%</td>
<td>24.0%</td>
<td>23.3%</td>
<td>19.6%</td>
</tr>
<tr>
<td>Clusterin</td>
<td>CLU</td>
<td>75</td>
<td>28.3%</td>
<td>28.0%</td>
<td>19.7%</td>
<td>24.1%</td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>CRP</td>
<td>72</td>
<td>8.2%</td>
<td>6.5%</td>
<td>44.1%</td>
<td>41.2%</td>
</tr>
<tr>
<td>Ig kappa chain C region</td>
<td>IGKC</td>
<td>61</td>
<td>27.9%</td>
<td>24.6%</td>
<td>23.0%</td>
<td>24.6%</td>
</tr>
<tr>
<td>Ig mu chain C region</td>
<td>IGHM</td>
<td>52</td>
<td>30.3%</td>
<td>25.6%</td>
<td>23.0%</td>
<td>21.0%</td>
</tr>
<tr>
<td>Apolipoprotein A-IV</td>
<td>APOA4</td>
<td>48</td>
<td>31.2%</td>
<td>29.2%</td>
<td>20.7%</td>
<td>18.9%</td>
</tr>
<tr>
<td>Lipopolysaccharide-binding protein</td>
<td>LBP</td>
<td>43</td>
<td>18.4%</td>
<td>16.7%</td>
<td>36.6%</td>
<td>28.3%</td>
</tr>
<tr>
<td>Complement C1q subcomponent subunit C</td>
<td>C1QC</td>
<td>32</td>
<td>21.8%</td>
<td>20.6%</td>
<td>29.9%</td>
<td>27.6%</td>
</tr>
<tr>
<td>Alpha-1-antitrypsin</td>
<td>SERPINA1</td>
<td>31</td>
<td>26.0%</td>
<td>23.2%</td>
<td>24.1%</td>
<td>26.7%</td>
</tr>
<tr>
<td>Apolipoprotein E</td>
<td>APOE</td>
<td>30</td>
<td>25.2%</td>
<td>21.2%</td>
<td>25.3%</td>
<td>28.3%</td>
</tr>
<tr>
<td>Galectin-3-binding protein</td>
<td>LGALS3BP</td>
<td>25</td>
<td>26.3%</td>
<td>24.0%</td>
<td>23.6%</td>
<td>26.1%</td>
</tr>
<tr>
<td>Alpha-1-antichymotrypsin</td>
<td>SERPINA3</td>
<td>24</td>
<td>19.0%</td>
<td>16.5%</td>
<td>25.8%</td>
<td>38.7%</td>
</tr>
<tr>
<td>C4b-binding protein alpha chain</td>
<td>C4BPA</td>
<td>14</td>
<td>26.2%</td>
<td>23.1%</td>
<td>24.6%</td>
<td>26.1%</td>
</tr>
<tr>
<td>Ig kappa chain V-I regions</td>
<td>not available</td>
<td>14</td>
<td>35.0%</td>
<td>24.0%</td>
<td>22.2%</td>
<td>18.8%</td>
</tr>
<tr>
<td>Trypsin (Pig)</td>
<td>not available</td>
<td>11</td>
<td>26.3%</td>
<td>24.3%</td>
<td>25.7%</td>
<td>23.7%</td>
</tr>
<tr>
<td>Streptavidin</td>
<td>not available</td>
<td>10</td>
<td>28.0%</td>
<td>23.1%</td>
<td>26.5%</td>
<td>22.4%</td>
</tr>
<tr>
<td>Decoy</td>
<td>not available</td>
<td>7</td>
<td>6.5%</td>
<td>40.8%</td>
<td>26.4%</td>
<td>26.3%</td>
</tr>
<tr>
<td>-------</td>
<td>---------------</td>
<td>---</td>
<td>------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>Alpha-2-macroglobulin &amp; Pregnancy Zone Protein</td>
<td>A2M &amp; PZP</td>
<td>6</td>
<td>28.3%</td>
<td>23.1%</td>
<td>25.0%</td>
<td>23.6%</td>
</tr>
<tr>
<td>Phosphatidylinositol-glycan-specific phospholipase D</td>
<td>GPLD1</td>
<td>6</td>
<td>30.6%</td>
<td>27.1%</td>
<td>24.8%</td>
<td>17.6%</td>
</tr>
<tr>
<td>Complement C1s subcomponent</td>
<td>C1s</td>
<td>4</td>
<td>26.0%</td>
<td>21.7%</td>
<td>24.5%</td>
<td>27.8%</td>
</tr>
<tr>
<td>Inter-alpha-trypsin inhibitor heavy chain H4</td>
<td>ITIH4</td>
<td>4</td>
<td>26.2%</td>
<td>19.8%</td>
<td>26.3%</td>
<td>27.7%</td>
</tr>
<tr>
<td>Apolipoprotein C-III</td>
<td>APOC3</td>
<td>3</td>
<td>25.9%</td>
<td>25.8%</td>
<td>22.0%</td>
<td>26.3%</td>
</tr>
<tr>
<td>Carboxypeptidase N catalytic chain</td>
<td>CPN1</td>
<td>3</td>
<td>17.3%</td>
<td>18.0%</td>
<td>36.2%</td>
<td>28.5%</td>
</tr>
<tr>
<td>Complement C1q subcomponent subunit B</td>
<td>C1QB</td>
<td>3</td>
<td>26.0%</td>
<td>23.7%</td>
<td>24.1%</td>
<td>26.2%</td>
</tr>
<tr>
<td>Decoy</td>
<td>not available</td>
<td>3</td>
<td>24.2%</td>
<td>25.2%</td>
<td>25.7%</td>
<td>24.9%</td>
</tr>
<tr>
<td>Decoy</td>
<td>not available</td>
<td>3</td>
<td>25.3%</td>
<td>29.0%</td>
<td>24.3%</td>
<td>21.4%</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>HP</td>
<td>3</td>
<td>25.1%</td>
<td>18.4%</td>
<td>34.3%</td>
<td>22.2%</td>
</tr>
<tr>
<td>Complement C1q subcomponent subunit A</td>
<td>C1QA</td>
<td>2</td>
<td>25.2%</td>
<td>30.1%</td>
<td>24.7%</td>
<td>20.0%</td>
</tr>
<tr>
<td>Complement C1r subcomponent</td>
<td>C1R</td>
<td>2</td>
<td>28.5%</td>
<td>23.5%</td>
<td>25.4%</td>
<td>22.7%</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>FN1</td>
<td>2</td>
<td>20.7%</td>
<td>26.1%</td>
<td>29.2%</td>
<td>24.0%</td>
</tr>
<tr>
<td>Vitamin K-dependent protein S</td>
<td>PROS1</td>
<td>2</td>
<td>27.0%</td>
<td>22.8%</td>
<td>24.9%</td>
<td>25.4%</td>
</tr>
<tr>
<td>Apolipoprotein M</td>
<td>APOM</td>
<td>1</td>
<td>25.9%</td>
<td>26.0%</td>
<td>27.4%</td>
<td>20.7%</td>
</tr>
<tr>
<td>C4b-binding protein beta chain</td>
<td>C4BPB</td>
<td>1</td>
<td>31.6%</td>
<td>24.6%</td>
<td>21.5%</td>
<td>22.3%</td>
</tr>
<tr>
<td>Coiled-coil domain-containing protein 40</td>
<td>CCDC40</td>
<td>1</td>
<td>23.2%</td>
<td>23.2%</td>
<td>25.2%</td>
<td>28.3%</td>
</tr>
<tr>
<td>Decoy</td>
<td>not available</td>
<td>1</td>
<td>28.9%</td>
<td>25.4%</td>
<td>21.6%</td>
<td>24.1%</td>
</tr>
<tr>
<td>E3 ubiquitin-protein ligase</td>
<td>HUWE1</td>
<td>1</td>
<td>28.9%</td>
<td>23.2%</td>
<td>16.9%</td>
<td>31.0%</td>
</tr>
<tr>
<td>Serum amyloid P-component</td>
<td>APCS</td>
<td>1</td>
<td>32.2%</td>
<td>30.0%</td>
<td>17.8%</td>
<td>20.0%</td>
</tr>
</tbody>
</table>
Appendix 4

The following are mass spectra corresponding to Chapters 2 and 3. Generally, two spectra are included for each peptide: a modified and unmodified (in that order). Some modified peptide spectra did not have unmodified counterparts. In some cases, both the 2+ and 3+ peptides were modified and thus spectra are included for both charge states.

<table>
<thead>
<tr>
<th>Mass Spectra Description</th>
<th>Page Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 2 (Peptide Enrichment) – Label 1</td>
<td>192-203</td>
</tr>
<tr>
<td>Chapter 2 (Peptide Enrichment) – Label 2</td>
<td>204-221</td>
</tr>
<tr>
<td>Chapter 2 (Peptide Enrichment) – Label 3</td>
<td>222-231</td>
</tr>
<tr>
<td>Chapter 2 (Protein Enrichment) – Label 2</td>
<td>232-234</td>
</tr>
<tr>
<td>Chapter 2 (Protein Enrichment) – Label 4</td>
<td>235-253</td>
</tr>
<tr>
<td>Chapter 3 – 25% spike sample</td>
<td>254-267</td>
</tr>
<tr>
<td>Chapter 3 – 50% spike sample</td>
<td>268-286</td>
</tr>
<tr>
<td>Chapter 3 – 75% spike sample</td>
<td>287-314</td>
</tr>
</tbody>
</table>
RHPYFYAPELFFAK, MH+ 1898.9952, m/z 633.6699

File: 20120529_09_BHZ_2.14811.14811.3, Scan: 14811, Exp. m/z: 634.06, Charge: 3

<table>
<thead>
<tr>
<th>Ion</th>
<th>m/z</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>a1+</td>
<td>157.1084</td>
<td>79.6578</td>
</tr>
<tr>
<td>a2+</td>
<td>786.1671</td>
<td>147.5873</td>
</tr>
<tr>
<td>a3+</td>
<td>391.2201</td>
<td>196.1137</td>
</tr>
<tr>
<td>b1+</td>
<td>554.1834</td>
<td>377.6433</td>
</tr>
<tr>
<td>b2+</td>
<td>761.3518</td>
<td>551.1795</td>
</tr>
<tr>
<td>b3+</td>
<td>664.4151</td>
<td>432.7112</td>
</tr>
<tr>
<td>c1+</td>
<td>915.4593</td>
<td>603.5871</td>
</tr>
<tr>
<td>c2+</td>
<td>1032.5050</td>
<td>566.7564</td>
</tr>
<tr>
<td>x1+</td>
<td>1161.5476</td>
<td>581.2774</td>
</tr>
<tr>
<td>x2+</td>
<td>1274.6317</td>
<td>637.8195</td>
</tr>
<tr>
<td>y1+</td>
<td>1387.7157</td>
<td>694.3615</td>
</tr>
<tr>
<td>y2+</td>
<td>1534.8541</td>
<td>762.4067</td>
</tr>
<tr>
<td>z1+</td>
<td>1681.8526</td>
<td>861.4299</td>
</tr>
<tr>
<td>z2+</td>
<td>1752.8897</td>
<td>876.9405</td>
</tr>
</tbody>
</table>

Neutral Loss:
- H2O (o)
- NH3 (*)

Mass Type:
- Mono
- Avg

Mass Tol: 0.5

Peak Assignment:
- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:
- Ion
- m/z
- None

Width: 650
Height: 400

COMET/Lorikeet Spectrum Viewer
(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
Ions:
- a
- b
- c
- x
- y
- z

Neutral Loss:
- H\textsubscript{2}O (o)
- NH\textsubscript{3} (*)

Mass Type:
- Mono
- Avg

Mass Tol: 0, 0

Peak Assignment:
- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:
- Ion
- m/z
- None

Width: 650
Height: 400

Click and drag in the plot to zoom X: Y: Zoom Out
Print: Enable tooltip

Click and drag in the plot to zoom X: Y: 1097

COMET/Lorikeet Spectrum Viewer
(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
Ions:

- a+ 1+
- b+ 1+
- c+ 1+
- x+ 1+
- y+ 1+
- z+ 1+

Neutral Loss:

- H₂O (o)
- NH₃ (*)

Mass Type:

- Mono
- Avg

Mass Tol: ±<value>

Peak Assignment:

- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:

- Ion
- m/z
- None

Width: 650
Height: 400

COMET/Lorikeet Spectrum Viewer
(TPP v4.6 OCCUPY rev 3, Build 20130724109 (MinGW))
**COMET/Lorikeet Spectrum Viewer**  
(TPP v.6.0 OCCUPY rev 3, Build 201307241109 (MinGW))
ALVLIAFAQYLQQ\EPFEDHVK, MH+ 2490.2849, m/z 830.7665
File: 20120529_22_LC_2.17580.17580.3, Scan: 17580, Exp. m/z: 831.25, Charge: 3

Ions:
a 1* 2* 3*
b 1* 2* 3*
c 1* 2* 3*
x 1* 2* 3*
y 1* 2* 3*
z 1* 2* 3*

Neutral Loss:
- H2O (o)
- NH3 (*)

Mass Type:
- Mono
- Avg

Peak Assignment:
- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:
- Ion
- m/z
- None

Width: 650
Height: 400

COMET/Lorikeet Spectrum Viewer
(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
### Ions:

- a
- b
- c
- x
- y
- z

### Neutral Loss:
- $\text{H}_2\text{O}$
- $\text{NH}_3$

### Mass Type:
- Mono
- Avg

### Mass Tol:
- Peak Assignment:
  - Most Intense
  - Nearest Match
  - Peak Detect

### Peak Labels:
- Ion
- m/z

### Width:
- 650

### Height:
- 400

### Peak Label:
- m/z

### Modifications:

### COMET/Lorikeet Spectrum Viewer

(Comet 4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
Ions:

- a
- b
- c
- x
- y
- z

Neutral Loss:

- H₂O
- NH₃

Mass Type:

- Mono
- Avg

Mass Tol: 0.5

Peak Assignment:

- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:

- Ion
- m/z
- None

Width: 650

Height: 400

Click and drag in the plot to zoom

X: 207

Y: 1300

Enable tooltip

File: 20120529_22_LC_2.20904.20904.3, Scan: 20904, Exp. m/z: 633.91, Charge: 3

RHPFYAPELLFAK, MH+ 1898.9952, m/z 633.6699

Peak Assignment:

Most Intense

Nearest Match

Peak Detect

Peak Labels:

Ion

m/z

None

Width: 650

Height: 400

Click and drag in the plot to zoom

X: 207

Y: 1300

Enable tooltip

COMET/Lorikeet Spectrum Viewer
(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))

157.1084 79.0578 1 R 15
191.2701 196.1137 3 P 13 1605.8352 403.4277
234.2684 237.6403 4 Y 12 1508.7824 754.8948
261.3158 351.3795 5 F 11 1345.7919 637.3542
264.4151 432.7112 6 Y 10 1198.6567 599.8299
315.4637 468.7294 7 A 9 501.5873 518.7973
302.3520 512.7564 8 P 8 964.9502 482.7298
1161.5476 581.2774 9 E 8 867.4975 434.2524
1274.6317 637.8188 10 L 7 918.4469 369.7311
1387.7157 694.3635 11 L 6 625.3708 313.1890
1354.7841 767.8957 12 K 6 912.2967 256.6470
1481.8532 861.4299 13 K 5 1151.2183 183.1528
1752.8897 878.9487 14 A 4 218.1499 109.5786
147.1128 74.0600

Click to move table
Ions:
- a
- b
- c
- x
- y
- z

Neutral Loss:
- H₂O
- NH₃

Mass Type:
- Mono
- Avg

Mass Tol:
- 0.5

Peak Assignment:
- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:
- Ion
- m/z
- None

Width:
- 650

Height:
- 400

COMET/Lorikeet Spectrum Viewer
(TPP v4.6, OCCUPY rev 3, Build 201307241109 (MinGW))
IONS:

- a
- b
- c
- x
- y
- z

NEUTRAL LOSS:
- H$_2$O
- NH$_3$

MASS TYPE:
- Mono
- Avg

MASS TOL:
- 0.5

PEAK ASSIGNMENT:
- Most Intense
- Nearest Match
- Peak Detect

PEAK LABELS:
- Ion
- m/z
- None

WIDTH: 650
HEIGHT: 400

COMET/Lorikeet Spectrum Viewer
(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
Ions:
- a
- b
- c
- x
- y
- z

Neutral Loss:
- H₂O (o)
- NH₃ (*)

Mass Type:
- Mono
- Avg

Mass Tol: 0.5

Peak Assignment:
- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:
- Ion
- m/z
- None

Width: 650
Height: 400

 modifications:
- C: 57.021415 [3]
- K: 411.230437 [13]
- Q: -17.026478 [1]

COMET/Lorikeet Spectrum Viewer
(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
Ions:

- a
- b
- c
- x
- y
- z

Neutral Loss:

- H₂O (o)
- NH₃ (*)

Mass Type:

- Mono
- Avg

Mass Tol: 0.5

Peak Assignment:

- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:

- Ion m/z
- None

Width: 650
Height: 400

Requirements:

- a
- b
- c
- x
- y
- z

Neutral Loss:

- H₂O (o)
- NH₃ (*)

Mass Type:

- Mono
- Avg

Mass Tol: 0.5

Peak Assignment:

- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:

- Ion m/z
- None

Width: 650
Height: 400

COMET/Lorikeet Spectrum Viewer
(TPP v.6. OCCUPY rev 3, Build 20130724109 (MinGW))
**IONS**

<table>
<thead>
<tr>
<th>Ion</th>
<th>b1</th>
<th>b2</th>
<th>y1</th>
<th>y2</th>
<th>z1</th>
<th>z2</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>1+</td>
<td>2+</td>
<td>3+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>1+</td>
<td>2+</td>
<td>3+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c</td>
<td>1+</td>
<td>2+</td>
<td>3+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>x</td>
<td>1+</td>
<td>2+</td>
<td>3+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>y</td>
<td>1+</td>
<td>2+</td>
<td>3+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>z</td>
<td>1+</td>
<td>2+</td>
<td>3+</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**NEUTRAL LOSS**

- H$_2$O (o)
- NH$_3$ (*)

**MSS MASS TYPE**

- Mono
- Avg

**MASS TOL**

- 0.5

**PEAK ASSIGNMENT**

- Most Intense
- Nearest Match
- Peak Detect

**PEAK LABELS**

- Ion
- m/z
- None

**Width**

- 650

**Height**

- 400

---

**COMET/Lorikeet Spectrum Viewer**

(PPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
**Ions:**
- a\(\pm 1^+\), b\(\pm 2^+\), c\(\pm 3^+\)
- x\(\pm 1^+\), y\(\pm 1^+\), z\(\pm 2^+\), 2\(\pm 3^+\)

**Neutral Loss:**
- H\(_2\)O (o)
- NH\(_3\) (*)

**Mass Type:**
- Mono
- Avg

**Mass Tol:** 0.5

**Peak Assignment:**
- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**
- Ion m/z
- None

**Width:** 650
**Height:** 400

**Modifications:**
- K: 411.230437 [K]

---

**COMET/Lorikeet Spectrum Viewer**
(TPP v4.6 OCCUPY rev 3, Build 20130724109 (MinGW))

---

<table>
<thead>
<tr>
<th>m/z</th>
<th>b+</th>
<th>b2+</th>
<th>#</th>
<th>Seq</th>
<th>#</th>
<th>y-</th>
<th>y2-</th>
</tr>
</thead>
<tbody>
<tr>
<td>258.1084</td>
<td>129.5579</td>
<td>2</td>
<td>Q1</td>
<td>1923.1028</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>371.1925</td>
<td>186.0999</td>
<td>1</td>
<td>L1</td>
<td>1995.0442</td>
<td>984.0378</td>
<td></td>
<td></td>
</tr>
<tr>
<td>910.5179</td>
<td>455.7626</td>
<td>3</td>
<td>K1</td>
<td>1881.9602</td>
<td>941.4817</td>
<td></td>
<td></td>
</tr>
<tr>
<td>981.5580</td>
<td>491.2812</td>
<td>5</td>
<td>A1</td>
<td>1342.1348</td>
<td>671.8210</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1080.6574</td>
<td>542.8154</td>
<td>7</td>
<td>V1</td>
<td>1271.5977</td>
<td>636.3025</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1711.6410</td>
<td>606.3356</td>
<td>9</td>
<td>M1</td>
<td>1177.5792</td>
<td>586.7651</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1326.6469</td>
<td>663.5647</td>
<td>13</td>
<td>D9</td>
<td>1041.4800</td>
<td>521.2480</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1441.7175</td>
<td>721.3625</td>
<td>15</td>
<td>D</td>
<td>926.4618</td>
<td>463.7345</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1591.7963</td>
<td>794.8967</td>
<td>17</td>
<td>F8</td>
<td>814.4340</td>
<td>406.2211</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1659.8223</td>
<td>835.4153</td>
<td>19</td>
<td>A6</td>
<td>664.3665</td>
<td>332.6869</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1730.8465</td>
<td>916.9329</td>
<td>21</td>
<td>A5</td>
<td>593.3293</td>
<td>297.1683</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1877.9389</td>
<td>939.4681</td>
<td>23</td>
<td>F4</td>
<td>522.2992</td>
<td>261.6498</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1976.9973</td>
<td>981.0023</td>
<td>25</td>
<td>V3</td>
<td>375.2238</td>
<td>188.1155</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2106.0089</td>
<td>1051.5324</td>
<td>27</td>
<td>E2</td>
<td>276.1954</td>
<td>138.5813</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2197.0707</td>
<td>1119.0707</td>
<td>29</td>
<td>E1</td>
<td>275.1954</td>
<td>138.5813</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2281.1311</td>
<td>1187.1311</td>
<td>31</td>
<td>E1</td>
<td>275.1954</td>
<td>138.5813</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2369.1925</td>
<td>1255.1925</td>
<td>33</td>
<td>E1</td>
<td>275.1954</td>
<td>138.5813</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2457.2539</td>
<td>1323.2539</td>
<td>35</td>
<td>E1</td>
<td>275.1954</td>
<td>138.5813</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2545.3153</td>
<td>1391.3153</td>
<td>37</td>
<td>E1</td>
<td>275.1954</td>
<td>138.5813</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2633.3766</td>
<td>1459.3766</td>
<td>39</td>
<td>E1</td>
<td>275.1954</td>
<td>138.5813</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Click and drag in the plot to zoom**
**X:** [ ] **Y:** [ ]

**Enable tooltip**

---

**MS1 scan:** 17447, RT 5688.30
**m/z:** 1126.5763

---

**File:** 20120529_22_LC_2.17450.17450.2, Scan: 17450, Exp. m/z: 1126.92, Charge: 2

---

**Click and drag in the plot to move table**

---

**Modifications:**
- K: 411.230437 [K]
**Ions:**

- Ions:  
  - a 1+ 2+ 3+  
  - b 1+ 2+ 3+  
  - c 1+ 2+ 3+  
  - x 1+ 2+ 3+  
  - y 1+ 2+ 3+  
  - z 1+ 2+ 3+  

**Neutral Loss:**
- H₂O (o)  
- NH₃ (*)

**Mass Type:**
- Mono Avg

**Mass Tol:** 0.5

**Peak Assignment:**
- Most Intense  
- Nearest Match  
- Peak Detect

**Peak Labels:**
- Ion m/z  
- None

**Width:** 650  
**Height:** 400

---

**COMET/Lorikeet Spectrum Viewer**
(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
Ions:

- \(a\) 1\(^+\) 2\(^+\) 3\(^+\)
- \(b\) 1\(^+\) 2\(^+\) 3\(^+\)
- \(c\) 1\(^+\) 2\(^+\) 3\(^+\)
- \(x\) 1\(^+\) 2\(^+\) 3\(^+\)
- \(y\) 1\(^+\) 2\(^+\) 3\(^+\)
- \(z\) 1\(^+\) 2\(^+\) 3\(^+\)

Neutral Loss:
- \(H_2O\) (o)
- \(NH_3\) (*)

Mass Type:
- Mono
- Avg

Mass Tol: 0.5

Peak Assignment:
- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:
- Ion
- m/z
- None

Width: 650
Height: 400

COMET/Lorikeet Spectrum Viewer
(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
**Ions:**

- a \(1^+\) \(2^+\) \(3^+\)
- b \(1^+\) \(2^+\) \(3^+\)
- c \(1^+\) \(2^+\) \(3^+\)
- x \(1^+\) \(2^+\) \(3^+\)
- y \(1^+\) \(2^+\) \(3^+\)
- z \(1^+\) \(2^+\) \(3^+\)

**Neutral Loss:**

- H\(_2\)O (\(\circ\))
- NH\(_3\) (\(*\))

**Mass Type:**

- Mono  Avg

**Mass Tol:**

- 0,5

**Peak Assignment:**

- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**

- Ion  m/z  None

**Width:** 650

**Height:** 400

**Modifications:**

- C: 57.021415 [14]
**Ions:**
- a: $a_1^+$, $a_2^+$, $a_3^+$
- b: $b_1^+$, $b_2^+$, $b_3^+$
- c: $c_1^+$, $c_2^+$, $c_3^+$
- x: $x_1^+$, $x_2^+$, $x_3^+$
- y: $y_1^+$, $y_2^+$, $y_3^+$
- z: $z_1^+$, $z_2^+$, $z_3^+$

**Neutral Loss:**
- $\text{H}_2\text{O} (~o)$
- $\text{NH}_3 (~*)$

**Mass Type:**
- Mono
- Avg

**Peak Assignment:**
- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**
- Ion
- $m/z$

**Width:** 650

**Height:** 400

**Modifications:**
- H: 545.288188 (2)

**COMET/Lorikeet Spectrum Viewer**
(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
Ions:
- a 1+, 2+, 3+
- b 1+, 2+, 3+
- c 1+, 2+, 3+
- x 1+, 2+, 3+
- y 1+, 2+, 3+
- z 1+, 2+, 3+

Neutral Loss:
- H$_2$O (o)
- NH$_3$ (*)

Mass Type:
- Mono
- Avg

Mass Tol: 0.5

Peak Assignment:
- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:
- Ion
- m/z
- None

Width: 650
Height: 400

File: 20120529_34_PEG_2.22921.22921.3, Scan: 22921, Exp. m/z: 634.09, Charge: 3

**RHPYFYAPELLFFAK**, MH$^+$ 1898.9952, m/z 633.6699

**Peak Assignment**:
- **Most Intense**
- **Nearest Match**
- **Peak Detect**

**Peak Labels**:
- **Ion**
- **m/z**
- **None**

Click and drag in the plot to zoom

Click to move table

COMET/Lorikeet Spectrum Viewer
(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
### Ions:

- a\(^+\)\(^+\)\(^+\)\(^+\)\(^+\)
- b\(^+\)\(^+\)\(^+\)\(^+\)\(^+\)
- c\(^+\)\(^+\)\(^+\)\(^+\)\(^+\)
- x\(^+\)\(^+\)\(^+\)\(^+\)\(^+\)
- y\(^+\)\(^+\)\(^+\)\(^+\)\(^+\)
- z\(^+\)\(^+\)\(^+\)\(^+\)\(^+\)

### Neutral Loss:
- H\(_2\)O (o)
- NH\(_3\) (*)

### Mass Type:
- Mono
- Avg

### Mass Tol:
- µ, γ

### Peak Assignment:
- Most Intense
- Nearest Match
- Peak Detect

### Peak Labels:
- Ion m/z
- None

### Width:
- 650

### Height:
- 400

### COMET/Lorikeet Spectrum Viewer

- TPP v4.6 OCCUPY rev 3, Build 20130724109 (MinGW)

