Isotope Ratio Mass Spectrometry - A Rapidly Developing Tool for Forensic Samples

A dissertation presented to
the faculty of
the College of Arts and Sciences of Ohio University

In partial fulfillment
of the requirements for the degree
Doctor of Philosophy

Zeland Muccio
March 2010

© 2010 Zeland Muccio. All Rights Reserved.
This dissertation titled
Isotope Ratio Mass Spectrometry - A Rapidly Developing Tool for Forensic Samples

by

ZELAND MUCCIO

has been approved for
the Department of Chemistry and Biochemistry
and the College of Arts and Sciences by

______________________________

Glen P. Jackson
Associate Professor of Chemistry and Biochemistry

______________________________

Benjamin M. Ogles
Dean, College of Arts and Sciences
ABSTRACT

MUCCIO, ZELAND, Ph.D., March 2010, Chemistry

Isotope Ratio Mass Spectrometry - A Rapidly Developing Tool for Forensic Samples

(236 pp.)

Director of Dissertation: Glen P. Jackson

The development of a dual detection system that provides simultaneous structural elucidation and isotopic analyses of forensic samples is described. A gas chromatograph (GC) was coupled to an isotope ratio mass spectrometer (IRMS) in parallel with a single quadrupole mass spectrometer (MS). The modification was achieved by using a low, dead volume x-connector to split the effluent coming from the column so that approximately 10% entered the EI MS and the remaining 90% entered the combustion interface for IRMS. The transfer line that connected the GC to the MS was fabricated to extend the line into the GC oven. Heat tape was tightly wrapped around the extension to maintain a predetermined, constant temperature by use of a manual heat controller. The modified instrumentation was then applied to forensic samples to simultaneously determine the structural elucidation and the isotopic ratios of individual compounds and impurities within the sample.

Illicit drugs are one of the most analyzed forensic samples in federal, state, and private forensic laboratories. Cocaine was analyzed and identification was confirmed using a NIST library. The probability scores from the NIST library for all of the cocaine samples ranged between 52.9% and 77.1%. Several cocaine samples were used to determine if the cocaine could have come from the same source using carbon isotopic
analysis. Marijuana was another illicit drug that was analyzed using this instrumentation method. We report the first application of GC-IRMS to individual components of Cannabis sativa L. to discriminate between different sources.

Different manufacturers, or lot numbers, of common household accelerants such as Goof Off, turpentine, charcoal lighter fluid and WD-40 were also analyzed. The analysis of accelerants demonstrated that this modification of instrumentation could be used for not only pure compounds but, also for very complex compounds. IRMS could distinguish between different sources of accelerants by analyzing trace residues remaining after combustion.

To further examine the versatility of this tool, individual amino acids in hair were analyzed. We have shown that it is possible to determine minimally nine individual amino acids within hair samples using a single step derivatization method. In the future, we would like to study single strands of hair and the possibilities of segmenting the hair into sections of monthly growth. The analysis of forensic samples using this modification is virtually unlimited.

Approved: 

Glen P. Jackson

Associate Professor of Chemistry and Biochemistry
ACKNOWLEDGMENTS

When I first started my graduate degree, I had the pleasure of working with Dr. Vladimir Alexeev. His time working with me was unfortunately cut short but, I do appreciate his wisdom and guidance during the time that we did have together. I will forever remember his carefree nature and his passion for life.

I was fortunate enough, during this difficult time, to become associated with Dr. Glen P. Jackson who took me into his research group with open arms. Dr. Jackson was the first one to reach out to me and encourage me to continue when doubts crept into my mind as to whether or not to continue. I truly appreciate the advice and guidance that Dr. Jackson has afforded me during my time in his research group. In addition, was it not for his far superior writing skills, this dissertation would not have come to fruition.

I want to thank, Dr. Hao Chen, Dr. Kenneth Brown, and Dr. Shigeru Okada for agreeing to serve on my dissertation committee. I also want to thank Dr. Glen P. Jackson for agreeing to serve as the chair for my committee. I appreciate the time that they took to review my dissertation and provide their learned expertise.

I also wish to thank my current lab mates, Shannon Cook, Carolyn Zimmermann, Yan An and former lab mates Derrell Hood, and Dr. Ünige Laskay, for their encouragement and camaraderie. It was always a tremendous help to have them around to help me and talk to. In addition, I had the pleasure of working with a number of very talented undergraduate and exchange students: Christopher Kanalas, Andreas Baum, and Claudia Wöckel. I would like to recognize the entire department of Chemistry and Biochemistry at Ohio University for all of their dedication and hard work, particularly
Bascom French and Paul Schmittauer for all of their assistance which allowed my research to progress as it did. I also want to acknowledge the financial assistance received from the National Science Foundation grant number (0745590).

Special thanks go out to Dr. Palmer Graves and Dr. Bruce McCord at Florida International University for encouraging me to continue in the field of chemistry and the support that they gave me when I encountered difficulties in my studies.

I want to thank Carl Fitzgerald for never losing faith in me and teaching me the values of a good education and the opportunities that it opened up for my future.

Finally, and most importantly, I want to sincerely thank Michael Martel, for his endless support and for always being there when I needed him. In the nine years that we have been together he has never once doubted my ability to see this through and I love him dearly for that. And, even if she doesn’t realize it, I want to thank my loving cat Tasha for always welcoming me home and keeping me company!
I would first like to dedicate this work to the loving memory of my mother who passed away in 1979. Had circumstances been different, I would have loved for her to have shared my success with me. I would next like to thank my father for continuously encouraging me throughout my education and life. He taught me many things throughout my lifetime and I would like to think that a part of my success was owed to him. Lastly, I want to sincerely thank my brother Frankie, for helping me realize that I could be a better person and make so much more of myself.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>........................................................................................................</td>
<td>3</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>................................................................................</td>
<td>5</td>
</tr>
<tr>
<td>Dedication</td>
<td>..................................................................................</td>
<td>7</td>
</tr>
<tr>
<td>List of Tables</td>
<td>...............................................................................</td>
<td>13</td>
</tr>
<tr>
<td>List of Figures</td>
<td>................................................................................</td>
<td>14</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>........................................................................</td>
<td>18</td>
</tr>
<tr>
<td>Chapter 1: Introduction</td>
<td>........................................................................</td>
<td>20</td>
</tr>
<tr>
<td>1.1 Objective</td>
<td>..............................................................................</td>
<td>20</td>
</tr>
<tr>
<td>1.2 Project Overview</td>
<td>.........................................................................</td>
<td>21</td>
</tr>
<tr>
<td>1.3 Instrumentation</td>
<td>...........................................................................</td>
<td>24</td>
</tr>
<tr>
<td>Chapter 2: Isotope Ratio Mass Spectrometry</td>
<td>........................................</td>
<td>26</td>
</tr>
<tr>
<td>2.1 Introduction</td>
<td>............................................................................</td>
<td>26</td>
</tr>
<tr>
<td>2.1.1 EA-IRMS</td>
<td>........................................................................</td>
<td>32</td>
</tr>
<tr>
<td>2.1.2 GC-IRMS</td>
<td>........................................................................</td>
<td>33</td>
</tr>
<tr>
<td>2.1.3 LC-IRMS</td>
<td>........................................................................</td>
<td>34</td>
</tr>
<tr>
<td>2.2 Origins of variations in isotopic abundances</td>
<td>................................</td>
<td>35</td>
</tr>
<tr>
<td>2.3 Applications</td>
<td>...........................................................................</td>
<td>38</td>
</tr>
<tr>
<td>2.3.1 Forensic</td>
<td>........................................................................</td>
<td>38</td>
</tr>
<tr>
<td>2.3.2 Food and Drugs</td>
<td>.....................................................................</td>
<td>42</td>
</tr>
<tr>
<td>2.3.3 Diet, Biochemistry and Metabolism</td>
<td>...........................................</td>
<td>45</td>
</tr>
</tbody>
</table>
2.3.4 Athletics and Doping ........................................................................................ 49
2.3.5 Environmental Pollution .............................................................................. 50
2.4 Summary .......................................................................................................... 51

Chapter 3: Isotope Ratio Mass Spectrometry – A New Tool for the Forensic Chemist... 53

3.1 Introduction ....................................................................................................... 53
3.1.1 IRMS ........................................................................................................... 55
3.1.2 EA-IRMS .................................................................................................... 58
3.1.3 GC-IRMS ................................................................................................... 59
3.2 Origins of Variations in Isotopic Abundances ............................................. 60
3.3 Forensic Applications ..................................................................................... 63
3.4 Summary .......................................................................................................... 68

Chapter 4: Simultaneous Identification and δ^{13}C Classification of Drugs Using GC with Concurrent Single Quadrupole and Isotope Ratio Mass Spectrometers ........................................ 70

4.1 Introduction ....................................................................................................... 70
4.2 Materials and Methods ................................................................................... 74
4.2.1 Chemicals .................................................................................................... 74
4.2.2 Gas Chromatography – Mass Spectrometry / Isotope Ratio Mass Spectrometry ......................................................................................................................... 76
4.3 Results and Discussion ................................................................................... 80
4.4 Summary .......................................................................................................... 86
Chapter 5: Simultaneous Identification and $\delta^{13}$C Classification of Cannabinol in Unknown Marijuana Samples Using GC with Concurrent Single Quadrupole and Isotope Ratio Mass Spectrometers

5.1 Introduction

5.2 Materials and Methods

5.2.1 Chemicals

5.2.2 Gas Chromatography – Mass Spectrometry / Isotope Ratio Mass Spectrometry

5.3 Results and Discussion

5.4 Summary

Chapter 6: Forensic Analysis of Accelerants Using Gas Chromatography with Parallel Isotope Ratio and Single Quadrupole Detectors

6.1 Introduction

6.2 Materials and Methods

6.2.1 Chemicals

6.2.2 Simulated fire debris

6.2.3 Gas Chromatography – Mass Spectrometry / Isotope Ratio Mass Spectrometry

6.2.4 Operating Conditions

6.3 Results and Discussion

6.3.1 Standard Accelerants

6.3.2 Scorched Samples
Chapter 7: Measurement of $^{13}$C/$^{12}$C Isotopic Composition of Individual Amino Acids in Human Hair for Forensic Comparisons

7.1 Introduction

7.2 Materials and Methods

7.2.1 Subjects

7.2.2 Reagents and Standards

7.2.3 Derivatization of Standards

7.2.4 Derivatization and Pulverization of Hair

7.2.5 Gas Chromatography – Mass Spectrometry / Isotope Ratio Mass Spectrometry

7.3 Preliminary Results

7.4 Summary

Chapter 8: Conclusion and Future work

Appendices

Appendix A.1 GC-IRMS Standard ($C_{11}$, $C_{15}$, $C_{20}$)

Appendix A.2 GC-IRMS Standard ($C_{25}$, $C_{30}$, $C_{36}$)

Appendix B.1 Identification and Individualization of Cocaine Samples Using Gas Chromatography Coupled Simultaneously to a Quadrupole Mass Spectrometer and an Isotope Ratio Mass Spectrometry

Appendix B.1.1 Isotope Ratio Mass Spectrometry Laboratory (pages 1-5)

Appendix B.1.2 Crime Lab Request for Evidence Examination Form
Appendix B.1.3 Receipt and Tracking of Physical Evidence Form ...................... 171
Appendix B.1.4 Official Crime Laboratory Report ........................................ 172
Appendix B.1.5 Example of an Actual RSS Lab Report (pages 1-11) ............. 173
Appendix C.1 Poster Presentation at MUACC hosted by the Michigan State University ................................................................................................................... 184
Appendix D.1 Poster Presentation at Ohio University Research and Creative Activity ........................................................................................................ 185
Appendix E.1 Institutional Review Board Approval Form (09X194) ............... 186
Appendix E.2 Institutional Review Board Consent Form (09X194) ................. 187
Supplements ........................................................................................................ 190
Supplement S.1 Supplemental Material Submitted with Publication to the Journal of Chemical Education with “Isotope Ratio Mass Spectrometry – A New Tool for the Forensic Chemist” (slides 1-19) ................................................................. 190
Supplement S.2 Differentiation Between Origins of Extra Virgin Olive Oils by GC/C/IRMS Using Principal Component Analysis, Linear Discriminant Analysis and Hierarchical Cluster Analysis (pages 1-17) ......................................................... 200
References ........................................................................................................... 220
LIST OF TABLES

Table 4.1  Cocaine sample, company, name of sample, and lot number.......................... 74

Table 4.2  F-calculated values for six cocaine samples; cocaine free base standard Samples A and B, cocaine HCl standard Sample C, and three street crack cocaine Samples D, E, and F. Cells with a white background are significantly different. .............................................. 85

Table 4.3  P-values for six cocaine samples; cocaine free base standard Samples A and B, cocaine HCl standard Sample C, and three street crack cocaine Samples D, E, and F. Cells with a white background are significantly different. ........................................................ 86

Table 5.1  F-calculated values for $\delta^{13}$C values of CBN in four marijuana samples; A, B, C, and D. Cells with a white background are significantly different. ................................................................. 103

Table 5.2  P-values for $\delta^{13}$C values of CBN in four marijuana samples; A, B, C, and D. Cells with a white background are significantly different. ..................................................................................................... 103

Table 5.3  T-values for $\delta^{13}$C values of CBN in four marijuana samples; A, B, C, and D. Cells with a white background are significantly different. ..................................................................................................... 104

Table 6.1  The number of fires, deaths, and dollar loss due to intentionally set structure fires that occurred from 1999 to 2008. ..................................................... 107

Table 6.2  F-calculated values for four accelerant samples; A, B, A(scorched), and B(scorched). Cells with a white background are significantly different at the 95% C.L................................................................. 128

Table 6.3  P-values for four accelerant samples; A, B, A(scorched), and B(scorched). Cells with a white background are significantly different ..................................................................................................... 128

Table 7.1  Carbon isotope ratios of individual amino acids as established by derivatization GC-C-IRMS (N=3) ........................................................................................................ 138

Table 7.2  Error at 95% CI of individual amino acids as established by derivatization GC-C-IRMS (N=3) ........................................................................................................ 138
LIST OF FIGURES

Figure 2.1   Histogram showing the number of publications per year containing the research topic 'Isotope ratio mass spectrometry'. The total number of hits for research topic, as entered, was 2050. Search performed using SciFinder Scholar 2006 on May 3, 2008. Reproduced with permission from Muccio and Jackson.1 ................. 27

Figure 2.2   Schematics to show how the three most common sample introduction systems/interfaces for carbon isotope measurements (as CO2) and an isotope ratio mass spectrometer. LC = liquid chromatography, EA = elemental analyzer, GC = gas chromatography. Reproduced with permission from Muccio and Jackson.1 ....................................................................................................... 31

Figure 2.3   Examples of variations of carbon isotopic abundances of plants and human diets. Reproduced with permission from Muccio and Jackson.1 ....................................................................................................... 36

Figure 2.4   The path of GHB/GBL, from spiked drink, to victim, to hair, and eventually to the GC/C/IRMS m/z 44 chromatogram. The peaks were obtained from the liquid injection of a standard of GBL and ε-caprolactone at 50 ng each (on column). The square-topped peaks represent pulses of CO2 reference gas. Reproduced with permission from Muccio and Jackson.1 ............................................... 41

Figure 2.5   Carbon and nitrogen isotope ratio analysis of wild elephants’ hair can be used to detect the occurrence of night-time raids on farmers’ crops. Reproduced with permission from Muccio and Jackson.1 ....................................................................................................... 47

Figure 3.1   Schematic of our current set up with the GC-IRMS coupled to a single quadrupole mass spectrometer to allow for a single injection of a sample and have results that simultaneously provide the retention time, characteristic EI fragmentation patterns used to confirm the identity of individual components within a sample and the carbon isotopic abundance ratio of each compound in the mixture............................................................................................. 57

Figure 3.2   Typical isotopic composition of an average built adult. "Artwork provided for use in this article only courtesy of W Meier-Augenstein; artwork is based on a figure in E. Wada and A. Hattori (1990)"75 ........................................................................................................... 61

Figure 3.3   Examples of variations of carbon isotopic abundances of plants and human diets........................................................................................................................................... 62
Figure 3.4  Comparison of hydrogen and carbon isotope ratios for match sticks from the crime scene, from the suspect’s possession, and other sources. Data points are the mean from triplicate analyses per isotope and error bars are ±1 s.d. These results did not find a common source between the matches at the crime scene and any of the other known samples. Reproduced with permission from reference 36. .......................................................... 65

Figure 4.1  (a) Schematic of our GC-MS set up with a transfer line extension and heat tape configuration and (b) the schematic of GC-MS-IRMS wiring to control the auto-sampler signal to both PC’s indicating that the sample injection has been made and the run should begin. ................................................................................................ 78

Figure 4.2  TIC chromatogram and mass spectrum of the peak at 26.52 minutes. The retention index and mass spectrum of this sample C confirm the identity of the sample as cocaine. The NIST head to tail output confirm the presence of cocaine with a 77.1% probability score. ......................................................................................... 81

Figure 4.3  Example of a concurrent IRMS output acquisition for the single injection of cocaine sample C. ........................................................................ 82

Figure 4.4  Average δ¹³C values of six cocaine samples. Results represent three aliquots of each sample with quadruplicate sampling and error bars indicate 95% confidence interval. ................................................ 84

Figure 5.1  Cannabinoid structures of the main active constituents in the Cannabis sativa L. .................................................................................. 89

Figure 5.2  TIC chromatogram and mass spectra with the NIST head to tail library output of samples A, B, (top row) C, and D (bottom row). Samples A and B containing the cannibinoid CBN and samples C and D having both CBD and CBN. ............................................ 97

Figure 5.3  Example of a concurrent IRMS output acquisition for the single injection of marijuana samples A, and B. .................................................... 99

Figure 5.4  Example of a concurrent IRMS output acquisition for the single injection of marijuana samples C, and D. .............................................. 100

Figure 5.5  Average δ¹³C values of CBN of four marijuana samples. Results represent three aliquots of each sample with quadruplicate sampling and error bars indicate 95% confidence interval. ....................... 102

Figure 6.1  Accelerant Classification System Table ..................................................... 111

Figure 6.2  Example of an IRMS chromatogram for one injection of an accelerant sample, Turpentine (B). The second CO₂ peak is designated as the internal standard in the instrument acquisition software. ........................................ 116
Figure 6.3  TIC chromatogram and NIST head to tail library output of Goof Off (A). ......................................................................................................................... 117
Figure 6.4  Example of an IRMS chromatogram for one injection of an accelerant sample, Goof Off (A). ......................................................................................................................... 118
Figure 6.5  Bar graph showing $\delta^{13}$C for 3 major peaks from Goof Off samples A & B with error bars representing a 95% C.I. ................................................................. 119
Figure 6.6  TIC chromatogram and NIST head to tail library output of WD-40 (B). ................................................................................................................................. 120
Figure 6.7  Example of an IRMS chromatogram for one injection of an accelerant sample, WD-40 (B). Peak 1 (902 s) is Nonane, peak 2 (1181 s) is Decane and peak 3 (1455 s) is Undecane ................................................................. 121
Figure 6.8  Bar graph showing $\delta^{13}$C for 3 major peaks from WD-40 samples A & B with error bars representing a 95% C.I. ................................................................. 122
Figure 6.9  TIC chromatogram and NIST head to tail library output of Turpentine (B). ................................................................................................................................. 123
Figure 6.10  Example of an IRMS chromatogram for one injection of an accelerant sample, Turpentine (B). Peak 1 (795 s) is Ethylbenzene, Peak 2 (842 s) is $\alpha$-pinene and peak 3 (883 s) is Camphene ................................................................................................................................. 124
Figure 6.11  Bar graph showing $\delta^{13}$C for 3 peaks from Turpentine samples A & B with error bars representing a 95% C.I. ................................................................. 125
Figure 6.12  Example of an IRMS chromatogram for one injection of an accelerant sample, Charcoal lighter fluid (B). Peak 1 (592 s) is Nonane, Peak 2 (856 s) is Decane and peak 3 (1134 s) is Undecane ................................................................................................................................. 126
Figure 6.13  Bar graph showing $\delta^{13}$C for 3 peaks from Charcoal Lighter Fluid samples A & B with error bars representing a 95% C.I. ................................................................. 127
Figure 6.14  Average $\delta^{13}$C values of turpentine aliquots; ($\alpha$-pinene sample A and scorched sample A(scorched)) and ($\alpha$-pinene sample B, and scorched sample B(scorched)) with error bars showing 95% C.I. ................................................................. 129
Figure 7.1  TIC chromatogram and mass spectra for a single injection of hair sample A0009 displaying individual amino acids. ............................................................................... 139
Figure 7.2  Concurrent IRMS output acquisition for the simultaneous injection of hair sample A0009 displaying individual amino acids ......................................................................................... 140
Figure 7.3  Comparison of $\delta^{13}$C values for six hair samples showing the results of Alanine, Valine, and Leucine with 95% C.I. (N=3) ............................................................................. 141
Figure 7.4  Comparison of $\delta^{13}C$ values for six hair samples showing the results of Isoleucine, Serine, and Aspartic Acid with 95% C.I. (N=3). ......................................................................................................... 142