### File: 20120529_34_PEG_2.16254.16254.3, Scan: 16254, Exp. m/z: 992.33, Charge: 3

<table>
<thead>
<tr>
<th>b+</th>
<th>b2+</th>
<th># Seq</th>
<th># y-</th>
<th>y2+</th>
</tr>
</thead>
<tbody>
<tr>
<td>88.0393</td>
<td>44.5333</td>
<td>5</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>225.0982</td>
<td>113.0327</td>
<td>6</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>385.1288</td>
<td>193.0680</td>
<td>7</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>498.2129</td>
<td>249.6101</td>
<td>8</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>569.2800</td>
<td>285.1266</td>
<td>9</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>698.2926</td>
<td>349.6499</td>
<td>10</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>767.3410</td>
<td>399.1841</td>
<td>11</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>926.4026</td>
<td>463.7954</td>
<td>12</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>1040.4445</td>
<td>520.7269</td>
<td>13</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>1165.4710</td>
<td>578.3403</td>
<td>14</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>1284.5161</td>
<td>642.7617</td>
<td>15</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>1415.5565</td>
<td>708.2819</td>
<td>16</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>1512.6093</td>
<td>756.8083</td>
<td>17</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>1843.6166</td>
<td>872.3126</td>
<td>18</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>1884.6473</td>
<td>891.6402</td>
<td>19</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>1981.7201</td>
<td>940.3234</td>
<td>20</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>1908.8102</td>
<td>954.9087</td>
<td>21</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>1995.8422</td>
<td>996.4024</td>
<td>22</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>2100.9063</td>
<td>1025.7258</td>
<td>23</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>2179.9634</td>
<td>1091.6463</td>
<td>24</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>2251.0005</td>
<td>1126.0039</td>
<td>25</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>2336.0275</td>
<td>1183.5174</td>
<td>26</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>2513.0595</td>
<td>1327.0516</td>
<td>27</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>2612.1643</td>
<td>1300.5853</td>
<td>28</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>2701.2089</td>
<td>1371.0711</td>
<td>29</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>2828.2359</td>
<td>1414.6214</td>
<td>30</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>3124.2527</td>
<td>1471.1128</td>
<td>31</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

**Click to move table**

**Modifications:**
- C: 57.021415 [3]

**Click and drag in the plot to zoom**

**X:**

**Y:**

**Enable tooltip**

**COMET/Lorikeet Spectrum Viewer**

(TPP v.6 OCCUPY rev 3, Build 20130724109 (MinGW))
**Ions:**

- a 1+ 2+ 3+
- b 1+ 2+ 3+
- c 1+ 2+ 3+
- x 1+ 2+ 3+
- y 1+ 2+ 3+
- z 1+ 2+ 3+

**Neutral Loss:**

- H₂O (o)
- NH₃ (*)

**Mass Type:**

- Mono
- Avg

**Mass Tol:**

- Update

**Peak Assignment:**

- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**

- Ion
- m/z
- None

**Width:** 650

**Height:** 400

---

**COMET/Lorikeet Spectrum Viewer**

(TPP v4.6 OCCUPY rev 3, Build 20130724109 (MinGW))

---

**Neutral Loss:**

- H₂O (o)
- NH₃ (*)

**Mass Type:**

- Mono
- Avg

**Mass Tol:**

- Update

**Peak Assignment:**

- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**

- Ion
- m/z
- None

**Width:** 650

**Height:** 400

---

**COMET/Lorikeet Spectrum Viewer**

(TPP v4.6 OCCUPY rev 3, Build 20130724109 (MinGW))
Ions:

- **a** 1+ 2+ 3+
- **b** 1+ 2+ 3+
- **c** 1+ 2+ 3+
- **x** 1+ 2+ 3+
- **y** 1+ 2+ 3+
- **z** 1+ 2+ 3+

Neutral Loss:

- H$_2$O (o)
- NH$_3$ (*)

Mass Type:

- Mono
- Avg

Mass Tol: 0.5

Peak Assignment:

- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:

- Ion
- m/z
- None

Width: 650

Height: 400

Click and drag in the plot to zoom X: Y: 7
dim Out Print Enable tooltip

File: 20120529_32_PEG_2.04893.04893.3, Scan: 4893, Exp. m/z: 555.62, Charge: 3

YKAAFTE C CQAADK, MH+ 1662.7250, m/z 554.9132

Neutral Loss:

- H$_2$O (o)
- NH$_3$ (*)

Mass Type:

- Mono
- Avg

Mass Tol: 0.5

Peak Assignment:

- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:

- Ion
- m/z
- None

Click and drag in the plot to zoom X: Y: 7
dim Out Print Enable tooltip

Table:

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>b</td>
<td>b+</td>
<td>y</td>
<td>y+</td>
<td>y2+</td>
</tr>
<tr>
<td>164.0706</td>
<td>82.5389</td>
<td>1</td>
<td>Y</td>
<td>14</td>
</tr>
<tr>
<td>792.8484</td>
<td>146.5864</td>
<td>2</td>
<td>K</td>
<td>13</td>
</tr>
<tr>
<td>363.0227</td>
<td>182.1050</td>
<td>3</td>
<td>A</td>
<td>12</td>
</tr>
<tr>
<td>436.2788</td>
<td>217.6325</td>
<td>4</td>
<td>A</td>
<td>11</td>
</tr>
<tr>
<td>581.3082</td>
<td>291.1577</td>
<td>5</td>
<td>F</td>
<td>10</td>
</tr>
<tr>
<td>682.3559</td>
<td>341.6791</td>
<td>6</td>
<td>T</td>
<td>9</td>
</tr>
<tr>
<td>815.3984</td>
<td>456.7909</td>
<td>7</td>
<td>E</td>
<td>8</td>
</tr>
<tr>
<td>975.4289</td>
<td>486.2182</td>
<td>8</td>
<td>C</td>
<td>7</td>
</tr>
<tr>
<td>1131.4971</td>
<td>566.2335</td>
<td>9</td>
<td>C</td>
<td>6</td>
</tr>
<tr>
<td>1299.5183</td>
<td>600.3147</td>
<td>10</td>
<td>Q</td>
<td>5</td>
</tr>
<tr>
<td>1330.5544</td>
<td>655.7813</td>
<td>11</td>
<td>A</td>
<td>4</td>
</tr>
<tr>
<td>1401.5925</td>
<td>705.2999</td>
<td>12</td>
<td>A</td>
<td>3</td>
</tr>
<tr>
<td>1515.6194</td>
<td>766.3173</td>
<td>13</td>
<td>D</td>
<td>2</td>
</tr>
<tr>
<td>1640.6563</td>
<td>826.3245</td>
<td>14</td>
<td>K</td>
<td>1</td>
</tr>
</tbody>
</table>

Average: 57.02145 [8, 9]

Click to move table

Click and drag in the plot to zoom X: Y: 7
dim Out Print Enable tooltip

Click and drag in the plot to zoom X: Y: 7
dim Out Print Enable tooltip

COMET/Lorikeet Spectrum Viewer

(TPP v6.6 OCCUPY rev 3, Build 201307241109 (MinGW))
Ions:

- **a**
- **b**
- **c**
- **x**
- **y**
- **z**

Neutral Loss:

- H₂O (o)
- NH₃ (*)

Mass Type:

- Mono
- Avg

Mass Tol: 0.5

Peak Assignment:

- Most Intense
- Nearest Match
- Peak Detect

Width: 650

Height: 400

Click and drag in the plot to zoom X: Y: Zoom Out: Print: Enable tooltip

Modifications:

- C: 57.021415 [3]
- K: 545.288237 [13]
- Q: -17.026478 [1]
RHPYFFAPELLFFAK, MH+ 2310.2256, m/z 770.7467

File: 20140712_09_Treated_B_2.17386.17386.3, Scan: 17386, Exp. m/z: 771.06, Charge: 3

Ions:
- a
- b
- c
- x
- y
- z

Neutral Loss:
- H₂O (o)
- NH₃ (*)

Mass Type:
- Mono
- Avg

Mass Tol: 0.5

Peak Assignment:
- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:
- Ion
- m/z
- None

Width: 650
Height: 400

Click and drag in the plot to zoom
X: Y:
70mm Cut

COMET/Lorikeet Spectrum Viewer
(Add: 4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
**Ions:**

- a
- b
- c
- x
- y
- z

**Neutral Loss:**

- H₂O (o)
- NH₃ (*)

**Mass Type:**

- Mono
- Avg

**Mass Tol:** 0.5

**Peak Assignment:**

- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**

- Ion
- m/z
- None

**Width:** 650
**Height:** 400

---

**Peak List: B+**

<table>
<thead>
<tr>
<th>B+</th>
<th>B2+</th>
<th>Seq</th>
<th>y+</th>
<th>y2+</th>
</tr>
</thead>
<tbody>
<tr>
<td>157,1084</td>
<td>79.6578</td>
<td>1</td>
<td>R</td>
<td>15</td>
</tr>
<tr>
<td>196,1671</td>
<td>147.5873</td>
<td>2</td>
<td>H</td>
<td>14</td>
</tr>
<tr>
<td>196,1137</td>
<td>147.5873</td>
<td>3</td>
<td>P</td>
<td>13</td>
</tr>
<tr>
<td>1605.8326</td>
<td>1605.8326</td>
<td>4</td>
<td>Y</td>
<td>12</td>
</tr>
<tr>
<td>1508.7824</td>
<td>754.8948</td>
<td>5</td>
<td>F</td>
<td>11</td>
</tr>
<tr>
<td>1345.7191</td>
<td>673.3632</td>
<td>6</td>
<td>Y</td>
<td>10</td>
</tr>
<tr>
<td>1198.6507</td>
<td>599.8290</td>
<td>7</td>
<td>E</td>
<td>9</td>
</tr>
<tr>
<td>681.8585</td>
<td>340.9292</td>
<td>8</td>
<td>A</td>
<td>8</td>
</tr>
<tr>
<td>642.3078</td>
<td>321.1539</td>
<td>9</td>
<td>L</td>
<td>7</td>
</tr>
<tr>
<td>607.9475</td>
<td>303.9238</td>
<td>10</td>
<td>F</td>
<td>6</td>
</tr>
<tr>
<td>562.8974</td>
<td>281.4487</td>
<td>11</td>
<td>A</td>
<td>5</td>
</tr>
<tr>
<td>516.7561</td>
<td>258.3781</td>
<td>12</td>
<td>P</td>
<td>4</td>
</tr>
<tr>
<td>462.3078</td>
<td>231.1539</td>
<td>13</td>
<td>Y</td>
<td>3</td>
</tr>
<tr>
<td>417.1128</td>
<td>208.5561</td>
<td>14</td>
<td>K</td>
<td>2</td>
</tr>
</tbody>
</table>

**COMET/Lorikeet Spectrum Viewer**

(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
**Ions:**

- **a**: $a^+ + 2a^2 + 3a^3$
- **b**: $b^+ + 2b^2 + 3b^3$
- **c**: $c^+ + 2c^2 + 3c^3$
- **x**: $x^+ + 2x^2 + 3x^3$
- **y**: $y^+ + 2y^2 + 3y^3$
- **z**: $z^+ + 2z^2 + 3z^3$

**Neutral Loss:**

- $H_2O$ (o)
- $NH_3$ (*)

**Mass Type:**

- Mono
- Avg

**Mass Tol:** 0.5

[Update]

**Peak Assignment:**

- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**

- Ion
- m/z
- None

**Width:** 650

**Height:** 400

**COMET/Lorikeet Spectrum Viewer**

(Comet v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
COMET/Lorikeet Spectrum Viewer
(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
File: 20140726_06_Bound_1.22522.22522.3, Scan: 22522, Exp. m/z: 1007.79, Charge: 3

**Ions:**
- a
- b
- c
- x
- y
- z

**Neutral Loss:**
- H₂O (Δ)
- NH₃ (*)

**Mass Type:**
- Mono
- Avg

**Mass Tol:** ±

**Peak Assignment:**
- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**
- Ion
- m/z
- None

**Width:** 650

**Height:** 400

---

**COMET/Lorikeet Spectrum Viewer**

(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
Ions:

a 1+ 2+ 3+

b 1+ 2+ 3+ 2+

c 1+ 2+ 3+ 2+

x 1+ 2+ 3+ 2+

y 1+ 2+ 3+ 2+

z 1+ 2+ 3+ 2+

Neutral Loss:

- H2O (o)
- NH3 (*)

Mass Type:

- Mono
- Avg

Mass Tol: ±

Peak Assignment:

- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:

- Ion
- m/z
- None

Width: 650

Height: 400

Click and drag on the plot to zoom

Click and drag to move table

Modifications:

C: 57.021415 [10]
**Ions:**
- a
- b
- c
- x
- y
- z

**Neutral Loss:**
- H₂O (o)
- NH₃ (*)

**Mass Type:**
- Mono
- Avg

**Mass Tol:** 0.5

**Peak Assignment:**
- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**
- Ion
- m/z
- None

**Width:** 650
**Height:** 400

---

**COMET/Lorikeet Spectrum Viewer**
(TPP v4.6 OCCUPY rev 3, Build 20130724109 (MinGW))
**Ions:**
a
b

c

x

y

z

**Neutral Loss:**

\( \text{H}_2\text{O}\) (o)

\( \text{NH}_3\) (*)

**Mass Type:**

- Mono
- Avg

**Mass Tol:**

Update

**Peak Assignment:**

- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**

- Ion
- m/z
- None

**Width:** 650

**Height:** 400

**Width:** 650

**Height:** 400

**COMET/Lorikeet Spectrum Viewer**

(TPP v4.6, OCCUPY rev 3, Build 201307241109 (MinGW))
**Ions:**
a 1 + 2 + 3 
b 1 + 2 + 3 
c 1 + 2 + 3 
x 1 + 2 + 3 
y 1 + 2 + 3 
z 1 + 2 + 3

**Neutral Loss:**
- H₂O (o)
- NH₃ (*)

**Mass Type:**
- Mono
- Avg

**Mass Tol:** 0.5

**Peak Assignment:**
- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**
- Ion
- m/z
- None

**Width:** 650
**Height:** 400

**Modifications:**

COMET/Lorikeet Spectrum Viewer
(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
RHPFYAPELLFAK, MH+ 1898.9952, m/z 633.6699