Figure 7.5  Comparison of $\delta^{13}C$ values for six hair samples showing the results of Proline, Tyrosine, and Cystine with 95% C.I. (N=3). .............. 143

Figure 7.6  Hypothetical example of how segmented analysis of $\delta^{13}C$ of amino acids in hair could be used to establish potential common origins and time since shedding. Sample Q1 came from the same donor as K1, but with a three-month offset. K2 came from a different donor. Other amino acids could be used to confirm or relate the positive comparison............................................................... 144
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAAS</td>
<td>American Association for the Advancement of Science</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis-of-variance</td>
</tr>
<tr>
<td>CAM</td>
<td>Crassulacean acid metabolism</td>
</tr>
<tr>
<td>CBC</td>
<td>Cannabichrome</td>
</tr>
<tr>
<td>CBD</td>
<td>Cannabidiol</td>
</tr>
<tr>
<td>CBG</td>
<td>Cannabigerol</td>
</tr>
<tr>
<td>CBN</td>
<td>Cannabinol</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>DEA</td>
<td>Drug Enforcement Administration</td>
</tr>
<tr>
<td>DFSA</td>
<td>Drug-facilitated sexual assault</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EA</td>
<td>Elemental analyzer</td>
</tr>
<tr>
<td>EI</td>
<td>Electron ionization</td>
</tr>
<tr>
<td>FBI</td>
<td>Federal Bureau of Investigation</td>
</tr>
<tr>
<td>FIRMS</td>
<td>Forensic isotope ratio mass spectrometry</td>
</tr>
<tr>
<td>GBL</td>
<td>$\gamma$-butyrolactone</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GC/C/IRMS</td>
<td>Gas chromatography combustion isotope ratio mass spectrometry</td>
</tr>
<tr>
<td>GHB</td>
<td>$\gamma$-hydroxybutyric acid</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSSR</td>
<td>Glutathione disulfide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IAEA</td>
<td>International Atomic Energy Agency</td>
</tr>
<tr>
<td>IRMM</td>
<td>Institute for reference materials and measurements</td>
</tr>
<tr>
<td>IRMS</td>
<td>Isotope ratio mass spectrometry</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass to charge ratio</td>
</tr>
<tr>
<td>MC-ICP-MS</td>
<td>Multiple collector inductively coupled plasma mass spectrometry</td>
</tr>
<tr>
<td>MS</td>
<td>Single quadrupole mass spectrometer</td>
</tr>
<tr>
<td>NACME</td>
<td>N-acetylmethyl</td>
</tr>
<tr>
<td>NAD</td>
<td>Nandrolone</td>
</tr>
<tr>
<td>NIST</td>
<td>National Institute of Standards and Technology</td>
</tr>
<tr>
<td>NPL</td>
<td>National physical laboratory</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic aromatic hydrocarbons</td>
</tr>
<tr>
<td>RSS</td>
<td>Randomly selected student</td>
</tr>
<tr>
<td>SWGDRUG</td>
<td>Scientific Working Group for the Analysis of Seized Drugs</td>
</tr>
<tr>
<td>TFAA</td>
<td>Trifluoroacetic anhydride</td>
</tr>
<tr>
<td>THC</td>
<td>$\Delta^9$-tetrahydrocannabinol</td>
</tr>
<tr>
<td>TIC</td>
<td>Total ion current</td>
</tr>
<tr>
<td>V-CDT</td>
<td>Vienna Canyon Diablo Troilite</td>
</tr>
<tr>
<td>VPDB</td>
<td>Vienna Pee Dee Belemnite</td>
</tr>
<tr>
<td>VSMOW</td>
<td>Vienna Standard Mean Ocean Water</td>
</tr>
<tr>
<td>$\delta$</td>
<td>Delta</td>
</tr>
<tr>
<td>$%o$</td>
<td>per mil</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION

1.1 Objective

The main objective of this research is to develop and improve on current analytical instrumentation for applications in the field of forensic science. This dissertation focuses on a modest adaptation of a commercial GC/C/IRMS system and an application for forensic chemistry. The ability to inject only one sample, one time and concurrently acquire mass spectrometric fragmentation pattern analyses, and isotopic ratios is very new. The parallel detectors make it possible to have two independent tests on one sample that would satisfy the requirements, laid out by the forensic community, to be used as a confirmatory method.

To begin, it was necessary to modify an already existing gas chromatography isotope ratio mass spectrometer (GC-IRMS) so that when the effluent eluted from the GC column, the effluent would be split, allowing the effluent to enter the single quadrupole mass spectrometer (MS) detector. The transfer line was modified so that it extended into the GC oven and then the capillary going to the transfer line was inserted into a low dead volume X-connector. By using a 0.10 mm I.D. capillary, approximately 10% of the effluent was allowed to enter into the transfer line. After completion, forensic samples were then introduced to demonstrate that this novel modification achieved the results that were expected. Forensic samples such as cocaine, marijuana, accelerants, olive oils and individual amino acids in human hair were all used to prove the versatility and reliability of using this type of instrumentation setup.
1.2 Project Overview

The primary goal of the initial research project was to modify an already existing Gas Chromatograph (Thermo Trace GC) – Isotope Ratio Mass Spectrometer (Thermo Finnigan Delta\textsuperscript{plus} Advantage) to a single quadrupole mass spectrometer (HP5970B Hewlett Packard). This would allow for a single sample injection to be simultaneously analyzed by two different detectors.

The original GC-IRMS set up, without modifications, can provide, both carbon and nitrogen isotopic analyses of volatile molecules eluting from the GC column (though not simultaneous). The downside to using a conventional GC-IRMS set-up is that the identity of the compound of interest cannot be confirmed. To obtain the identity of the compound, a separate injection would have to be made on a separate system. This would require a separate GC column and another injection of the sample, thereby adding to the possibilities of systematic error or human errors in sample preparation.

To eliminate the need for two separate injections, a low dead volume X-connector was used to split the effluent coming from the GC column. This allowed for approximately 10% going to the single quadrupole mass spectrometer for structural elucidation and 90% going to the IRMS for isotopic analysis. The capillary leading through to the transfer line needs to be heated in order to prevent compounds from condensing before they reach the single quadrupole mass spectrometer detector.

The transfer line going to the mass spectrometer could only enter through the right side of the GC oven due to the fact that the oxidation chamber was on the left side, therefore requiring the appropriate hole to be drilled into the plastic casing of the GC.
Enclosed just inside the casing, were a circuit board panel that also needed to be drilled and then the metal frame of the actual GC oven itself. Once the hole was drilled all the way through, the instrument measurements were made to determine how much of an extension had to be added to the transfer line to reach inside the GC oven. Details of the dimensions and components are discussed in Chapter 4. The extension inside the oven as well as the original transfer line was heated but, there was area between the two components that were not.

Heat tape was wrapped around the outside of the transfer line extension to maintain the appropriate temperature. An external heater was marked at the appropriate voltage using an external thermocouple to mark the temperature at that position. The external heater was then placed at the voltage reading necessary to maintain a constant heat in the same amount of the set transfer line temperature. The capillary was then inserted into both the extension and the transfer line so that the effluent from the GC column could flow into the mass spectrometer for structural elucidation. The complete description of the instrument modification can also be found in Chapter 4.

To test the effectiveness of the modification, several controlled drugs were analyzed. Cocaine was the first drug that was tested using our modified instrumentation, results of which can be found in Chapter 4. A simultaneous analysis by both detectors was proven possible using a single injection of a cocaine sample. Several samples of individual cocaine were then tested to determine whether or not isotopic analysis could match or differentiate between samples based on their carbon $\delta^{13}C$ values.
The ease of use of the instrument was also a question of concern. To test the ease of usability, a forensic lab experiment using this equipment was introduced to the senior forensic chemistry majors in the spring of 2008 and again in the spring of 2009. The students were able to get hands-on experience setting up the instrument methods/sequences and preparing samples for testing. They were also able to explore several statistical analyses on the results and learned how to write-up an official laboratory report. The explanation of the lab and the results are discussed in Appendix B.1. Also included in the appendices is an example of a randomly selected students (RSS) results and report, Appendix B.1.5.

To further investigate the efficiency of our instrument modification, unknown marijuana samples were analyzed to determine individual cannabinoids within the samples. We were able to isolate and identify two cannabinoids: cannabinol (CBN) and cannabidiol (CBD) based on the typical fragmentation patterns collected using the MS. The carbon δ^{13}C values were then analyzed to determine if they could have come from the same source. Results can be found in Chapter 5.

One of the main disadvantages of IRMS is the need to have baseline resolution of each component. When a complex mixture has overlapping peaks, complications in determining peak areas for use of carbon δ^{13}C ratios can occur. Accelerants generally contain several hydrocarbons that are very hard to baseline resolve. We were able to use several of the peaks within the complex mixtures to compare the isotopic ratios for origin comparison. We tested both liquid samples and burnt carpet samples using the known
accelerants Goof Off, WD-40, Lighter Fluid, and Turpentine. Results can be found in Chapter 6.

Amino acids, which are found in abundance in human hair, can also have similar problems with baseline resolution. We were able to successfully separate many of the amino acids so that carbon isotopic analysis could be used to distinguish between the hair samples using the individual amino acids, carbon content. Human hair were hydrolyzed in acid and then derivatized so that the individual amino acids could be identified using the NIST library, results of which can be found in Chapter 7.

Olive oils have become a common food stuff that is being artificially enhanced with less expensive ingredients. IRMS analysis was performed on several olive oils to determine the carbon isotopes in order to differentiate between the samples. Chemometric analysis and testing was done on the results to show how well the peaks of individual compounds could be separated. The results can be found in the supplement section S.2.

1.3 Instrumentation

Isotope ratio mass spectrometry (IRMS) instruments require a somewhat steady stream of a fixed gas (such as He) for precise analysis. The sample first elutes from the GC column into an oxidation chamber, where the samples are combusted at elevated temperatures into a combination of gases such as CO$_2$, NO$_x$, and H$_2$O. For $\delta^{13}$C measurements, the combusted sample is then carried into a reduction chamber where nitrous oxides are converted into N$_2$ and any excess O$_2$ is removed. To avoid H$_2$O from protonating CO$_2$ in the MS source—and causing deleterious isobaric interference of
$^{12}\text{CO}_2\text{H}^+$ with the $^{13}\text{CO}_2^+$ peak at m/z 45—the analyte stream is passed through a semi-permeable membrane, such as Nafion™. The gas molecules are carried into the ion source where small magnets focus electrons produced from a heated filament into a tight beam. Electrons collide with the gas molecules and strip away an electron creating ions. The ions are kicked into an electric field by an ion repeller, and then accelerated into a magnetic field by focusing lenses. The heavier mass ions are deflected less than the lighter ion masses, so by varying the current through the electromagnet, it is possible to change the trajectory to detect multiple ions. The incident ions strike the dynode surface which emits electrons and induces an ion current that is amplified and recorded by a computer. There are five main sections of a typical IRMS sector instrument: 1) a sample introduction system, 2) an electron ionization source, 3) a magnetic sector analyzer, 4) a Faraday-collector detector array, and 5) a computer-controlled data acquisition system. More detailed descriptions of IRMS and the introduction systems are discussed in Chapters 2 and 3.
2.1 Introduction

Isotope ratio mass spectrometry (IRMS) is a technique which finds increasingly widespread use in disciplines such as archaeology, medicine, geology, biology, food authenticity, and forensic science. The histogram plot in Figure 2.1 shows the number of publications per year containing the research topic “Isotope ratio mass spectrometry” using SciFinder Scholar 2006 (searched on May 3, 2008) and reflects the rapid growth in applications since the introduction of commercially-available instrumentation approximately ten years ago. The fastest growth is arguably in forensic applications, where the ability to differentiate substances by their geographical origins provides information that is difficult or unattainable by any other technique.
Disciplines which stand to benefit from IRMS are those which require the ability to accurately and precisely measure variations in the abundance of isotopic ratios of light elements such as $^{13}\text{C}/^{12}\text{C}$, $^{18}\text{O}/^{16}\text{O}$, $^{2}\text{H}/^{1}\text{H}$, $^{15}\text{N}/^{14}\text{N}$, and $^{34}\text{S}/^{32}\text{S}$. The ratios of these isotopes are always measured relative to an isotopic standard in order to eliminate any bias or systematic error in the measurements. These standards are, or can be linked to, internationally recognized standards such as Vienna Pee Dee Belemnite (VPDB) for carbon, Vienna Canyon Diablo Troilite meteorite (V-CDT) for sulfur, Vienna Standard Mean Ocean Water (VSMOW) for oxygen and hydrogen, and laboratory air for nitrogen. As primary standards can become environmentally depleted, secondary standards must sometimes be used in their place. Several of these secondary standards are discussed in detail by Valkiers et al. The International Atomic Energy Agency (IAEA, Vienna, Austria) and the National Institute of Standards and Technology (NIST, Washington, DC, USA) both supply a range of natural abundance standards. Isotope
ratios of samples of interest are measured relative to universal standards and are reported in the delta notation, $\delta$:

$$\delta = \frac{1000(R_{\text{sample}} - R_{\text{standard}})}{R_{\text{standard}}} \quad (1)$$

The value $R_{\text{sample}}$ is the abundance ratio of the minor, heavier isotope of the element to the major, lighter isotope (e.g. $^{13}\text{C}/^{12}\text{C}$). Samples which establish the $R_{\text{standard}}$ values are usually selected because they represent a stable material which is highly enriched in the heavy (minor) isotopes. Most analyzed substances are depleted in the heavy-isotope relative to the standard and will therefore have negative delta values. Guidelines for the selection of working standards and a review of strategies to institute universal isotopic referencing procedures have been reported by Werner et al.\textsuperscript{6}

Commonly used mass spectrometers such as single quadrupoles, ion traps, and time-of-flight mass spectrometers typically do not provide the sensitivity or precision required to detect the subtle differences in naturally-occurring isotopic abundances. It should be noted that these instruments can be useful when used with isotope dilution\textsuperscript{7} – a technique in which the heavier isotopes are deliberately enriched well beyond their natural levels. However, the measurement of natural isotopic abundances requires a specialized instrument such as a multi-collector magnetic sector mass spectrometer, also known as an isotope ratio mass spectrometer (IRMS).

Several authors have investigated the precision and accuracy of IRMS. Continuous flow IRMS instruments have shown precisions of 0.1‰, with the lowest
reported detection limits for monoaromatic compounds between 0.07 and 0.35 ug/L. In general, detection limits vary according to the analyte: for example, halogenated hydrocarbons are reported between 0.76 and 27 ug/L, which is significantly higher than the limits seen for monoaromatics. Although the analyte is also the most important variable in instrumental performance, certain benchmarks in accuracy and precision can be reasonably anticipated. Wong et al. tested three commercially available GC-IRMS instruments to determine differences in precision and accuracy. The average precision was 0.12‰ with reproducibility of 1.48‰ and accuracy of -1.11±2.16‰. Additional experimental variables such as the stability of the ion current, dead time or bit board size dependencies, and even the possibilities of sample vial influences can all effect precision and accuracy on individual instruments.

Another technique which can be used is known as multiple collector inductively coupled plasma mass spectrometry (MC-ICP-MS). MC-ICP-MS is a technique which has undergone extensive research to enhance the accuracy and precision of stable isotopic measurement. Clough et al. have demonstrated that MC-ICP-MS can be used as a high throughput tool for the \( \delta_{34}^{S} \) measurements of bulk aqueous and solid samples, using Si as an internal standard for correction of instrumental mass bias effects in both pure solutions and in samples with high matrix content. This technique is limited, by plasma instabilities and the performance of data acquisition in sequential mode, to the identification of large variations in isotopic abundances.

There are five main sections of an IRMS instrument; a sample introduction system, an electron ionization source, a magnetic sector analyzer, a Faraday-collector
detector array, and a computer-controlled data acquisition system. Several different interfaces are used to introduce samples into the IRMS, the two most common being elemental analyzers (EA-IRMS) and gas chromatographs (GC-IRMS). Figure 2.2 demonstrates how each of these sample introduction systems can be coupled to the same mass spectrometer. Although liquid chromatographs (LC-IRMS) have recently gained interest for some applications, there are only a limited number of publications that have shown this technique to be successful. Here, we examine the present state of research involving IRMS and explore some of the most interesting and unusual applications.
Figure 2.2  Schematics to show how the three most common sample introduction systems/interfaces for carbon isotope measurements (as CO$_2$) and an isotope ratio mass spectrometer. LC = liquid chromatography, EA = elemental analyzer, GC = gas chromatography. Reproduced with permission from Muccio and Jackson.$^1$
2.1.1 **EA-IRMS**

EA-IRMS is a bulk measurement technique which provides representative data for the average isotopic signal of the entire sample. Without significant sample preparation, this method cannot divulge how each constituent of the sample contributes to the total average value. In order to measure the average isotope ratios for non-volatile liquids or solids, the bulk sample can simply be weighed and placed in a tin or silver capsule. The sample capsule is lowered into a combustion furnace through an autosampler carousel, at which time the sample is combusted at elevated temperatures under a flow of oxygen into NOx, CO2, SO2, or H2O. Depending on the isotopes of interest, the combustion products may need to be specifically treated to reduce interferences. In carbon isotope ratio analysis—by far the most common application—the combusted sample is carried by a helium gas stream into a reduction chamber where nitrous oxides are converted into N2 and excess O2 is removed. The analyte is next carried through a chemical trap to remove water that was produced from combustion, and then into the gas chromatograph where separation of CO2 and N2 is performed. Effluent from the elemental analyzer is then sent to the IRMS. Because the isotope ratios for questioned samples are reported relative to a reference gas standard, best results are obtained when the signal intensities for the two samples are of similar magnitude and are analyzed as closely together in time as possible. The flow of each reference gas is regulated using a dedicated sample-introduction interface system which toggles quickly between the reference gas and the sample using pneumatic actuators.
2.1.2 GC-IRMS

By performing a separation prior to isotope ratio analysis, hyphenated techniques such as GC-IRMS and LC-IRMS can provide isotopic analysis of a complex mixture, thereby providing additional information and higher discriminatory power. IRMS instruments require a somewhat steady stream of a fixed gas (such as CO₂) for precise analysis. The sample first elutes from the GC column into an oxidation chamber, usually housed on the side of the GC oven. The oxidation chamber is normally a non-porous alumina tube that contains three separate twisted wires made of copper, nickel, and platinum. The samples are combusted at elevated temperatures into a combination of gases such as CO₂, NOₓ, and H₂O. For δ¹³C measurements, the combusted sample is then carried into a reduction chamber where nitrous oxides are converted into N₂ and any excess O₂ is removed. Since CO₂, NOₓ, and H₂O will not condense at room temperature, the transfer line from the oxidation chamber to the reduction chamber does not need to be heated. The reduction chamber and subsequent valves, splitters, and pneumatic actuators etc. are contained in a stand-alone interface system. To avoid H₂O from protonating CO₂ in the MS source—and causing deleterious isobaric interference of ¹²CO₂H⁺ with the ¹³CO₂⁺ peak at m/z 45—the analyte stream is passed through a semi permeable membrane such as Nafion™. Here, a dry helium counter-flow is used to remove the H₂O. The flow rate of the subsequent sample stream is carefully controlled to provide a stable flow rate to the IRMS ion source of approximately 0.5 mL/min. Deactivated fused silica capillaries are used throughout the interface systems to restrict the analyte flow to the required flow rates. The interface system also uses electronically-controlled pneumatic
actuators to toggle the flow of the effluent stream between that of the analyte and that of a reference gas, such as a cylinder of CO₂.