File: 20140726_07_Bound_2.22770.22770.3, Scan: 22770, Exp. m/z: 633.93, Charge: 3

Ions:
- a
  - 1
  - 2
  - 3
- b
  - 1
  - 2
  - 3
- c
  - 1
  - 2
  - 3
- x
  - 1
  - 2
  - 3
- y
  - 1
  - 2
  - 3
- z
  - 1
  - 2
  - 3

Neutral Loss:
- H₂O (o)
- NH₃ (*)

Mass Type:
- Mono
- Avg

Mass Tol: 0.5

Peak Assignment:
- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:
- Ion
- m/z
- None

Width: 650
Height: 400

Click and drag in the plot to zoom
X: Y:
Zoom Out Print Enable tooltip

COMET/Lorikeet Spectrum Viewer
(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
COMET/Lorikeet Spectrum Viewer
(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
SHC.LevendempadlpslaadvKvesk, NHI+ 2974.3444, m/z 992.1197
File: 20140726_06_Bound_1, 26479.16479.3, Scan: 26479, Exp. m/z: 992.5, Charge: 3

Ions:
a 1+ 2+ 3+
b 1+ 2+ 3+
c 1+ 2+ 3+
y 1+ 2+ 3+
z 1+ 2+ 3+

Neutral Loss:
H2O (o)
NH3 (*)

Mass Type:
Mono Avg

Peak Assignment:
Most Intense Nearest Match Peak Detect

Peak Labels:
Ion m/z None

Width: 650
Height: 400

Modifications:
C: 57.021415 [3]
**IONS:**

- a
- b
- c
- x
- y
- z

**Neutral Loss:**

- H₂O
- NH₃

**Mass Type:**

- Mono
- Avg

**Mass Tol:** 0.5

**Peak Assignment:**

- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**

- Ion
- m/z
- None

**Width:** 650

**Height:** 400

**Modifications:**

- K: 369.147037 [1]
**Ions:**
- a 1+ 2+ 3+
- b 1+ 2+ 3+
- c 1+ 2+ 3+
- x 1+ 2+ 3+
- y 1+ 2+ 3+
- z 1+ 2+ 3+

**Neutral Loss:**
- H2O (o)
- NH3 (*)

**Mass Type:**
- Mono
- Avg

**Mass Tol:** 0.5

**Peak Assignment:**
- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**
- Ion
- m/z
- None

**Width:** 650
**Height:** 400

---

### Neutral Loss Table

<table>
<thead>
<tr>
<th>Loss</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O</td>
<td>114,5890</td>
</tr>
<tr>
<td>NH3</td>
<td>131,5791</td>
</tr>
</tbody>
</table>

---

### Mass Type Table

<table>
<thead>
<tr>
<th>Mass Type</th>
<th>Mono Avg</th>
</tr>
</thead>
</table>

---

### Mass Tol Table

<table>
<thead>
<tr>
<th>Mass Tol</th>
<th>Peak Assignment</th>
<th>Peak Labels</th>
</tr>
</thead>
</table>

---

### Peak Labels Table

<table>
<thead>
<tr>
<th>Ion</th>
<th>m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>151,1842</td>
</tr>
<tr>
<td>V</td>
<td>118,7630</td>
</tr>
<tr>
<td>P</td>
<td>152,7744</td>
</tr>
<tr>
<td>Q</td>
<td>141,7216</td>
</tr>
<tr>
<td>S</td>
<td>131,5791</td>
</tr>
<tr>
<td>T</td>
<td>121,4523</td>
</tr>
</tbody>
</table>

---

### Peak Assignments

<table>
<thead>
<tr>
<th>Peak</th>
<th>Seq</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>129,1022</td>
<td>b+</td>
<td>55,0548</td>
</tr>
<tr>
<td>137,1707</td>
<td>b2+</td>
<td>116,1153</td>
</tr>
<tr>
<td>143,3280</td>
<td>b3+</td>
<td>161,1560</td>
</tr>
<tr>
<td>151,3054</td>
<td>y+</td>
<td>131,5791</td>
</tr>
<tr>
<td>163,3824</td>
<td>y2+</td>
<td>108,8394</td>
</tr>
<tr>
<td>175,4610</td>
<td>y3+</td>
<td>501,7849</td>
</tr>
<tr>
<td>187,4829</td>
<td>y4+</td>
<td>900,5149</td>
</tr>
<tr>
<td>199,5306</td>
<td>y5+</td>
<td>402,2347</td>
</tr>
<tr>
<td>211,5646</td>
<td>y6+</td>
<td>603,4621</td>
</tr>
<tr>
<td>223,6080</td>
<td>y7+</td>
<td>703,6445</td>
</tr>
<tr>
<td>235,6530</td>
<td>y8+</td>
<td>703,6445</td>
</tr>
<tr>
<td>247,7126</td>
<td>y9+</td>
<td>890,2820</td>
</tr>
<tr>
<td>259,7794</td>
<td>y10+</td>
<td>988,3294</td>
</tr>
<tr>
<td>271,8452</td>
<td>y11+</td>
<td>1361,2194</td>
</tr>
<tr>
<td>283,9104</td>
<td>y12+</td>
<td>1561,1510</td>
</tr>
<tr>
<td>295,9756</td>
<td>y13+</td>
<td>1751,1919</td>
</tr>
</tbody>
</table>

---

### COMET/Lorikeet Spectrum Viewer

(TPP v.6.6 OCCUPY rev 3, Build 201307241109 (MinGW))

---

**Click and drag in the plot to zoom**

**X:**

<table>
<thead>
<tr>
<th>Mass Type</th>
<th>Peak Assignment</th>
<th>Peak Labels</th>
</tr>
</thead>
</table>

---

**Click to move table**

**Enable tooltip**

---

**File:** 20140726_07_Bound_2.15301.15301.3, **Scan:** 15301, **Exp. m/z:** 547.5, **Charge:** 3
**Ions:**

- a
- b
- c
- x
- y
- z

**Neutral Loss:**

- H₂O (o)
- NH₃ (*)

**Mass Type:**

- Mono
- Avg

**Mass Tol:** 0.5

**Peak Assignment:**

- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**

- Ion
- m/z
- None

**Width:** 650

**Height:** 400

---

**LAKYETTEK, MH+ 1665.8516, m/z 555.9554**

File: 20140726_08_Bound_1.0965.0965.3, Scan: 9965, Exp. m/z: 556.29, Charge: 3

<table>
<thead>
<tr>
<th>b+</th>
<th>b2+</th>
<th>#</th>
<th>Seq</th>
<th>y+</th>
<th>y2+</th>
</tr>
</thead>
<tbody>
<tr>
<td>114.0913</td>
<td>57.5493</td>
<td>1</td>
<td>L</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>184.1045</td>
<td>93.0679</td>
<td>2</td>
<td>A</td>
<td>10</td>
<td>1552.7675</td>
</tr>
<tr>
<td>1481.7304</td>
<td>1441.7444</td>
<td>3</td>
<td>K</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>783.4181</td>
<td>392.2127</td>
<td>4</td>
<td>T</td>
<td>8</td>
<td>984.4686</td>
</tr>
<tr>
<td>492.7478</td>
<td>442.2240</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>546.4815</td>
<td>473.7444</td>
<td>5</td>
<td>Y</td>
<td>7</td>
<td>883.4467</td>
</tr>
<tr>
<td>433.2023</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1075.5241</td>
<td>538.2637</td>
<td>6</td>
<td>E</td>
<td>6</td>
<td>720.3774</td>
</tr>
<tr>
<td>360.6923</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1176.5747</td>
<td>588.7895</td>
<td>7</td>
<td>T</td>
<td>5</td>
<td>796.1146</td>
</tr>
<tr>
<td>756.1710</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1277.6194</td>
<td>629.3133</td>
<td>8</td>
<td>T</td>
<td>4</td>
<td>890.2075</td>
</tr>
<tr>
<td>245.6472</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1390.7035</td>
<td>695.8554</td>
<td>9</td>
<td>L</td>
<td>3</td>
<td>389.2395</td>
</tr>
<tr>
<td>195.1234</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1519.7461</td>
<td>760.3793</td>
<td>10</td>
<td>E</td>
<td>2</td>
<td>274.1515</td>
</tr>
<tr>
<td>138.5813</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>K</td>
<td>1</td>
<td>147.1128</td>
<td>74.0600</td>
<td></td>
</tr>
</tbody>
</table>

**Click to move table**

**Modifications:**

- K: 369.147037 [3]

---

**COMET/Lorikeet Spectrum Viewer**

(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
## Ions:

- **a**: $1^+$, $2^+$, $3^+$
- **b**: $1^+$, $2^+$, $3^+$
- **c**: $1^+$, $2^+$, $3^+$
- **x**: $1^+$, $2^+$, $3^+$
- **y**: $1^+$, $2^+$, $3^+$
- **z**: $1^+$, $2^+$, $3^+$

## Neutral Loss:

- H$_2$O ($\text{o}$)
- NH$_3$ ($\text{*}$)

## Mass Type:

- Mono
- Avg

## Mass Tol:

- 0.5

## Peak Assignment:

- Most Intense
- Nearest Match
- Peak Detect

## Peak Labels:

- Ion
- m/z
- None

## Width:

- 650

## Height:

- 400

---

**COMET/Lorikeet Spectrum Viewer**

(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
Ions:

- a
- b
- c
- x
- y
- z

Neutral Loss:
- H₂O (o)
- NH₃ (*)

Mass Type:
- Mono
- Avg

Mass Tol: 0.5

Peak Assignment:
- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:
- Ion
- m/z
- None

Width: 650
Height: 400

<table>
<thead>
<tr>
<th>b-</th>
<th>b²+</th>
<th>#</th>
<th>Seq. #</th>
<th>y-</th>
<th>y²+</th>
</tr>
</thead>
<tbody>
<tr>
<td>112.0394</td>
<td>113.5048</td>
<td>11</td>
<td>20</td>
<td>2471.2387</td>
<td>1236.1330</td>
</tr>
<tr>
<td>146.1179</td>
<td>153.6901</td>
<td>18</td>
<td>39</td>
<td>2357.1958</td>
<td>1179.1015</td>
</tr>
<tr>
<td>215.1555</td>
<td>222.6932</td>
<td>16</td>
<td>19</td>
<td>2197.1652</td>
<td>1099.0868</td>
</tr>
<tr>
<td>628.2396</td>
<td>635.7754</td>
<td>17</td>
<td>17</td>
<td>2068.1226</td>
<td>1034.5649</td>
</tr>
<tr>
<td>775.3080</td>
<td>782.8438</td>
<td>16</td>
<td>16</td>
<td>1955.0385</td>
<td>978.0229</td>
</tr>
<tr>
<td>912.1562</td>
<td>919.6909</td>
<td>15</td>
<td>15</td>
<td>1807.9701</td>
<td>904.4887</td>
</tr>
<tr>
<td>1052.4071</td>
<td>1059.9409</td>
<td>14</td>
<td>14</td>
<td>1678.9275</td>
<td>833.9674</td>
</tr>
<tr>
<td>1145.4932</td>
<td>1152.9830</td>
<td>13</td>
<td>13</td>
<td>1550.8669</td>
<td>775.9381</td>
</tr>
<tr>
<td>1302.5147</td>
<td>1309.9485</td>
<td>12</td>
<td>12</td>
<td>1437.7849</td>
<td>749.3941</td>
</tr>
<tr>
<td>1311.5373</td>
<td>1318.9711</td>
<td>11</td>
<td>11</td>
<td>1380.7634</td>
<td>690.8853</td>
</tr>
<tr>
<td>1444.4304</td>
<td>1451.8642</td>
<td>10</td>
<td>10</td>
<td>1251.7208</td>
<td>624.3640</td>
</tr>
<tr>
<td>1622.7156</td>
<td>1630.1494</td>
<td>9</td>
<td>9</td>
<td>1088.6575</td>
<td>544.8324</td>
</tr>
<tr>
<td>1769.7840</td>
<td>1777.2178</td>
<td>8</td>
<td>8</td>
<td>960.5623</td>
<td>480.7849</td>
</tr>
<tr>
<td>1897.8425</td>
<td>1905.2763</td>
<td>7</td>
<td>7</td>
<td>813.4941</td>
<td>407.2507</td>
</tr>
<tr>
<td>2011.8855</td>
<td>2019.3193</td>
<td>6</td>
<td>6</td>
<td>685.4255</td>
<td>343.2214</td>
</tr>
<tr>
<td>2082.7026</td>
<td>2089.1364</td>
<td>5</td>
<td>5</td>
<td>557.3579</td>
<td>286.1999</td>
</tr>
<tr>
<td>2196.0067</td>
<td>2203.4405</td>
<td>4</td>
<td>4</td>
<td>500.2891</td>
<td>250.6814</td>
</tr>
<tr>
<td>2309.0907</td>
<td>2316.5245</td>
<td>3</td>
<td>3</td>
<td>357.2207</td>
<td>194.1394</td>
</tr>
<tr>
<td>2408.1581</td>
<td>2415.5920</td>
<td>2</td>
<td>2</td>
<td>274.1547</td>
<td>137.9073</td>
</tr>
</tbody>
</table>

Width: 650
Height: 400

Click and drag in the plot to zoom
X: 888
Y: 888

Enable tooltip

COMET/Lorikeet Spectrum Viewer
(TPP v.4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
**Ion:**

- a
- b
- c
- x
- y
- z

**Neutral Loss:**
- H₂O (o)
- NH₃ (*)

**Mass Type:**
- Mono
- Avg

**Mass Tol:**
- u.d

**Peak Assignment:**
- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**
- Ion
- m/z
- None

**Width:** 650

**Height:** 400

---

**VFDEF PLVEEPQNLK**, MH+ 2414.2424, m/z 805.4190

File: 20140726_07_Bound_2,24031.24031.3, Scan: 24031, Exp. m/z: 805.6, Charge: 3

<table>
<thead>
<tr>
<th>Peak</th>
<th>Seq</th>
<th>y+</th>
<th>y2+</th>
</tr>
</thead>
<tbody>
<tr>
<td>640.6609</td>
<td>L 10</td>
<td>1182.6729</td>
<td>591.8401</td>
</tr>
<tr>
<td>664.7293</td>
<td>V 9</td>
<td>1069.9288</td>
<td>535.2980</td>
</tr>
<tr>
<td>1702.8145</td>
<td>E 7</td>
<td>841.4778</td>
<td>421.2325</td>
</tr>
<tr>
<td>1799.8672</td>
<td>F 6</td>
<td>712.3313</td>
<td>356.7259</td>
</tr>
<tr>
<td>1927.9258</td>
<td>Q 5</td>
<td>613.3825</td>
<td>308.1949</td>
</tr>
<tr>
<td>2041.9688</td>
<td>M 4</td>
<td>487.3219</td>
<td>244.1656</td>
</tr>
<tr>
<td>2155.0286</td>
<td>L 3</td>
<td>373.2809</td>
<td>187.1441</td>
</tr>
<tr>
<td>2268.1369</td>
<td>K 2</td>
<td>260.1969</td>
<td>130.6021</td>
</tr>
<tr>
<td>2381.2458</td>
<td>J 1</td>
<td>147.1128</td>
<td>74.0600</td>
</tr>
</tbody>
</table>

**Modifications:**
- K: 369.147037

---

**COMET/Lorikeet Spectrum Viewer**

*(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))*. 