2.1.3 LC-IRMS

LC-IRMS applications are typically dedicated to carbon isotope ratio analyses. When the solution elutes from a high pressure liquid chromatograph (HPLC) column, the solution is directly injected onto or into one of two interfaces. These two interfaces are 1) a moving wire interface and 2) a wet chemical oxidation interface. The wet chemical oxidation interface is currently being offered as a commercial instrument by Thermo (LC IsoLink) and shows somewhat more promise than the moving wire interface, which only has one prototype. The wet chemical oxidation method converts organic compounds present in the effluent of the HPLC column into CO₂ gas directly in the mobile phase. To reduce interferences, the HPLC mobile phase must be void of any organic or oxidizable components that could interfere with the results. It should be noted that because most HPLC separations are greatly enhanced with organic solvents or modifiers, this requirement poses significant restrictions on the potential application of LC-IRMS. The effluent from the HPLC column is then mixed with an oxidizing stream composed of an oxidizing agent such as ammonium peroxodisulfate, and a catalyst such as phosphoric acid and silver nitrate. The mobile phase and the combined reagents pass through a capillary oxidation reactor where the organic compounds are converted into CO₂. A membrane exchanger separates CO₂ gases from the other gases (water vapor, oxygen, argon, etc.) that originate from the liquid phase. The CO₂ is then transferred
through a gas permeable membrane\textsuperscript{23} into a counterflow of helium. The CO\textsubscript{2} in the helium stream is then dried in an online gas drying semi permeable membrane (Nafion\textsuperscript{TM}) and admitted to the isotope ratio mass spectrometer via an open split. The wet-chemical oxidation interface allows for the $^{13}\text{C}/^{12}\text{C}$ determination of organic compounds with a completely automated online high precision method.

2.2 Origins of variations in isotopic abundances

Although the average isotope ratio of each terrestrial element was fixed around the time of the earth’s formation, localized variations occur based on selective enrichment/depletion of the heavier isotopes relative to the average values. For example, even though all plants use atmospheric or dissolved CO\textsubscript{2} as a source of carbon, various factors can influence a plant’s ability to enrich or deplete $^{13}\text{C}$ from these common sources in a process known as fractionation. One such fractionation factor is genetic.

Monocotyledonous plants (C\textsubscript{4} plants), such as sugar cane, corn, tropical grasses, desert plants and marine plants, utilize the Hatch-Slack photosynthetic cycle.\textsuperscript{22} These plants typically have $\delta^{13}\text{C}$ values varying from -8 to -20‰.\textsuperscript{24} Most dicotyledons plants (C\textsubscript{3} plants), such as flowering plants, wheat, rice, rye and cotton employ the Calvin-Benson photosynthetic cycle and have $\delta^{13}\text{C}$ values varying -22 to -35‰.\textsuperscript{24} Crassulacean acid metabolism (CAM) plants, such as pineapple, cactus, and orchids, can utilize either the C\textsubscript{3} or C\textsubscript{4} metabolic systems, depending on sunlight, and therefore have $\delta^{13}\text{C}$ values ranging between -10 to -34‰.\textsuperscript{24} Because animals can only incorporate carbon through the ingestion of plant or animal matter, the carbon isotope ratios in an animal will reflect the
isotope ratios of the food source; i.e. “you are what you eat”. This fact can be used to
great advantage, as shown in Figure 2.3. For example, human European diets are richer in
C₃ plants (wheat, barley, and rye), whereas human North American diets are richer in C₄
plants (corn, sugar cane and millet). Therefore, a person living in North America will
have body matter with isotope ratios more similar to C₄ plants and will have lower ¹³C
levels (i.e. less negative delta values) relative to Europeans.

Figure 2.3  Examples of variations of carbon isotopic abundances of plants and human
diets. Reproduced with permission from Muccio and Jackson.¹

In addition to genetic factors, environmental factors such as temperature, rainfall,
and hours of sunlight also influence fractionation. These factors can influence kinetic
processes such as the diffusion of CO₂ through the stomata in plant leaves. Clear
evidence for environmental sensitivity to fractionation was presented by Ehleringer et al. in 2000, wherein they demonstrated the ability to determine the local geographic farming regions in South America from which different cocaine plants were obtained. Fractionation also occurs in common elements such as sulfur, hydrogen, oxygen and nitrogen. In the case of sulfur, fractionation occurs in an equilibrium (between reactants and products) and non-equilibrium (kinetic) mode. Kinetic effects are due to fast, incomplete, or unidirectional processes, typically resulting in a preferential enrichment of the lighter isotope in the reaction products. Grassineau studied fractionation of both carbon and sulfur and concluded that it is possible to limit the effects of fractionation with careful attention to detail. Hydrogen fractionation was studied by Maruoka et al., who showed that hydrogen comparatively has the most extreme fractionation effects. Bond strength also plays an important role in kinetic effects due to the greater strength of a deuterium-carbon bond relative to a hydrogen-carbon bond. Oxygen fractionation is largely due to the combustion of the sample, with temperature a deciding factor as to whether or not the sample is completely combusted. If the sample is only partially combusted, or if the oxygen levels are depleted in the oxidation chamber, this can affect the results of the isotopic ratio. It has also been shown that oxygen fractionation can occur within a sample vial. Additionally, oceanic vapors have had a large effect on the oxygen content. Nitrogen fractionations in nature are due to kinetic effects: there are also two non-biological fractionation effects, dissolution in water and diffusion in water. Bacteria, in particular, display several fractionation processes; nitrification, denitrification, and nitrogen fixation.
General fractionation also occurs with ambient diffusion.\textsuperscript{30} Chemical reactions and physical processes like evaporation and condensation create products that are isotopically distinct from their starting materials.\textsuperscript{31} For example, in the hydrologic cycle, snow falling at the poles is depleted in \( ^2\text{H} \) and \( ^{18}\text{O} \) content with respect to rainfall at the equator.\textsuperscript{31} Fractionation effects are also observed in purely chemical reactions. As a result, any simple or complex substance will be composed of isotope ratios that provide a key in unravelling the history and origins of its precursor elements. This fact has been pivotal in solving a variety of interesting and important problems, as described below. For additional information, a complete description of isotope fractionation effects for \( ^{13}\text{C}/^{12}\text{C}, \ ^{18}\text{O}/^{16}\text{O}, \ ^{3}\text{H}/^{2}\text{H}, \ ^{15}\text{N}/^{14}\text{N}, \ ^{34}\text{S}/^{32}\text{S} \), and several others not mentioned in this highlight, was written by Mook and Vries.\textsuperscript{30}

2.3 Applications

2.3.1 Forensic

Forensic science researchers have long-recognized the need to distinguish between different sources of evidential material. Such determinations were formerly difficult or impossible in cases where two samples had identical physical or chemical properties. However, isotopic analysis now provides a means to look beyond the chemical composition of matter to the level of the nucleus. To help monitor and disseminate the developing forensic applications of isotope ratio mass spectrometry, a specialized network called the Forensic Isotope Ratio Mass Spectrometry (FIRMS)
Network was developed in January 2002. This voluntary network is mostly composed of British and European members from universities and forensic laboratories. FIRMS have also organized several inter-laboratory studies as well as the European Institute for Reference Materials and Measurements (IRMM) and the National Physical Laboratory (NPL). An extensive review of analytical chemistry inter-laboratory studies was written by Hund et al. while an excellent example of an inter-comparison study was that done on tetramethylurea by Breas et al.

Until recently, the notion that the carbon isotopic ratio of soil could be used to determine whether soil recovered from a suspect matched a crime scene would have been regarded as implausible. The use of carbon and nitrogen EA-IRMS analysis of soil to partially exonerate a suspect is thus yet another example of how far the forensic applications of IRMS have advanced. In another application, it was proven that a carbonate rock was switched during transit from a supplier’s factory in South Africa to a client’s factory in Israel. Isotopic analysis of the carbon in the sample indicated that the origin of the carbonate rock was likely the client’s site in Israel, which substantiated the claim that the rock was switched after arrival. Further elemental flexibility of EA-IRMS was exhibited by the analysis of carbon, oxygen, and hydrogen in matches. The results of these tests indicated that matches recovered from a suspects’ house and crime scene were different, which underscores the potential importance of this technique to criminal justice. EA-IRMS has also been used to measure the carbon isotopic abundance of twenty eight samples of white architectural paint. Specifically, the effects of drying time, layering, ageing, and homogeneity on discriminatory results were reported.
As a result of constant legal demand, the analysis of controlled drugs is one of the largest areas of active development. For example, the presence of elevated levels of γ-hydroxybutyric acid (GHB) in blood, urine, or hair samples is often necessary to support claims of drug-facilitated sexual assault (DFSA). GC-IRMS of carbon isotopic ratios has been used to discriminate between endogenous production of GHB and exogenous ingestion levels. Figure 2.4 shows the IRMS results of such an analysis. The figure shows the m/z 44 peak for the esterified version of GHB, γ-butyrolactone (GBL) and the internal standard ε-caprolactone. In a broader sense, IRMS may also be useful in the struggle against drug trafficking. A notable illustration of this application is the nitrogen isotopic comparison of twenty samples of heroin seized from a North Korean-flagged cargo vessel versus a database of more than 200 authentic samples. It was determined that the samples from the seizure had not originated from a source currently on record and were therefore likely to have come from a new source.
Figure 2.4  The path of GHB/GBL, from spiked drink, to victim, to hair, and eventually to the GC/C/IRMS m/z 44 chromatogram. The peaks were obtained from the liquid injection of a standard of GBL and ε-caprolactone at 50 ng each (on column). The square-topped peaks represent pulses of CO₂ reference gas. Reproduced with permission from Muccio and Jackson.¹

With the threat of terrorism omnipresent in our modern lives, techniques are needed to address unique problems associated with mass-disasters. For example, the ability to reconstruct a victim’s remains following an event of mass destruction is useful from both a forensic point of view and to provide closure to the families and friends of deceased victims. When deoxyribonucleic acid (DNA) cannot be collected, or when DNA reference samples are not available, IRMS is a potential option to link recovered body parts to each other or to a geographic location. EA-IRMS has been used to analyze the hydrogen and oxygen isotope ratios of human scalp hair and fingernails.⁴⁰ In another case,⁴¹ isotope ratio analysis of oxygen and hydrogen from an unknown deceased person
in Ireland was used to verify that the person almost certainly had not lived in the local geographic region for a significant time. Isotopic analysis instead suggested that the person was more likely from Eastern Europe or Scandinavia. Further studies by the same authors proved that hydrogen isotope ratios provided much more valuable information when analyzing human hair than when analyzing nails. In a separate study, a model was developed to predict the geographical region of North America from which a person resides by cross-referencing the hydrogen and oxygen isotopic abundances of scalp hair with tap water. For the initial data in this model, individual hair samples were collected from 65 cities across the United States, while tap water samples were collected from 18 states. The accuracy of this model was confirmed by collecting hair samples from local barbershops in the same cities and comparing them with previous samples. The findings showed an agreement of 85% with the model, which relies upon the assumption that all hair in the local barbershops originated from indigenous citizens. A more-thorough review on forensic applications of IRMS, including earlier studies of explosives and synthetic drugs, was written by Bensen et al.