---

250
**IONs:**
- a
- b
- c
- x
- y
- z

**Neutral Loss:**
- H₂O (o)
- NH₃ (*)

**Mass Type:**
- Mono
- Avg

**Mass Tol:** 0.5

**Peak Assignment:**
- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**
- Ion
- m/z

**Width:** 650
**Height:** 400

### COMET/Lorikeet Spectrum Viewer
(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))

<table>
<thead>
<tr>
<th>m/z</th>
<th>Charge</th>
<th># Seq</th>
<th>Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>533.1656</td>
<td>20</td>
<td>F</td>
<td>77.1145</td>
</tr>
<tr>
<td>581.2057</td>
<td>19</td>
<td>E</td>
<td>65.7600</td>
</tr>
<tr>
<td>626.2358</td>
<td>18</td>
<td>D</td>
<td>54.3094</td>
</tr>
<tr>
<td>667.2660</td>
<td>17</td>
<td>C</td>
<td>43.8580</td>
</tr>
<tr>
<td>704.2961</td>
<td>16</td>
<td>B</td>
<td>33.4066</td>
</tr>
<tr>
<td>741.3262</td>
<td>15</td>
<td>A</td>
<td>22.9552</td>
</tr>
<tr>
<td>778.3563</td>
<td>14</td>
<td></td>
<td>12.5038</td>
</tr>
</tbody>
</table>

**VFDEFKLPVEEPQNLIK, MH+ 2045.0954, m/z 682.3700**

File: 20140726_Ob_Bound_1.23687.23687.3, Scan: 23687, Exp. m/z: 682.3, Charge: 3
YKAffteCCQADK, NH+ 2031.8720, m/z 677.9622
File: 20140726_08_Bound_3.09858.09858.3, Scan: 9858, Exp. m/z: 678.25, Charge: 3

Ions:
a 1+ 2+ 3+  
b 1+ 2+ 3+  
c 1+ 2+ 3+  
x 1+ 2+ 3+  
y 1+ 2+ 3+  
z 1+ 2+ 3+  
[Clear All]

Neutral Loss:
H2O (o)  	NH3 (*)

Mass Type:
Mono  Avg
Mass Tol: 0.5

Peak Assignment:
Most Intense
Nearest Match
Peak Detect

Peak Labels:
Ion  m/z  None

Width: 650
Height: 400

Click and drag in the plot to zoom
X:  Y:  Zoom Out  Point  Enable tooltip

Table:
<table>
<thead>
<tr>
<th>Peak</th>
<th>b+</th>
<th>b2+</th>
<th># Seq.</th>
<th>#</th>
<th>y+</th>
<th>y2+</th>
</tr>
</thead>
<tbody>
<tr>
<td>1640.0706</td>
<td>82.5389</td>
<td>1</td>
<td>Y</td>
<td>1-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>644.1159</td>
<td>331.1599</td>
<td>2</td>
<td>K</td>
<td>3-4</td>
<td>1868.8087</td>
<td>74.0600</td>
</tr>
<tr>
<td>603.3868</td>
<td>402.1971</td>
<td>4</td>
<td>A</td>
<td>1-2</td>
<td>1371.5667</td>
<td>486.2870</td>
</tr>
<tr>
<td>930.4592</td>
<td>475.7313</td>
<td>5</td>
<td>F</td>
<td>2-3</td>
<td>1229.4924</td>
<td>651.2499</td>
</tr>
<tr>
<td>1051.5029</td>
<td>536.2551</td>
<td>6</td>
<td>T</td>
<td>1-4</td>
<td>1082.4240</td>
<td>541.7156</td>
</tr>
<tr>
<td>1140.5445</td>
<td>580.7754</td>
<td>7</td>
<td>E</td>
<td>1-4</td>
<td>984.1341</td>
<td>541.1618</td>
</tr>
<tr>
<td>1340.5761</td>
<td>670.7917</td>
<td>8</td>
<td>C</td>
<td>1-5</td>
<td>852.2338</td>
<td>426.6705</td>
</tr>
<tr>
<td>1500.4675</td>
<td>750.8070</td>
<td>9</td>
<td>C</td>
<td>1-4</td>
<td>692.3012</td>
<td>346.6552</td>
</tr>
<tr>
<td>1628.8652</td>
<td>814.8363</td>
<td>10</td>
<td>Q</td>
<td>1-2</td>
<td>533.2796</td>
<td>266.6399</td>
</tr>
<tr>
<td>1699.7014</td>
<td>850.3558</td>
<td>11</td>
<td>A</td>
<td>1-2</td>
<td>404.2140</td>
<td>202.6106</td>
</tr>
<tr>
<td>1770.7395</td>
<td>880.8773</td>
<td>12</td>
<td>A</td>
<td>1-2</td>
<td>322.1749</td>
<td>167.0821</td>
</tr>
<tr>
<td>1885.7665</td>
<td>940.3869</td>
<td>13</td>
<td>D</td>
<td>1-5</td>
<td>662.1387</td>
<td>321.5735</td>
</tr>
<tr>
<td>2014.7037</td>
<td>14</td>
<td>147.1128</td>
<td>74.0600</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Modifications:
C: 57.021415  [8, 9]  
K: 369.147037  [2]  

COMET/Lorikeet Spectrum Viewer  
(TPP v.6.6 OCCUPY rev 3, Build 201307241109 (MinGW))
YKAASFE CCQADK, MH+ 1662.7250, m/z 554.9132
File: 20140726_DB_Bound_3.07239.07239.3, Scan: 7239, Exp. m/z: 555.3, Charge: 3

Ions:
- a: m/z 1, 2, 3
- b: m/z 1, 2, 3
- c: m/z 1, 2, 3
- x: m/z 1, 2, 3
- y: m/z 1, 2, 3
- z: m/z 1, 2, 3

Neutral Loss:
- H2O (o)
- NH3 (*)

Mass Type:
- Mono
- Avg

Mass Tol: 0.5

Peak Assignment:
- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:
- Ion m/z
- None

Width: 650
Height: 400

Comet/Lorikeet Spectrum Viewer
(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
Ions:

- a
- b
- c
- x
- y
- z

Neutral Loss:
- H_2O (o)
- NH_3 (*)

Mass Type:
- Mono
- Avg

Mass Tol: 0.5

Peak Assignment:
- Most Intense
- Nearest Match

Peak Labels:
- Ion
- m/z

Width: 650

Height: 400

Modifications:
- C: 57.021415 (14)
ALVLIAFAQYQQPFEDHVK, MH+ 2490.2849, m/z 830.7665
File: 20150202_09_25Percent_Beads_2_28410.28410.3, Scan: 28410, Exp. m/z: 831.04, Charge: 3

Ions:
a 1+ 2+ 3+
b 1+ 2+ 3+
c 1+ 2+ 3+
x 1+ 2+ 3+
y 1+ 2+ 3+
z 1+ 2+ 3+

Neutral Loss:
H2O (o)
NH3 (*)

Mass Type:
Mono Avg
Mass Tol: 0.5
Update

Peak Assignment:
Most Intense Nearest Match Peak Detect

Peak Labels:
Ion m/z None

Width: 650
Height: 400

Click and drag in the plot to zoom X: Y: Enable tooltip

COMET/Lorikeet Spectrum Viewer
(TTP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
**Ions:**
- a
- b
- c
- x
- y
- z

**Neutral Loss:**
- H₂O (o)
- NH₃ (*)

**Mass Type:**
- Mono
- Avg

**Mass Tol:**

**Peak Assignment:**

**Peak Labels:**
- Ion
- m/z

**Width:** 650

**Height:** 400
**Ions:**
- a
- b
- c
- x
- y
- z

**Neutral Loss:**
- H₂O (o)
- NH₃ (*)

**Mass Type:**
- Mono
- Avg

**Mass Tol:**
- 0.5

**Peak Assignment:**
- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**
- Ion
- m/z
- None

**Width:** 650
**Height:** 400

---

**RHPYFAPPELLFFAK, M+ 1898.9952, m/z 950.0012**

File: 20150202_08_25Percent_Beads_1.23029.2, Scan: 23029, Exp. m/z: 950.22, Charge: 2

<table>
<thead>
<tr>
<th>b+</th>
<th>b2+</th>
<th># Seq</th>
<th>#</th>
<th>y+</th>
<th>y2+</th>
</tr>
</thead>
<tbody>
<tr>
<td>157.1084</td>
<td>79.0578</td>
<td>1</td>
<td>R</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>196.1671</td>
<td>147.5871</td>
<td>2</td>
<td>H</td>
<td>2</td>
<td>6747.8041</td>
</tr>
<tr>
<td>391.2201</td>
<td>196.1137</td>
<td>3</td>
<td>P</td>
<td>10</td>
<td>NH₃ [*]</td>
</tr>
<tr>
<td>554.2834</td>
<td>227.6453</td>
<td>4</td>
<td>Y</td>
<td>12</td>
<td>1508.7824</td>
</tr>
<tr>
<td>701.3518</td>
<td>351.1795</td>
<td>5</td>
<td>F</td>
<td>15</td>
<td>1345.7119</td>
</tr>
<tr>
<td>864.4151</td>
<td>432.7112</td>
<td>6</td>
<td>Y</td>
<td>19</td>
<td>1198.8507</td>
</tr>
<tr>
<td>915.4577</td>
<td>468.7398</td>
<td>7</td>
<td>A</td>
<td>10</td>
<td>NH₃ [*]</td>
</tr>
<tr>
<td>1032.5050</td>
<td>516.7561</td>
<td>8</td>
<td>P</td>
<td>9</td>
<td>NH₃ [*]</td>
</tr>
<tr>
<td>1161.5476</td>
<td>581.2774</td>
<td>9</td>
<td>E</td>
<td>7</td>
<td>867.4975</td>
</tr>
<tr>
<td>1374.6149</td>
<td>637.8195</td>
<td>10</td>
<td>L</td>
<td>5</td>
<td>718.8448</td>
</tr>
<tr>
<td>1587.7157</td>
<td>694.3615</td>
<td>11</td>
<td>L</td>
<td>6</td>
<td>425.3708</td>
</tr>
<tr>
<td>1824.7841</td>
<td>762.4087</td>
<td>12</td>
<td>F</td>
<td>9</td>
<td>812.2867</td>
</tr>
<tr>
<td>1681.8526</td>
<td>804.4299</td>
<td>13</td>
<td>F</td>
<td>8</td>
<td>505.2183</td>
</tr>
<tr>
<td>1752.8297</td>
<td>856.4905</td>
<td>14</td>
<td>A</td>
<td>7</td>
<td>218.1499</td>
</tr>
<tr>
<td>1560.9380</td>
<td>780.4690</td>
<td>15</td>
<td>K</td>
<td>6</td>
<td>147.1128</td>
</tr>
</tbody>
</table>

Click to move table

COMET/Lorikeet Spectrum Viewer
(TPP v.4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
Ions:

- a 1+
- b 1+
- c 1+
- x 1+
- y 1+
- z 1+

Neutral Loss:
- H₂O (o)
- NH₃ (*)

Mass Type:
- Mono
- Avg

Mass Tol: 0.5

Peak Assignment:
- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:
- Ion
- m/z
- None

Width: 650
Height: 400

COMET/Lorikeet Spectrum Viewer
(TPP v.4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
<table>
<thead>
<tr>
<th>Ions</th>
<th>b+</th>
<th>b2+</th>
<th># Seq</th>
<th>#</th>
<th>y+</th>
<th>y2+</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>100.0757</td>
<td>50.5415</td>
<td>1</td>
<td>V</td>
<td>18</td>
<td>8</td>
</tr>
<tr>
<td>b</td>
<td>247.1441</td>
<td>124.0757</td>
<td>7</td>
<td>F</td>
<td>16</td>
<td>100.0757</td>
</tr>
<tr>
<td>c</td>
<td>140.1715</td>
<td>181.5892</td>
<td>3</td>
<td>D</td>
<td>15</td>
<td>615.3824</td>
</tr>
<tr>
<td>x</td>
<td>74.0600</td>
<td>147.1128</td>
<td>4</td>
<td>K</td>
<td>12</td>
<td>1701.177737 [6]</td>
</tr>
<tr>
<td>y</td>
<td>417.1885</td>
<td>219.1093</td>
<td>5</td>
<td>E</td>
<td>14</td>
<td>400.1367</td>
</tr>
<tr>
<td>z</td>
<td>74.0600</td>
<td>147.1128</td>
<td>5</td>
<td>K</td>
<td>12</td>
<td>1701.177737 [6]</td>
</tr>
</tbody>
</table>

Neutral Loss: 
- H₂O (o) 
- NH₃ (*)