2.3.2 Food and Drugs

In the food and drug industries, it is necessary to determine whether or not a product’s actual contents agree with the labeled contents. Although food and drinks are sometimes laced with relatively innocuous substances such as artificial sweeteners, they are not always marked as such. Isotope ratio analysis can be used to establish whether or not the product contains natural sweeteners (from the original food source), or artificial
sweeteners (such as corn syrup). A recent study showed that sugars could be analyzed using LC-IRMS to obtain carbon isotopic ratio abundances of each sugar of interest.\textsuperscript{21} Because of their similar carbohydrate contents, cheap beet sugars are sometimes undetected when added to honey products as sweeteners. However, through the isotopic analysis of each sugar within the questioned honey samples, it is possible to determine which honeys are altered and which are not. Moreover, the detection of other modifiers such as corn, sugarcane, or a sweetener other than high fructose corn syrup is possible. In a similar vein, an internal standard of malic acid, found only in very low abundances in commercial sweeteners, was employed to study which of fifty six selected maple syrup samples were unsweetened.\textsuperscript{22}

Other food products known to be altered are fruit juices and sparkling drinks. Authentic sparkling drinks are usually pressurized with CO\textsubscript{2} through a fermentation process, whereas a cheaper and easier method for carbonation is to pressurize the drinks with CO\textsubscript{2} from an external cylinder. GC-IRMS can detect the different modes of carbonation by testing the carbon and hydrogen isotope ratios of natural versus injected CO\textsubscript{2}.\textsuperscript{44} In a different application to food produce, IRMS was used for the quality assessment of apple aroma profiles in apple juices.\textsuperscript{45} Additions of food additives, in this case citric acid, for the authentication of fruit juices were also studied using 20 commercial citric acids and 79 citric acids extracted from fruit juices.\textsuperscript{46} A disadvantage encountered in this study was that the exchangeable hydrogen sites bound to the oxygen atoms are included in the overall D/H result. Therefore, an offline preparation step was
necessary. The capability of the developed method to detect an addition of citric acid was confirmed by spiking an orange juice sample with known amounts of citric acid.

The increasing popularity of organic produce has been accompanied by prices that are significantly higher than non-organic produce. To validate whether or not more expensive produce is truly organic, and thus warrants the higher prices, isotope ratio analyses have been used to authenticate organic produce. Based on the premise that “you are what you eat”, the isotope ratios of flesh (meat) from animals such as cows are determined by their feed source. Bahar et al. investigated the seasonal variations of beef using EA-IRMS to study the carbon, nitrogen, and sulfur isotopic ratios of two hundred and forty two beef samples. Between the months of December and June an isotopic shift was apparent, most likely due to indoor winter feeding practices. By applying the shift during these months, it was possible to use isotopic ratios to determine whether or not a beef composition was indeed organic or merely conventional Irish beef. In a related study, the accuracy of beef rearing labeling was questioned in part by measuring the hydrogen and oxygen isotopic ratios of lipid fractions with EA-IRMS. Carbon and nitrogen isotope composition of beef defatted dry mass comprised the other component of the analysis. The study suggested that it was not only possible to determine where the beef was reared, but also to validate the accuracy of the information on the labels.

Although the above analyses require that the animal be sacrificed prior to analysis, it is also possible to test for isotope ratios without these invasive measures. For example, a non-invasive method to test carbon and nitrogen isotopic abundances has been used using urine and milk from cattle. Specifically, isotope ratio analysis helped
determine which type of feed was being used during what season. Further, from the identity of the feed, it was possible to distinguish whether or not the beef production was organic or conventional.

2.3.3 Diet, Biochemistry and Metabolism

Isotopic abundance ratios can also establish dietary patterns and movements of cattle. One such study reconstructed the dietary history of cattle by measuring the carbon and nitrogen isotopic abundances of bovine hooves with EA-IRMS. Specifically, the keratin within the hoof was used in order to establish the short term dietary changes and history of the cattle. This was taken a step further using a three dimensional growth of the bovine hoof to study the seasonal or ontogenetic feeding patterns, as well as the movement, of the cattle. Feeding patterns have also been characterized using bone collagen to perform palaeodietary reconstruction and distinction between marine and C4 based diets. To make this contrast, LC-IRMS was used to separate eighteen amino acids from modern protein and archeological bone collagen hydrolysates taken from human and faunal bone collagen. Bone collagen has also provided evidence that indicated maize as the primary source of sustenance in many regions of the Central Andes during the era of Inka hegemony. The effects of preferential fertilization of maize relative to manure were determined by using the carbon and nitrogen isotopic ratios within the bone collagen. To augment these findings, muscle and skin from mummies dating back to the late prehistoric early colonial (AD1490-1640) time from Peru’s Ayacucho valley was analyzed as well.
When dealing with non-volatile compounds such as amino acids or fatty acids, extensive sample preparation or derivatization is needed prior to introduction into the GC-IRMS. This adds complexity to the data interpretation, particularly if the number or type of atoms in the molecule is increased through the derivatization process. Corr et al. demonstrated a less extensive derivatization method using 4 novel derivatives on 15 amino acids. N-acetylmethyl (NACME) esters were shown to add the fewest amount of carbon atoms and resulted in the smallest δ^{13}C errors relative to the underivatized amino acids. The same authors later confirmed these findings by utilizing this derivatization technique to study amino acids from rat tissue (bone collagen).

Zoology has also been the beneficiary of IRMS research, such as in the study of the dietary and migration patterns of elephants by Cerling et al. This project used an EA-IRMS analyzer to determine the carbon and nitrogen isotopic ratios of hair taken from wild elephants, as shown in Figure 2.5. Segmented analysis of the hair from the elephants was then used to generate a chronological history of the elephants’ eating habits, and even their feeding locations. The isotope ratios for the average of all the elephants in the study showed gradual changes from season to season. The results also showed that an individual elephant’s hair could be significantly different from the control group, if the elephant in question was involved in night-time crop raiding. Such differences are only possible when the crops under discussion are of a different metabolic class than the control group’s native diet.
Figure 2.5 Carbon and nitrogen isotope ratio analysis of wild elephants’ hair can be used to detect the occurrence of night-time raids on farmers’ crops. Reproduced with permission from Muccio and Jackson.¹
Another zoological study examined the migration patterns of wild birds.\textsuperscript{57} Using feathers from vertebrate and invertebrate species of birds, the migrating patterns as a function of isotopic carbon and hydrogen abundance ratios were developed. Additional analysis of the birds’ summer plover feathers established the bird’s origin within several kilometers of their known origin. An earlier, related report of migratory patterns of other species can be found in an article by Bowen et al.\textsuperscript{58} on global applications of stable hydrogen and oxygen isotopes in wildlife forensics. In short, isotope grids were used to statistically constrain the unknown origin of North American and European feathers and water isotopes.

Medical problems such as digestion patterns and mechanisms have also been an area of interest. A recent study examined carbohydrate digestion and glucose absorption by measuring plasma glucose with EA-IRMS after oral administration of naturally occurring $^{13}$C enriched carbohydrates.\textsuperscript{59} By using Saccharomyces cerevisiae (yeast) to convert the samples into CO$_2$, the magnitude of glucose digestion was determined. Another biochemical investigation used a tracer from blood to measure the fractional synthesis rate of glutathione (GSH) after infusion of (1-$^{13}$C) – glycine.\textsuperscript{60} Amino acids from low birth weight infants admitted into the neonatal intensive care unit were analyzed using LC-IRMS. To achieve higher resolution, the oxidized form of glutathione, glutathione disulfide (GSSG), was used: this tracer made it possible to determine both the individual carbon isotopic ratio of GSSG and the fractional synthesis rate of glutathione.

Given current circumstances, environmental investigations may be among the most important IRMS applications. Environmental effects are known to contribute to
carbon isotopic abundance ratios of plant matter, although the manner and extent of this interaction is still a mystery. To partially address this question, the effects of turbulent water on the CO₂ flux of herbarium material from members of fresh water torrenticolous families have been studied. Fast flowing water removes the boundary layer of CO₂ on the plant surface, which in turn causes faster diffusion rates and thus noticeable differences in the isotopic ratios of fixed CO₂. In this application, EA-IRMS was used to analyze the levels of carbon isotope ratio variability of the torrenticolous families Podostemaceae and Hydrostachyaceae.

2.3.4 Athletics and Doping

Doping in the athletics world has been a problem for decades. Many sports governing bodies rely on isotope ratio analyses to determine elevated levels of exogenous sources of illicit steroids versus elevated levels of endogenous hormones, which is possibly indicative of a genetic anomaly. Because of its specificity, IRMS is the preferred analytical techniques to confirm steroid use. GC-IRMS has been used to analyze urine for the carbon isotope ratios of nandrolone (NAD), an endogenous steroid hormone metabolite used to enhance the performance of race horses and athletes. One of the most abundant metabolites of the synthetic steroid 19-nortestosterone is 19-norandrosterone. Utilizing reference compounds to compare isotopic carbon ratios to distinguish between endogenous and exogenous concentration levels, EA-IRMS determined the origin of urinary norandrosterone traces. Urine samples can also be a vehicle to detect steroid use. To detect the effects of undecanoate over a 4 week period,
GC-IRMS was used to measure the carbon isotope ratios of androsterone and etiocholanolone metabolites in seven Caucasian male volunteers.