Mass Type: Mono Avg

Mass Tol: 0.000001

Peak Assignment: 
- Most Intense 
- Nearest Match 
- Peak Detect

Peak Labels: 
- Ion o m/z 
- None

Width: 650

Height: 400

Click and drag in the plot to zoom X: Y: 

Print 
Enable tooltip

COMET/Lorikeet Spectrum Viewer
(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
ions:
- $a^1_1$, $a^2_1$, $a^3_1$
- $b^1_2$, $b^2_2$, $b^3_2$
- $c^1_3$, $c^2_3$, $c^3_3$
- $x^1_1$, $x^2_2$, $x^3_3$
- $y^1_2$, $y^2_2$, $y^3_2$
- $z^1_2$, $z^2_2$, $z^3_2$

Neutral Loss:
- $H_2O$ (£)
- $NH_3$ (*)

Mass Type:
- Mono
- Avg
- Mass Tol: 0.5

Peak Assignment:
- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:
- Ion
- m/z
- None

Width: 650
Height: 400

COMET/Lorikeet Spectrum Viewer
(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))

Modifications:
- C: 391.177715 [14]
Ions:

- a = 1^+ 2^- 3^- 
- b = 1^- 2^- 3^- 
- c = 1^- 2^- 3^- 
- x = 1^- 2^- 3^- 
- y = 1^- 2^- 3^- 
- z = 1^- 2^- 3^- 

Neutral Loss:

- H₂O (o) 
- NH₃ (*)

Mass Type:

- Mono 
- Avg

Mass Tol: 0.5

Peak Assignment:

- Most Intense 
- Nearest Match 
- Peak Detect

Peak Labels:

- Ion 
- m/z 
- None

Width: 650 
Height: 400

COMET/Lorikeet Spectrum Viewer

(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
**IONS:**
- a: $\pm$ $1^+$, $2^+$, $3^+$
- b: $\pm$ $1^+$, $2^+$, $3^+$
- c: $\pm$ $1^+$, $2^+$, $3^+$
- x: $\pm$ $1^+$, $2^+$, $3^+$
- y: $\pm$ $1^+$, $2^+$, $3^+$
- z: $\pm$ $1^+$, $2^+$, $3^+$

**Neutral Loss:**
- H$_2$O (o)
- NH$_3$ (*)

**Mass Type:**
- Mono
- Avg

**Mass Tol:**
- 0.5

**Peak Assignment:**
- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**
- Ion
- m/z
- None

**Width:** 650

**Height:** 400

---

**COMET/Lorikeet Spectrum Viewer**

(TPP v.4.6 OCCUPY rev 3, Build 201307241109 (MinGW))

---

**Table:**

<table>
<thead>
<tr>
<th>File</th>
<th>Scan</th>
<th>Exp. m/z</th>
<th>Charge</th>
<th>ALVLIAFAQYLQQ, MH+ 2490.2849, m/z 830.7665</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Peak</th>
<th>m/z</th>
<th>Intensity</th>
<th>Width</th>
<th>Height</th>
<th>ALVLIAFAQYLQQ, MH+ 2490.2849, m/z 830.7665</th>
</tr>
</thead>
<tbody>
<tr>
<td>b+</td>
<td>185.1285</td>
<td>93.0679</td>
<td>L 20</td>
<td>2419.2478</td>
<td>1210.1275</td>
</tr>
<tr>
<td>b2+</td>
<td>199.1441</td>
<td>199.1441</td>
<td>L 18</td>
<td>2207.0953</td>
<td>1004.0513</td>
</tr>
<tr>
<td>y+</td>
<td>219.2047</td>
<td>219.2047</td>
<td>L 17</td>
<td>2094.0113</td>
<td>1047.5093</td>
</tr>
<tr>
<td>y2+</td>
<td>291.7189</td>
<td>291.7189</td>
<td>F 16</td>
<td>1980.9272</td>
<td>990.9672</td>
</tr>
<tr>
<td>x+</td>
<td>364.7319</td>
<td>364.7319</td>
<td>F 15</td>
<td>1909.8901</td>
<td>945.4487</td>
</tr>
<tr>
<td>x2+</td>
<td>400.2575</td>
<td>400.2575</td>
<td>F 14</td>
<td>1762.8217</td>
<td>881.9145</td>
</tr>
<tr>
<td>z+</td>
<td>464.2867</td>
<td>464.2867</td>
<td>Q 13</td>
<td>1691.7846</td>
<td>846.3959</td>
</tr>
<tr>
<td>z2+</td>
<td>545.1834</td>
<td>545.1834</td>
<td>Q 12</td>
<td>1563.7260</td>
<td>782.3644</td>
</tr>
<tr>
<td>727.5662</td>
<td>602.3640</td>
<td>L 11</td>
<td>1406.4627</td>
<td>700.8350</td>
<td></td>
</tr>
<tr>
<td>666.3897</td>
<td>666.3897</td>
<td>Q 10</td>
<td>1287.5786</td>
<td>641.9359</td>
<td></td>
</tr>
<tr>
<td>722.4190</td>
<td>722.4190</td>
<td>Q 9</td>
<td>1159.5200</td>
<td>580.2636</td>
<td></td>
</tr>
<tr>
<td>815.4345</td>
<td>815.4345</td>
<td>Q 8</td>
<td>1031.4614</td>
<td>516.2344</td>
<td></td>
</tr>
<tr>
<td>858.9607</td>
<td>858.9607</td>
<td>Q 7</td>
<td>871.4208</td>
<td>436.2191</td>
<td></td>
</tr>
<tr>
<td>922.4949</td>
<td>922.4949</td>
<td>Q 6</td>
<td>724.3281</td>
<td>387.4927</td>
<td></td>
</tr>
<tr>
<td>997.0162</td>
<td>997.0162</td>
<td>E 5</td>
<td>607.3097</td>
<td>314.1585</td>
<td></td>
</tr>
<tr>
<td>1287.5786</td>
<td>1287.5786</td>
<td>Q 4</td>
<td>1054.5297</td>
<td>700.8350</td>
<td></td>
</tr>
<tr>
<td>1234.1794</td>
<td>1234.1794</td>
<td>Q 3</td>
<td>871.4208</td>
<td>436.2191</td>
<td></td>
</tr>
<tr>
<td>147.1128</td>
<td>147.1128</td>
<td>K 2</td>
<td>607.3097</td>
<td>314.1585</td>
<td></td>
</tr>
</tbody>
</table>

---

**Modifications:**
- C: 57.021415 [14]
**Ions:**
- a [1+ 2+ 3+]
- b [1+ 2+ 3+]
- c [1+ 2+ 3+]
- x [1+ 2+ 3+]
- y [1+ 2+ 3+]
- z [1+ 2+ 3+]

**Neutral Loss:**
- H$_2$O (o)
- NH$_3$ (*)

**Mass Type:**
- Mono
- Avg

**Mass Tol:** 0.5

**Peak Assignment:**
- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**
- Ion
- m/z
- None

**Width:** 650

**Height:** 400

---

**COMET/Lorikeet Spectrum Viewer**

(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
**Ions:**
- a: $^{1+}$ $^{2+}$ $^{3+}$
- b: $^{1+}$ $^{2+}$ $^{3+}$
- c: $^{1+}$ $^{2+}$ $^{3+}$
- x: $^{1+}$ $^{2+}$ $^{3+}$
- y: $^{1+}$ $^{2+}$ $^{3+}$
- z: $^{1+}$ $^{2+}$ $^{3+}$

**Neutral Loss:**
- $\text{H}_2\text{O}$ (o)
- $\text{NH}_3$ (*)

**Mass Type:**
- Mono
- Avg
**Mass Tol:** 0.5

**Peak Assignment:**
- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**
- Ion
- m/z
- None

**Width:** 650
**Height:** 400

**Click to move table**

**Modifications:**
- C: 39.994915 [1]
- C: 57.021415 [2, 10]

**COMET/Lorikeet Spectrum Viewer**

(TPP v4.6, OCCUPY rev 3, Build 201307241109 (MinGW))
Ions:

\[ a_{1+}^{2+} \quad b_{2+}^{3+} \quad c_{1+}^{2+} \quad d_{3+} \]

Neutral Loss:

- H$_2$O (o)
- NH$_3$ (*)

Mass Type:
- Mono
- Avg

Mass Tol: 0.5

Peak Assignment:
- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:
- Ion
- m/z
- None

Width: 650
Height: 400

COMET/Lorikeet Spectrum Viewer
(TPP v4.6 OCCUPY rev 3, Build 20130724109 (MinGW))
**Ions:**

- **a**
  - m/z 1
  - m/z 2
  - m/z 3
- **b**
  - m/z 1
  - m/z 2
  - m/z 3
- **c**
  - m/z 1
  - m/z 2
  - m/z 3
- **x**
  - m/z 1
  - m/z 2
  - m/z 3
- **y**
  - m/z 1
  - m/z 2
  - m/z 3
- **z**
  - m/z 1
  - m/z 2
  - m/z 3

**Neutral Loss:**
- H$_2$O (o)
- NH$_3$ (*)

**Mass Type:**
- Mono
dAvg

**Mass Tol:**
- 0.5

**Peak Assignment:**
- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**
- Ion
- m/z
- None

**Width:** 650

**Height:** 400

**Click to move table**

**Modifications:**
- H: 391.177688 [2]
**Ions:**

- a + 1
- b + 2
- c + 3
- x + 2
- y + 3
- z + 3

**Neutral Loss:**

- H₂O (°)
- NH₃ (*)

**Mass Type:**

- Mono
- Avg

**Mass Tol:** 0.5

[Update]

**Peak Assignment:**

- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**

- Ion m/z
- None

**Width:** 650

**Height:** 400

---

**COMET/Lorikeet Spectrum Viewer**

(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))

---

**RHPYFYAPELFFAK, MH+ 1898.9952, m/z 950.0012**

File: 20141212_18_50Percent_Labeled_3_19164_19164-2, Scan: 19164, Exp. m/z: 950.08, Charge: 2

**Peak Assignment:**

- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**

- Ion m/z
- None

**Width:** 650

**Height:** 400

---

**Neutral Loss:**

- H₂O (°)
- NH₃ (*)

**Mass Type:**

- Mono
- Avg

**Mass Tol:** 0.5

[Update]

**Peak Assignment:**

- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**

- Ion m/z
- None

**Width:** 650

**Height:** 400

---

**COMET/Lorikeet Spectrum Viewer**

(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))

---

**Neutral Loss:**

- H₂O (°)
- NH₃ (*)

**Mass Type:**

- Mono
- Avg

**Mass Tol:** 0.5

[Update]

**Peak Assignment:**

- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**

- Ion m/z
- None

**Width:** 650

**Height:** 400

---

**COMET/Lorikeet Spectrum Viewer**

(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))

---

**Neutral Loss:**

- H₂O (°)
- NH₃ (*)

**Mass Type:**

- Mono
- Avg

**Mass Tol:** 0.5

[Update]

**Peak Assignment:**

- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**

- Ion m/z
- None

**Width:** 650

**Height:** 400

---

**COMET/Lorikeet Spectrum Viewer**

(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
**IONS:**

- a
- b
- c
- x
- y
- z

**Neutral Loss:**

- H₂O
- NH₃

**Mass Type:**

- Mono
- Avg

**Mass Tol:** 0.5

**Peak Assignment:**

- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**

- Ion m/z
- None

**Width:** 650

**Height:** 400

---

**MAS Spectrum Viewer**

File: 20141212_16_50Percent_Labeled_1.18830.18830.3, Scan: 18830, Exp. m/z: 633.76, Charge: 3

**Peaks:**

<table>
<thead>
<tr>
<th>m/z</th>
<th>Intensity</th>
<th>Charge</th>
<th>Mass Type</th>
<th>Mass Tol</th>
<th>Peak Assignment</th>
<th>Peak Labels</th>
</tr>
</thead>
<tbody>
<tr>
<td>157.1084</td>
<td>79.0578</td>
<td>1</td>
<td>R</td>
<td>0.5</td>
<td>R†</td>
<td>None</td>
</tr>
<tr>
<td>147.5873</td>
<td>79.0578</td>
<td>2</td>
<td>H</td>
<td>0.5</td>
<td>H†</td>
<td>None</td>
</tr>
<tr>
<td>1508.7824</td>
<td>79.0578</td>
<td>3</td>
<td>P</td>
<td>0.5</td>
<td>P†</td>
<td>None</td>
</tr>
<tr>
<td>1345.7191</td>
<td>79.0578</td>
<td>4</td>
<td>F</td>
<td>0.5</td>
<td>F†</td>
<td>None</td>
</tr>
<tr>
<td>1300.7564</td>
<td>79.0578</td>
<td>5</td>
<td>A</td>
<td>0.5</td>
<td>A†</td>
<td>None</td>
</tr>
<tr>
<td>1274.6317</td>
<td>79.0578</td>
<td>6</td>
<td>P</td>
<td>0.5</td>
<td>P†</td>
<td>None</td>
</tr>
<tr>
<td>1252.7157</td>
<td>79.0578</td>
<td>7</td>
<td>E</td>
<td>0.5</td>
<td>E†</td>
<td>None</td>
</tr>
<tr>
<td>1238.6957</td>
<td>79.0578</td>
<td>8</td>
<td>L</td>
<td>0.5</td>
<td>L†</td>
<td>None</td>
</tr>
<tr>
<td>1224.6556</td>
<td>79.0578</td>
<td>9</td>
<td>F</td>
<td>0.5</td>
<td>F†</td>
<td>None</td>
</tr>
<tr>
<td>1206.6357</td>
<td>79.0578</td>
<td>10</td>
<td>L</td>
<td>0.5</td>
<td>L†</td>
<td>None</td>
</tr>
<tr>
<td>1192.6159</td>
<td>79.0578</td>
<td>11</td>
<td>E</td>
<td>0.5</td>
<td>E†</td>
<td>None</td>
</tr>
<tr>
<td>1178.5960</td>
<td>79.0578</td>
<td>12</td>
<td>L</td>
<td>0.5</td>
<td>L†</td>
<td>None</td>
</tr>
<tr>
<td>1162.5761</td>
<td>79.0578</td>
<td>13</td>
<td>F</td>
<td>0.5</td>
<td>F†</td>
<td>None</td>
</tr>
<tr>
<td>1146.5573</td>
<td>79.0578</td>
<td>14</td>
<td>A</td>
<td>0.5</td>
<td>A†</td>
<td>None</td>
</tr>
<tr>
<td>1132.5384</td>
<td>79.0578</td>
<td>15</td>
<td>K</td>
<td>0.5</td>
<td>K†</td>
<td>None</td>
</tr>
</tbody>
</table>

**Click and drag in the plot to zoom**

**X:**

**Y:**

**Zoom Out**

**Print**

**Enable tooltip**

---

**COMET/Lorikeet Spectrum Viewer**

(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
Ions:

- a
- b
- c
- x
- y
- z

Neutral Loss:

- H₂O (o)
- NH₃ (*)

Mass Type:

- Mono
- Avg

Mass Tol: 0.5

Update

Peak Assignment:

- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:

- Ion
- m/z
- None

Width: 650

Height: 400

Click and drag in the plot to zoom X: Y:

7mm Cut

Print

Enable tooltip

Click to move table

Modifications:


COMET/Lorikeet Spectrum Viewer

(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
VFDEF PVEPQNLIK, MH+ 2436.2731, m/z 812.7626
File: 20150202_16_30Percent_Beads_3.25071.25071.3, Scan: 25071, Exp. m/z: 813.66, Charge: 3

Neutral Loss:
- H₂O (o)
- NH₃ (*)

Mass Type:
- Mono
- Avg
Mass Tol: 0.5

Peak Assignment:
- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:
- Ion m/z
- None

Modifications:

COMET/LoriReet Spectrum Viewer
(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
Ions:

- a
- b
- c
- x
- y
- z

Neutral Loss:
- H₂O (o)
- NH₃ (*)

Mass Type:
- Mono
- Avg

Mass Tol: 0.5

Update

Peak Assignment:
- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:
- Ion
- m/z
- None

Width: 650
Height: 400

Click and drag in the plot to zoom

X:

Y:

Enable tooltip

Click to move table
ALVLIAFAQYLQQFEDHVK, MH+ 2824.4412, m/z 942.1519
File: 20141212_25_75Percent_Labeled_1.21760.21760.3, Scan: 21760, Exp. m/z: 942.83, Charge: 3

<table>
<thead>
<tr>
<th>Ion</th>
<th>m/z</th>
<th>Charge</th>
<th>Peak Assignment</th>
<th>Modifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>185.1285</td>
<td>2</td>
<td>L</td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>145.1285</td>
<td>2</td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>c</td>
<td>118.1285</td>
<td>2</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>x</td>
<td>118.1285</td>
<td>2</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>y</td>
<td>118.1285</td>
<td>2</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>z</td>
<td>118.1285</td>
<td>2</td>
<td>A</td>
<td></td>
</tr>
</tbody>
</table>

**Neutral Loss:**
- H2O (o)
- NH3 (*)

**Mass Type:**
- Mono
- Avg

**Peak Labels:**
- Ion
- m/z
- None

**Width:** 650
**Height:** 400

**COMET/Lorikeet Spectrum Viewer**
(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
ALVLIAFAQYQQEPFEDHK, MH+ 2490.2849, m/z 830.7665
File: 20141212_23_75Percent_Labeled_1.20853.20853.3, Scan: 20853, Exp. m/z: 831.18, Charge: 3

Ions:
- a
- b
- c
- x
- y
- z
Neutral Loss:
- H2O (o)
- NH3 (*)
Mass Type:
- Mono
- Avg
Mass Tol: 0.5
Peak Assignment:
- Most Intense
- Nearest Match
- Peak Detect
Peak Labels:
- Ion
- m/z
- None
Width: 650
Height: 400

 Modifications:
- C: 57.021415 [14]
### Ions:

<table>
<thead>
<tr>
<th>Ion</th>
<th>1&lt;sup&gt;+&lt;/sup&gt;</th>
<th>2&lt;sup&gt;+&lt;/sup&gt;</th>
<th>3&lt;sup&gt;+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>z</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Neutral Loss:

- H<sub>2</sub>O (o)
- NH<sub>3</sub> (*

### Mass Type:

- Mono
- Avg

### Mass Tol: 0.5

### Peak Assignment:

- Most Intense
- Nearest Match
- Peak Detect

### Peak Labels:

- Ion
- m/z
- None

### Width: 650

### Height: 400

### Modifications:

- C: 57.021415 [14]

---

**COMET/Lorikeet Spectrum Viewer**

(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
C CAAADP HE CYAK, MH+ 1926.7488, m/z 963.8780
File: 20150124_21_79Percent_Beads_2.14931.14931.2, Scan: 14931, Exp. m/z: 963.84, Charge: 2

**Ions:**
- a 1+ 2+ 3+
- b 1+ 2+ 3+
- c 1+ 2+ 3+
- x 1+ 2+ 3+
- y 1+ 2+ 3+
- z 1+ 2+ 3+

**Neutral Loss:**
- H₂O (o)
- NH₃ (*)

**Mass Type:**
- Mono
- Avg

**Mass Tol:** 0.5

**Peak Assignment:**
- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**
- Ion
- m/z
- None

**Width:** 650
**Height:** 400

**Modifications:**
- C: 39.994915 [1]
- C: 57.021415 [2, 10]
- H: 391.177688 [8]

**Neutral Loss:**
- H₂O (o)
- NH₃ (*)

**Mass Type:**
- Mono
- Avg

**Mass Tol:** 0.5

**Peak Assignment:**
- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**
- Ion
- m/z
- None

**Width:** 650
**Height:** 400

**Modifications:**
- C: 39.994915 [1]
- C: 57.021415 [2, 10]
- H: 391.177688 [8]

**Peak Assignment:**
- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**
- Ion
- m/z
- None

**Width:** 650
**Height:** 400

**Modifications:**
- C: 39.994915 [1]
- C: 57.021415 [2, 10]
- H: 391.177688 [8]
CCEAAADPCYAK, MH+ 1535.5711, m/z 768.2892

File: 20150124_21_75Percent_Beads_2_12259.12259.2, Scan: 12259, Exp. m/z: 768.61, Charge: 2

### Ions:
- a $^{1+}$, $^{2+}$, $^{3+}$
- b $^{1+}$, $^{2+}$, $^{3+}$
- c $^{1+}$, $^{2+}$, $^{3+}$
- x $^{1+}$, $^{2+}$, $^{3+}$
- y $^{1+}$, $^{2+}$, $^{3+}$
- z $^{1+}$, $^{2+}$, $^{3+}$

### Neutral Loss:
- H$_2$O (6)
- NH$_3$ (*)

### Mass Type:
- Mono
- Avg

### Mass Tol:
- 0.5

### Peak Assignment:
- Most Intense
- Nearest Match
- Peak Detect

### Peak Labels:
- Ion
- m/z
- None

### Width:
- 650

### Height:
- 400

### Modifiers:
- C: 39.9949
- C: 57.0214

---

**COMET/Lorikeet Spectrum Viewer**

(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
**IONS:**

- **a**
  - 1
  - 2
  - 3
- **b**
  - 1
  - 2
  - 3
- **c**
  - 1
  - 2
  - 3
- **x**
  - 1
  - 2
  - 3
- **y**
  - 1
  - 2
  - 3
- **z**
  - 1
  - 2
  - 3

**NEUTRAL LOSS:**

- H₂O (o)
- NH₃ (*)

**MASS TYPE:**

- Mono
- Avg

**MASS TOL:**

0.5

**PEAK ASSIGNMENT:**

- Most Intense
- Nearest Match
- Peak Detect

**PEAK LABELS:**

- Ion
- m/z
- None

**WIDTH:**

650

**HEIGHT:**

400

---

**COMET/Lorikeet Spectrum Viewer**

(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
SLHTLFGDK, MH+ 1017.5364, m/z 509.2718
File: 20150124_20_75Percent_Beads_1-15346, Exp. m/z: 509.77, Charge: 2

Ions:
- a 1' 2' 3'
- b 1' 2' 3'
- c 1' 2' 3'
- x 1' 2' 3'
- y 1' 2' 3'
- z 1'

Neutral Loss:
- H₂O (o)
- NH₃ (*)

Peak Assignment:
- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:
- Ion
- m/z
- None

Mass Type:
- Mono
- Avg

Mass Tol: 0.5

Update

Click and drag in the plot to zoom:
- X:
- Y:

Width: 650
Height: 400

COMET/Lorikeet Spectrum Viewer
(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
** ions:**
- a: 1, 2, 3
- b: 1, 2, 3
- c: 1, 2, 3
- x: 1, 2, 3
- y: 1, 2, 3
- z: 1, 2, 3

**Neutral Loss:**
- H₂O (o)
- NH₃ (*)

**Mass Type:**
- Mono
- Avg

**Mass Tol:** 0.5

**Peak Assignment:**
- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**
- Ion
- m/z
- None

**Width:** 650
**Height:** 400

**Modifications:**
- H: 391.177688 [2]
**COMET/Lorikeet Spectrum Viewer**
(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
Ions:

- a
- b
- c
- x
- y
- z

Neutral Loss:
- H₂O (o)
- NH₃ (*)

Mass Type:
- Mono
- Avg

Mass Tol: ±

Peak Assignment:
- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:
- Ion
- m/z
- None

Width: 650
Height: 400

Click and drag in the plot to zoom X: Y:

Enable tooltip

File: 20150202_22_75Percent_Beads_3.25469.25469.3, Scan: 25469, Exp. m/z: 992.35, Charge: 3

Mass Type: Mono

Mass Tol: ±

Peak Assignment:
- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:
- Ion
- m/z
- None

Width: 650
Height: 400

Click and drag in the plot to zoom X: Y:

Enable tooltip

COMET/Lorikeet Spectrum Viewer
(TPP v4.6 OCCUPY rev 3, Build 20130724109 (MinGW))
**Ions:**

- a
- b
- c
- x
- y
- z

**Neutral Loss:**

- H₂O (o)
- NH₃ (*)

**Mass Type:**

- Mono
- Avg

**Mass Tol.:** 0.5

**Peak Assignment:**

- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**

- Ion
- m/z
- None

**Width:** 650

**Height:** 400

**COMET/Lorikeet Spectrum Viewer**

(TPP v4.6 OCCUPY rev 3, Build 20130724109 (MinGW))

**File:** 20141212_25_79Percent_Labelled_1, 17939, 17939.3, Scan: 17939, Exp. m/z: 884.29, Charge: 3

**LVRPVEVDVMCTAFHDNEETFLK, M+ 2650.2639, m/z 884.0928**

**Peak Assignment:**

- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**

- Ion
- m/z
- None

**Width:** 650

**Height:** 400

**COMET/Lorikeet Spectrum Viewer**

(TPP v4.6 OCCUPY rev 3, Build 20130724109 (MinGW))
## Ions

<table>
<thead>
<tr>
<th>a</th>
<th>b^+</th>
<th>c^+</th>
<th>x</th>
<th>y^+</th>
<th>z^+</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>2^+</td>
<td>3*</td>
<td>1^*</td>
<td>2^*</td>
<td>3^*</td>
</tr>
<tr>
<td>1^*</td>
<td>2^+</td>
<td>3*</td>
<td>1^*</td>
<td>2^*</td>
<td>3^*</td>
</tr>
<tr>
<td>1*</td>
<td>2^+</td>
<td>3*</td>
<td>1^*</td>
<td>2^*</td>
<td>3^*</td>
</tr>
<tr>
<td>1*</td>
<td>2^+</td>
<td>3*</td>
<td>1^*</td>
<td>2^*</td>
<td>3^*</td>
</tr>
<tr>
<td>1*</td>
<td>2^+</td>
<td>3*</td>
<td>1^*</td>
<td>2^*</td>
<td>3^*</td>
</tr>
</tbody>
</table>

## Neutral Loss

- H\(_2\)O (o)
- NH\(_3\) (*)

## Mass Type

- Mono
- Avg

## Mass Tol

1.5

## Peak Assignment

- Most Intense
- Nearest Match
- Peak Detect

## Peak Labels

- Ion
- m/z
- None

## Width

650

## Height

400

---

**COMET/Lorikeet Spectrum Viewer**  
(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))
Ions:
- **a** 1+ 2+ 3+
- **b** 1+ 2+ 3+
- **c** 1+ 2+ 3+
- **x** 1+ 2+ 3+
- **y** 1+ 2+ 3+
- **z** 1+ 2+ 3+

Neutral Loss:
- H₂O (o)
- NH₃ (*)

Mass Type:
- Mono
- Avg

Mass Tol: 0.5

Peak Assignment:
- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:
- Ion
- m/z
- None

Width: 650
Height: 400

Click to move table

Modifications:
- C: 57.021415 [9, 10, 18]
Ions:
a 1+ 2+ 3+
b 1+ 2+ 3+
c 1+ 2+ 3+
x 1+ 2+ 3+
y 1+ 2+ 3+
z 1+ 2+ 3+

Neutral Loss:
H2O (o)  NH3 (*)

Mass Type:
Mono Avg

Peak Assignment:
Most Intense Nearest Match Peak Detect

Peak Labels:
Ion m/z None

Width: 650
Height: 400

Click and drag in the plot to zoom X: Y:

VFDEFPLVEEPQNLK, MH+ 2436.2731, m/z 812.7626
File: 20150202_22_75Percent_Beads_3.24162.24162.3, Scan: 24162, Exp. m/z: 813.61, Charge: 3

VFDEFPLVEEPQNLK, MH+ 2436.2731, m/z 812.7626
File: 20150202_22_75Percent_Beads_3.24162.24162.3, Scan: 24162, Exp. m/z: 813.61, Charge: 3

VFDEFPLVEEPQNLK, MH+ 2436.2731, m/z 812.7626
File: 20150202_22_75Percent_Beads_3.24162.24162.3, Scan: 24162, Exp. m/z: 813.61, Charge: 3