2.3.5 Environmental Pollution

For obvious public health reasons, the effects of pollutants on the environment are an area of great research importance. In a common post-industrial scenario, sediment with elevated polycyclic aromatic hydrocarbons (PAH) found in a lakebed near a former gas manufacturing plant was studied. Carbon isotopic analysis of the sediment samples were measured using a GC-IRMS to prove that the samples were not the same as the tarry soil samples recovered from the gas plant. Instead, results indicated that the hydrocarbons most likely came from a mixture of PAH sources such as coal tars and carbureted water gas tars. Perhaps the most significant contemporary environmental issue is that of global warming. Keppler et al. found that it was possible to use lignin methoxyl groups within wood to determine past climatic changes. Lignin methoxyl is a major component of wood (up to 3%) contained in cellulose cell walls and is produced by secondary metabolic processes. The researchers discovered that converting lignin methoxyl into CH₃I made determination of hydrogen isotopic ratios via EA-IRMS straightforward. In the future, it is hoped that this method could reconstruct annual climate histories and assist in ecophysiological research.
2.4 Summary

IRMS has been shown to have both wide applicability and the versatility to be coupled with several different interfaces. In determining which interface would be best suited for coupling to the IRMS, the sample itself is the most important determining factor. Non-volatile substances such as foods, drugs, amino acids, and fatty acids can be most easily measured with EA-IRMS, even though this technique only provides an average isotope ratio value for the entire sample. Analysis can typically be performed on samples as small as 0.5 mg and often avoids the complex sample preparation procedures that are usually needed for GC- or LC-IRMS analysis. With that said, it is important to note that GC-IRMS can be used for most volatile organic substances without sample preparation. LC-IRMS is still the least mature sample introduction method. As such, it seems that the most important obstacle in this technique is ensuring adequate baseline-resolution, in the absence of organic modifiers, in the chromatographic stage of the analysis.

Regardless of the sample introduction method, IRMS has great potential for forensic applications in high volume or high value crimes such as burglary, homicide, and drug dealing cases. In the future, the analysis of drugs using IRMS will undoubtedly become more common for both controlled and illegal drugs. A particularly key benefit is the possibility of linking trace amounts of drugs to a bulk source in order to determine trafficking routes. A noticeable gap in the market exists for compound-specific isotope ratio standards, although several suppliers are available for bulk isotope ratio standards such as polyethylene, sugar, and flour. Thus, the availability of IRMS standards and
standardized methods are both important goals. Another major current and future issue is the rendering of compatible isotopic measurement referencing strategies. To address this issue, Serra et al.\textsuperscript{67} have already begun development of a standardization method for inter-laboratory $\delta^{13}$C elemental analysis and gas chromatography combustion isotope ratio mass spectrometry measurements. Several suitable compounds for GC-IRMS isotopic reference materials were investigated in order to comprise a standardized Grob-test. For forensic applications, validated sampling protocols and sampling kits would be additionally advantageous.

Improvements continue to be made as researchers find new ways to utilize this technique. IRMS offers the potential of unlimited applications for nonvolatile and volatile compounds while achieving higher accuracy and precision via increased automation. Through continued progress in fundamental understanding and application development, IRMS should be able to transition from its current status as a specialized practice into a more routine method. Future instrumentation goals could then focus on shrinking the footprint and cost of the instrumentation, reducing analysis times, obtaining higher resolution data, and perhaps even looking towards miniaturization or portable instruments.
CHAPTER 3: ISOTOPE RATIO MASS SPECTROMETRY – A NEW TOOL FOR
THE FORENSIC CHEMIST

This chapter was submitted for publication in the Journal of Chemical Education in September, 2009. Appendix 3.1 is a previous version of this chapter was submitted to the Journal of Chemical Education and rejected.

3.1 Introduction

In the past decade, forensic-drama television shows such as CSI, NCIS, Forensic Files, etc. have captured the interest and imagination of young people and have encouraged students to pursue forensic science degrees at Universities. An efficacious way to engage student interest in forensic programs is to provide them with hands-on experience using modern analytical instrumentation. By so doing, students learn how to correctly prepare samples, to carefully consider experimental design (such as sampling, negative controls and positive controls), to operate the instrumentation, to construct methods, and to carefully and objectively calculate and analyze results.

Students also gain a better perspective on the actual size of a sample that is needed for analyses, as well as the length of time that it takes to both prepare and analyze samples. Due to the time constraints of network television, forensic dramas typically over-simplify or ignore the care, thoughtfulness and quality assurance that goes into preparing samples to obtain high-quality, objective results. When such instrumentation is not available for hands-on instruction, such is often the case for isotope ratio mass spectrometers, captivating examples of the instrument’s use can help maintain student interest in the subject matter.
One fascinating, and potentially far-reaching, technique at the cutting edge of forensic chemistry is isotope ratio mass spectrometry (IRMS). IRMS typically employs a magnetic sector mass spectrometer to measure natural variations of isotope ratios of light elements such as carbon, nitrogen, hydrogen, oxygen, and sulfur. IRMS has specific potential benefits within the forensic community where the need to classify, differentiate, or exclude questioned and known samples may be complicated or unachievable by any other technique. For example, the composition of a substance, such as cocaine, will often have the same organic makeup as another historically unrelated sample. IRMS has the capability to look beyond the organic makeup to identify the relative isotopic abundances of the elements within these materials.

Kinetic and thermodynamic factors are major contributors to the enrichment or depletion of carbon, hydrogen, oxygen, sulfur, and nitrogen isotope ratios. For example, plants fixing CO$_2$ through one metabolic pathway can have significantly different $^{13}$C ratios in the anabolized plant matter than plants fixing the same CO$_2$ through a different pathway. The ability to measure isotope ratios provides a means to differentiate between samples (eg. Cocaine), that otherwise have indistinguishable chemical compositions.

The terrestrial average natural abundance of $^{12}$C is 98.89% and $^{13}$C is 1.11%, but the isotope abundances of $^{13}$C may vary on the local level by as much as 0.4%. When measuring isotopic ratios, it is necessary to have a uniform reporting method to limit any bias or systematic errors between systems. To ensure that this is done correctly, the ratios should be reported relative to common standards in the delta notation, \(\delta\):
\[
\delta = \frac{1000(R_{\text{sample}} - R_{\text{standard}})}{R_{\text{standard}}}
\]  

(1)

where \(R_{\text{sample}}\) is the abundance ratio for the heavier isotope (\(^{13}\text{C}\)) over the lighter isotope (\(^{12}\text{C}\)). The \(R_{\text{standard}}\) values are normally selected to represent a stable material which is highly enriched in the minor, heavy isotopes. Negative delta values are more common since a larger percentage of analyzed substances are depleted in the minor isotope relative to the standard being used.\(^1\) Well-known standards, such as Vienna Standard Mean Ocean Water (VSMOW) for oxygen and hydrogen, Vienna Pee Dee Belemnite (VPDB) for carbon, Vienna Canyon Diablo Troilite meteorite (V-CDT) for sulfur, and laboratory air for nitrogen, provide for easier comparisons.\(^2\) Secondary standards, available from certain chemical suppliers, are frequently used in place of the primary standards because some of the primary standards have become environmentally depleted.

3.1.1 IRMS

There are five main sections of a typical IRMS sector instrument: 1) a sample introduction system, 2) an electron ionization source, 3) a magnetic sector analyzer, 4) a Faraday-collector detector array, and 5) a computer-controlled data acquisition system.

There are three types of introduction systems, although the two most common are gas chromatographs (GC-IRMS) and elemental analyzers (EA-IRMS). Figure 3.1 depicts how a GC-IRMS sample introduction system, housed in the laboratory of the authors, is currently coupled simultaneously to a single quadrupole mass spectrometer and an IRMS. Liquid chromatography (LC-IRMS) is the third type of introduction system. LC-IRMS
has seen significant growth in the number of applications in the last few years, but with relatively less success than GC-IRMS. We will not be discussing LC-IRMS in this article due to the lack of demonstrated applicability to forensic science.
Figure 3.1  Schematic of our current set up with the GC-IRMS coupled to a single quadrupole mass spectrometer to allow for a single injection of a sample and have results that simultaneously provide the retention time, characteristic EI fragmentation patterns used to confirm the identity of individual components within a sample and the carbon isotopic abundance ratio of each compound in the mixture.
3.1.2 **EA-IRMS**

Elemental analyzer-IRMS (EA-IRMS) provides representative data for the average isotopic signal of the total sample and is therefore considered to be a bulk measurement technique. Tin or silver capsules are used as sample holders for non-volatile liquids and solids. After the sample is weighed or a specific volume is measured out, the sample can then simply be placed into one of these capsules. The auto-sampler carousel will drop the sample capsule into the combustion furnace where the helium purge will change over to oxygen, to enable flash combustion of the sample to occur. After combustion is conducted under a specific volume flow of oxygen, elementary gases such as NO\textsubscript{x}, CO\textsubscript{2}, SO\textsubscript{2}, and H\textsubscript{2}O are produced, as shown in equation 2.

\[
C_kH_lN_MS_N \rightarrow k\text{CO}_2 + \frac{1}{2}l\text{H}_2\text{O} + m\text{NO}_2 + n\text{SO}_2
\]  

After combustion, the oxygen stops flowing and continuous helium flow carrying the sample, continues to a reduction chamber. The primary role of the reduction chamber is to convert the nitrous oxide by-products into N\textsubscript{2}, and remove surplus O\textsubscript{2}. The sample then flows through chemical traps, which are used to remove the H\textsubscript{2}O that was produced from the combustion process prior to the introduction into the packed GC column. After separation of CO\textsubscript{2} and N\textsubscript{2}, these gases continue onto the IRMS for isotopic analysis.
3.1.3 GC-IRMS

Unlike EA-IRMS, gas chromatography-IRMS (GC-IRMS) performs a separation prior to combustion. Through the use of this introduction system, it is possible to perform isotopic analyses of each component within a complex mixture. With this increased discriminatory power, more pertinent information can be acquired without extensive sample preparation. To ensure precise analysis, helium is most commonly used for the carrier gas and is held at a constant flow rate. The helium carries the sample from the GC column into an oxidation chamber, provided by the manufacturer, which is mounted on the side of the GC oven. A non-porous alumina tube containing three separate copper, nickel, and platinum twisted wires is housed in the oxidation chamber. The supply of oxygen comes from NiO and CuO in the oxidized surfaces, which can be replenished as needed. Elevated temperatures as high as 1000°C are used to combust the sample into the same combination of elemental gases as EA-IRMS; CO₂, NOₓ, and H₂O. Following combustion, the mixture enters the reduction chamber via an ambient temperature deactivated fused silica transfer line. It is not necessary to heat the transfer line because CO₂, NOₓ, and H₂O do not condense/sublime at room temperature. The purpose of the reduction chamber is the same as it is for EA-IRMS; to convert nitrous oxides into N₂ and remove any excess O₂. It is also important to remove H₂O from the analyte stream to remove any possibility of protonation of CO₂ in the MS source, which would lead to an isobaric interference of ¹²CO₂⁺, with the ¹³CO₂⁺ peak at m/z 45. H₂O is removed by using a dry helium flow to pass the analyte stream through a Nafion™ semi-permeable membrane made of a hygroscopic material. The IRMS ion source needs to have a very
specific and stable flow rate, approximately 0.5 mL min$^{-1}$. These stable flow rates are obtained by using deactivated fused silica capillaries with specific inner diameters throughout the entire system. Electronically-controlled pneumatic actuators are used for the sample, the reference gas, and the back flush (re-oxidation) system to ensure the correct gas is being introduced into the IRMS source at the desired flow rate. In general, commercially available systems do not require much, if any, optimization beyond default settings.