VFDEFPLVEEPQNLK, MH+ 2436.2731, m/z 812.7626
File: 20150202_22_75Percent_Beads_3.24162.24162.3, Scan: 24162, Exp. m/z: 813.61, Charge: 3

VFDEFPLVEEPQNLK, MH+ 2436.2731, m/z 812.7626
File: 20150202_22_75Percent_Beads_3.24162.24162.3, Scan: 24162, Exp. m/z: 813.61, Charge: 3

VFDEFPLVEEPQNLK, MH+ 2436.2731, m/z 812.7626
File: 20150202_22_75Percent_Beads_3.24162.24162.3, Scan: 24162, Exp. m/z: 813.61, Charge: 3

VFDEFPLVEEPQNLK, MH+ 2436.2731, m/z 812.7626
File: 20150202_22_75Percent_Beads_3.24162.24162.3, Scan: 24162, Exp. m/z: 813.61, Charge: 3

VFDEFPLVEEPQNLK, MH+ 2436.2731, m/z 812.7626
File: 20150202_22_75Percent_Beads_3.24162.24162.3, Scan: 24162, Exp. m/z: 813.61, Charge: 3

VFDEFPLVEEPQNLK, MH+ 2436.2731, m/z 812.7626
File: 20150202_22_75Percent_Beads_3.24162.24162.3, Scan: 24162, Exp. m/z: 813.61, Charge: 3

VFDEFPLVEEPQNLK, MH+ 2436.2731, m/z 812.7626
File: 20150202_22_75Percent_Beads_3.24162.24162.3, Scan: 24162, Exp. m/z: 813.61, Charge: 3

VFDEFPLVEEPQNLK, MH+ 2436.2731, m/z 812.7626
File: 20150202_22_75Percent_Beads_3.24162.24162.3, Scan: 24162, Exp. m/z: 813.61, Charge: 3

VFDEFPLVEEPQNLK, MH+ 2436.2731, m/z 812.7626
File: 20150202_22_75Percent_Beads_3.24162.24162.3, Scan: 24162, Exp. m/z: 813.61, Charge: 3

VFDEFPLVEEPQNLK, MH+ 2436.2731, m/z 812.7626
File: 20150202_22_75Percent_Beads_3.24162.24162.3, Scan: 24162, Exp. m/z: 813.61, Charge: 3

VFDEFPLVEEPQNLK, MH+ 2436.2731, m/z 812.7626
File: 20150202_22_75Percent_Beads_3.24162.24162.3, Scan: 24162, Exp. m/z: 813.61, Charge: 3

VFDEFPLVEEPQNLK, MH+ 2436.2731, m/z 812.7626
File: 20150202_22_75Percent_Beads_3.24162.24162.3, Scan: 24162, Exp. m/z: 813.61, Charge: 3
**VFDEFKPLVEEPQLIK**, MH+ 2045.0954, m/z 682.3700

File: 20150202_20_75Percent_Beads_1.23748.23748.3, Scan: 23748, Exp. m/z: 683, Charge: 3

**Ions:**
a 1
b 1
1
1
3
2
x 1
y 1
z 1
3
2
2
3
2
2
3
2
3
3

**Neutral Loss:**
- H₂O (o)
- NH₃ (*)

**Mass Type:**
- Mono
- Avg

**Mass Tol:**
- 0.5
- Update

**Peak Assignment:**
- Most Intense
- Nearest Match
- Peak Detect

**Peak Labels:**
- Ion
- m/z
- None

**Width:** 650
**Height:** 400

Click and drag in the plot to zoom

**COMET/Lorikeet Spectrum Viewer**
(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))

**Peak Table:**

<table>
<thead>
<tr>
<th>b</th>
<th>b2+</th>
<th>#</th>
<th>Seq</th>
<th>#</th>
<th>y+</th>
<th>y2+</th>
</tr>
</thead>
<tbody>
<tr>
<td>100.075</td>
<td>50.545</td>
<td>1</td>
<td>V</td>
<td>17</td>
<td>1946.0270</td>
<td>271.5175</td>
</tr>
<tr>
<td>167.177</td>
<td>30.209</td>
<td>3</td>
<td>D</td>
<td>15</td>
<td>1798.9585</td>
<td>399.9070</td>
</tr>
<tr>
<td>491.313</td>
<td>246.110</td>
<td>4</td>
<td>E</td>
<td>14</td>
<td>1683.9316</td>
<td>483.4694</td>
</tr>
<tr>
<td>638.328</td>
<td>319.644</td>
<td>5</td>
<td>F</td>
<td>13</td>
<td>1554.8890</td>
<td>577.9481</td>
</tr>
<tr>
<td>766.377</td>
<td>383.692</td>
<td>6</td>
<td>K</td>
<td>12</td>
<td>1407.8206</td>
<td>704.4139</td>
</tr>
<tr>
<td>863.429</td>
<td>432.219</td>
<td>7</td>
<td>P</td>
<td>11</td>
<td>1374.7796</td>
<td>440.3563</td>
</tr>
<tr>
<td>976.513</td>
<td>488.760</td>
<td>8</td>
<td>L</td>
<td>10</td>
<td>1182.6729</td>
<td>591.8401</td>
</tr>
<tr>
<td>1075.542</td>
<td>539.194</td>
<td>9</td>
<td>V</td>
<td>9</td>
<td>1069.5880</td>
<td>535.2586</td>
</tr>
<tr>
<td>1101.614</td>
<td>607.816</td>
<td>10</td>
<td>E</td>
<td>8</td>
<td>947.4304</td>
<td>488.7638</td>
</tr>
<tr>
<td>1333.667</td>
<td>667.337</td>
<td>11</td>
<td>E</td>
<td>8</td>
<td>841.4778</td>
<td>511.2823</td>
</tr>
<tr>
<td>1430.702</td>
<td>715.861</td>
<td>12</td>
<td>P</td>
<td>8</td>
<td>712.0392</td>
<td>554.7212</td>
</tr>
<tr>
<td>1558.758</td>
<td>799.890</td>
<td>13</td>
<td>Q</td>
<td>7</td>
<td>615.3824</td>
<td>399.1949</td>
</tr>
<tr>
<td>1672.821</td>
<td>836.914</td>
<td>14</td>
<td>W</td>
<td>6</td>
<td>887.3239</td>
<td>344.1656</td>
</tr>
<tr>
<td>1785.805</td>
<td>897.434</td>
<td>15</td>
<td>L</td>
<td>5</td>
<td>775.2809</td>
<td>287.4441</td>
</tr>
<tr>
<td>1898.801</td>
<td>955.449</td>
<td>16</td>
<td>I</td>
<td>4</td>
<td>660.1949</td>
<td>330.6021</td>
</tr>
<tr>
<td>17</td>
<td>K</td>
<td>3</td>
<td>147.1128</td>
<td>74.0600</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
YKAFTEC CQAADK, MH+ 2053.9027, m/z 685.3057

Ions:
- a 1+ 2+ 3+
- b 1+ 2+ 3+
- c 1+ 2+ 3+
- x 1+ 2+ 3+
- y 1+ 2+ 3+
- z 1+ 2+ 3+

Neutral Loss:
- H2O (o)
- NH3 (*)

Mass Type:
- Mono
- Avg

Mass Tol: 0.5

Peak Assignment:
- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:
- Ion m/z
- None

Width: 650
Height: 400

Modifications:
C: 57.021415 [8, 9]
K: 391.177737 [2]

COMET/Lorikeet Spectrum Viewer
(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))

File: 20150124_22_79Percent_Beads_1,14185,14185,3, Scan: 14185, Exp. m/z: 685.54, Charge: 3

<table>
<thead>
<tr>
<th>b+</th>
<th>b2+</th>
<th>#</th>
<th>Seq</th>
<th>y+</th>
<th>y2+</th>
</tr>
</thead>
<tbody>
<tr>
<td>164.0706</td>
<td>82.5389</td>
<td>1</td>
<td>Y</td>
<td>1-4</td>
<td></td>
</tr>
<tr>
<td>683.3433</td>
<td>342.1753</td>
<td>2</td>
<td>K</td>
<td>1-3, 1090.3944</td>
<td>608.9373</td>
</tr>
<tr>
<td>174.1248</td>
<td>87.0624</td>
<td>3</td>
<td>A</td>
<td>2-5, 617.6678</td>
<td>686.2870</td>
</tr>
<tr>
<td>823.4175</td>
<td>413.2124</td>
<td>4</td>
<td>A</td>
<td>3, 1300.3268</td>
<td>650.7684</td>
</tr>
<tr>
<td>972.4859</td>
<td>486.7466</td>
<td>5</td>
<td>F</td>
<td>2, 1229.4924</td>
<td>615.2499</td>
</tr>
<tr>
<td>1073.5336</td>
<td>537.2705</td>
<td>6</td>
<td>T</td>
<td>1, 1082.4240</td>
<td>541.7156</td>
</tr>
<tr>
<td>1303.6723</td>
<td>652.3365</td>
<td>7</td>
<td>E</td>
<td>1, 991.5461</td>
<td>451.1918</td>
</tr>
<tr>
<td>1362.8068</td>
<td>681.4030</td>
<td>8</td>
<td>C</td>
<td>1, 852.3318</td>
<td>426.6705</td>
</tr>
<tr>
<td>1522.6374</td>
<td>761.8223</td>
<td>9</td>
<td>C</td>
<td>1-3, 692.3032</td>
<td>346.6552</td>
</tr>
<tr>
<td>1650.8960</td>
<td>825.4417</td>
<td>10</td>
<td>Q</td>
<td>1-3, 533.2796</td>
<td>266.6399</td>
</tr>
<tr>
<td>1721.7331</td>
<td>861.3702</td>
<td>11</td>
<td>A</td>
<td>1, 404.2140</td>
<td>202.8106</td>
</tr>
<tr>
<td>1792.7702</td>
<td>895.3887</td>
<td>12</td>
<td>A</td>
<td>1, 322.1749</td>
<td>167.0921</td>
</tr>
<tr>
<td>1907.2972</td>
<td>954.4052</td>
<td>13</td>
<td>D</td>
<td>1, 662.1337</td>
<td>331.5735</td>
</tr>
<tr>
<td>1946.0154</td>
<td>974.0016</td>
<td>14</td>
<td>X</td>
<td>1-3, 147.1128</td>
<td>74.0600</td>
</tr>
</tbody>
</table>

Click to move table

Mass scan: 14183, RT 4569.79
Ions:

a 1+ 2+ 3+  
b 1+ 2+ 3+  
c 1+ 2+ 3+  
x 1+ 2+ 3+  
y 1+ 2+ 3+  
z 1+ 2+ 3+  

Neutral Loss:

- H₂O (o)
- NH₃ (*)

Mass Type:
- Mono
- Avg

Mass Tol: 0.5

Peak Assignment:
- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:
- Ion
- m/z
- None

Width: 650

Height: 400

COMET/Lorikeet Spectrum Viewer
(TPP v4.6 OCCUPY rev 3, Build 201307241109 (MinGW))

File: 20150124_21_75Percent_Beads_2.12413.12413.3, Scan: 12413, Exp. m/z: 555.55, Charge: 3

Peak Assignments:

<table>
<thead>
<tr>
<th>b+</th>
<th>b2+</th>
<th>#</th>
<th>Seq.</th>
<th>y+</th>
<th>y2+</th>
</tr>
</thead>
<tbody>
<tr>
<td>164.0706</td>
<td></td>
<td>1</td>
<td>Y</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>146.5864</td>
<td></td>
<td>2</td>
<td>K</td>
<td>13</td>
<td>1499.6616</td>
</tr>
<tr>
<td>182.1050</td>
<td>3</td>
<td>A</td>
<td>12</td>
<td>1371.5670</td>
<td></td>
</tr>
<tr>
<td>217.6235</td>
<td>4</td>
<td>A</td>
<td>11</td>
<td>1300.5296</td>
<td>650.7684</td>
</tr>
<tr>
<td>291.1976</td>
<td>5</td>
<td>F</td>
<td>10</td>
<td>1229.4924</td>
<td>615.2499</td>
</tr>
<tr>
<td>341.6816</td>
<td>6</td>
<td>T</td>
<td>9</td>
<td>1082.4240</td>
<td>541.7156</td>
</tr>
<tr>
<td>456.7039</td>
<td>7</td>
<td>E</td>
<td>8</td>
<td>981.3743</td>
<td>481.1918</td>
</tr>
<tr>
<td>486.2192</td>
<td>8</td>
<td>C</td>
<td>7</td>
<td>852.3318</td>
<td>456.6395</td>
</tr>
<tr>
<td>566.2335</td>
<td>9</td>
<td>C</td>
<td>6</td>
<td>692.3012</td>
<td>446.6552</td>
</tr>
<tr>
<td>590.3438</td>
<td>10</td>
<td>Q</td>
<td>5</td>
<td>933.2734</td>
<td>246.6399</td>
</tr>
<tr>
<td>615.7813</td>
<td>11</td>
<td>A</td>
<td>4</td>
<td>1004.2145</td>
<td>202.4106</td>
</tr>
<tr>
<td>1291.5924</td>
<td>12</td>
<td>A</td>
<td>3</td>
<td>1331.7748</td>
<td>147.0921</td>
</tr>
<tr>
<td>1330.5554</td>
<td>13</td>
<td>D</td>
<td>2</td>
<td>1622.1397</td>
<td>131.5715</td>
</tr>
<tr>
<td>1401.5925</td>
<td></td>
<td>K</td>
<td>1</td>
<td>147.1128</td>
<td>74.0600</td>
</tr>
</tbody>
</table>

Modifications:
- C: 57.021415 [8, 9]
Ions:
a  1+  2+  3+
b  1+  2+  3+
c  1+  2+  3+
x  1+  2+  3+
y  1+  2+  3+
z  1+  2+  3+

Neutral Loss:
- H₂O (o)
- NH₃ (*)

Neutral Loss:
- H₂O (o)
- NH₃ (*)

Mass Type:
- Mono
- Avg

Mass Tol: 0.0

Peak Assignment:
- Most Intense
- Nearest Match
- Peak Detect

Peak Labels:
- Ion
- m/z

None

Width: 650

Height: 400