3.2 Origins of Variations in Isotopic Abundances

*Fractionation* is a term that is used to describe the enrichment or depletion of an isotope based on a physical, chemical or biochemical process. Although all terrestrial plants use a common pool of atmospheric CO$_2$ as their source of carbon, fractionation can occur in plants depending on the type of plant and the growth conditions of the plant fixing the CO$_2$. For example, a genetic form of fractionation occurs in monocotyledonous plants (C$_4$ plants), such as sugar cane, corn, tropical grasses, desert plants and marine plants. These plants utilize the Hatch–Slack photosynthetic cycle and typically have $\delta^{13}$C values varying from $-8$ to $-20\%$. Dicotyledons plants (C$_3$ plants), such as flowering plants, wheat, rice, rye and cotton employ the Calvin–Benson photosynthetic cycle and have $\delta^{13}$C values varying from $-22$ to $-35\%$. In contrast, crassulacean acid metabolism (CAM) plants, such as pineapple, cactus, and orchids, can utilize either the C$_3$ or C$_4$ metabolic systems, depending on sunlight, and therefore have $\delta^{13}$C values ranging between $-10$ and $-34\%$. The carbon isotope ratios in animals will reflect the isotope
ratios of their food source; \textit{i.e.} “you are what you eat”, as animals will incorporate carbon through the ingestion of plant or animal matter\textsuperscript{74} The typical isotope of interest in human is provided in Figure 3.2.

With this information, it is possible to establish the geographical origin or residence of humans based on their tissue isotopic ratios, which is determined by food intake, as shown in Figure 3.3. For example, the diets of humans in Europe are richer in C\textsubscript{3} plants (wheat, barley, and rye), whereas the diets of humans in North America are richer in C\textsubscript{4} plants (corn, sugar cane and millet). A person living in North America will clearly have body matter with isotope ratios more similar to C\textsubscript{4} plants and will have lower
$^{13}$C levels; i.e. less negative $\delta$ values, relative to Europeans. Permanent tissues, such as bone and teeth, will retain the isotope ratio information of the person’s dietary intake during formation. Comparison of bone isotope ratios to soft-tissue isotope ratios can determine whether or not a person grew up in the same geographic area in which they currently reside.

Figure 3.3 Examples of variations of carbon isotopic abundances of plants and human diets.
In addition to genetic botanical fractionation (based on CO₂ fixation), there are many environmental factors that can further influence \(^{13}\)C ratios. These include temperature, rainfall, and the daily exposure to sunlight.

3.3 Forensic Applications

Forensic science researchers have long recognized the need to distinguish between different sources of evidentiary samples. Until recently, such determinations were nearly impossible in cases where two samples had identical physical or chemical properties. With isotopic analysis, we are now provided with a means to look beyond the chemical composition of matter to the level of the nucleus. Pye et al.\(^{34}\) were able to use EA-IRMS analysis of soil evidence samples to show the capability to clear a suspect of the charges being brought against him. One soil sample was recovered from the footwear of the suspect and another was collected from the crime scene. The results showed that the soil samples could not have come from the same source based on the \(^{13}\)C and \(^{15}\)N ratios and therefore, could not support (or relate) the claim that the suspect had been at the scene of the crime.

Investigators, at a large mining company called Anglo Platinum, became concerned about security breaches after a rock containing precious deposits was apparently switched somewhere in transit.\(^{35}\) It was necessary to discover where the rock was switched, either after transit from a supplier's factory in South Africa, or at the client's factory in Israel. The replacement rock was identified as being a finely crystalline dolomite and isotopic analysis of the carbon in the sample indicated that the origin of the
carbonate rock was likely from the client's site in Israel. The investigators concluded that the rock was switched post-transit.

Arson cases involving accelerants have shown how isotope analysis can help determine the source of ignitable liquid residues. Jasper et al.\textsuperscript{76} investigated how three different types of gasoline compounds differed. The isotopic composition of each component in the mixture of the combusted sample was shown to remain in the region of the original neat gasoline samples. Jasper et al.\textsuperscript{77} also provide real-life examples of cases where GC-IRMS could have been used to connect ignitable liquid residue to a source. In addition to linking the accelerants themselves, it is also possible to compare other organic materials that were involved in arson, such as the carbon, oxygen, and hydrogen isotope ratios of matches,\textsuperscript{36} as shown in Figure 3.4. Matches were recovered from a suspect's house and also collected at the scene of the crime. Isotopic analyses indicated that the matches recovered from the suspect's house and at the crime scene were not likely to have had a common source.
Figure 3.4 Comparison of hydrogen and carbon isotope ratios for match sticks from the crime scene, from the suspect’s possession, and other sources. Data points are the mean from triplicate analyses per isotope and error bars are ±1 s.d. These results did not find a common source between the matches at the crime scene and any of the other known samples. Reproduced with permission from reference 36.

Adhesive tape is another material that is frequently collected from crime scenes. Tape is used in activities such as in the packaging of drugs, to enclose explosive devices, and as a means to restrain victims. Isotopic analyses was able to distinguish between tapes of different brands, and also to differentiate between tapes of the same brand from different production batches, even in the presence of contaminants. In this study, the authors separated the adhesive layer from the backing before analyzing the $^{13}$C content of each layer. Separating the two layers added discriminating power. In another example EA-IRMS was also used on 28 samples of white architectural paint to measure their carbon isotopic abundance. Here, researchers analyzed the effects of drying time, layering, aging, and homogeneity on discriminatory results. They found that it was
possible to discriminate between the paint samples, and that the above factors did not have a significant effect on the measured ratios. The above examples help to underscore the potential importance of IRMS to the criminal and civil justice systems.

As a result of the sheer volume of cases being handled by our legal system, the analysis of controlled drugs has been one of the largest areas of development for IRMS. When allegations of drug-facilitated sexual assault (DFSA) are raised into question, it is necessary to test for the presence of drugs. One of the most common drugs in DFSA is γ - hydroxybutyric acid (GHB), which can be tested in blood, urine or hair samples. GC-IRMS of the carbon isotope ratios has been used to discriminate between endogenous production of GHB and exogenous ingestion levels. On a much larger scale, IRMS may also be a key tool when dealing with the war against drugs and drug trafficking. In fact, the DEA and FBI labs already use IRMS to help determine world-wide drug trafficking routes. In one published example, twenty samples of heroin were seized from a North Korean cargo vessel. To determine whether or not the samples had originated from any known source, they were compared to an existing database. This database contained more than 200 known/authentic samples and, after careful analysis, it was determined that the seized samples had not originated from a previously known source.

Due to the modern threat of terrorist attacks, techniques are necessary to address the unique problems associated with mass-disasters. For example, in the case of an event of mass destruction where massive casualties are suffered, the ability to identify human remains would prove beneficial from both a forensic standpoint and from a humanity standpoint, by providing closure for the families and friends affected. There have been
cases where it was not possible to collect DNA and other situations where there was not enough evidence to warrant a DNA test. IRMS can serve as a potential option to link recovered body parts to a single victim or to a geographic location from which victims might have originated. Human scalp hair and fingernails have shown to be good candidates for hydrogen and oxygen isotope ratio analysis.\textsuperscript{40} Meier-Augenstein et al. were able to use isotope ratio analysis of oxygen and hydrogen from an unidentified body found in Ireland to verify that the woman had not lived in that specific local geographical region for a significant amount of time.\textsuperscript{41} When looking at the isotopic analyses, results suggested that the woman was most probably from either Eastern Europe or Scandinavia. This evidence assisted in the eventual identification of the deceased person (based on immigration) by authorities. In another case,\textsuperscript{79} a man’s body was found near an expressway in Germany. The hair, teeth, cranium, and soil from the body were analyzed to determine his origin. The results determined that the body was most likely from Romania, which subsequently directed the criminal investigation department to look for missing males in that country. Not only was the family later found and a genetic relationship confirmed through the use of DNA analysis, but it was also determined that two of the man’s friends had murdered him and buried the body. In another interesting case,\textsuperscript{80} the investigating officer of a crime scene-where the mutilated body of a man who had been completely dismembered was found-took DNA samples from possible relatives. The combined isotopic analysis of hair, nails, and bone established the victim’s recent life history spanning 200 days prior to his death and allowed investigators to narrow
down the search to Dublin. Ultimately, as a result of this analysis, the victim’s killers were apprehended.

3.4 Summary

When considering isotopic analysis of forensic samples, it is important to decide if individual components of the sample need to be analyzed or if the entire sample can be analyzed. Ultimately, this will determine which interface is the most suitable, GC or EA respectively. When analyzing compounds that are non-volatile-like foods, drugs, amino and fatty acids, and even rocks-the introduction method is usually EA. When analyzing volatile organics such as petroleum distillates, methyl esters of fatty acids on scheduled drugs, they can often be separated and introduced to the IRMS via a GC-combustion interface. There is unlimited potential offered by the use of IRMS with equally unlimited applications for non-volatile and volatile compounds. However, forensic applications that stand to benefit most by the use of IRMS include high-volume or high-value crimes such as burglary, homicide, and drug cases. Future benefits of IRMS in the realm of drug control include the ability to link trace amounts of controlled or illegal drugs to bulk sources, leading eventually to the determination of trafficking routes.

The obvious down-side to the technique is that there are almost no crime laboratories, outside of the federal government, who own or have access to IRMS instrumentation. Almost all advances are being made by instrument manufacturers and academicians, in consultation with investigators. The technique clearly exceeds Daubert requirements for admissibility in court, is well documented in scientific literature, is
commonly used in other scientific fields of study, and has precedent for admission in civil and criminal cases. If databases could be developed for frequency distribution of isotope ratios of interest for high-frequency or high-profile applications, and if instrument vendors could improve reliability and robustness, then regional, state, and local crime labs should be able to make a strong case for implementation of IRMS in their labs.
CHAPTER 4: SIMULTANEOUS IDENTIFICATION AND $\delta^{13}$C CLASSIFICATION OF DRUGS USING GC WITH CONCURRENT SINGLE QUADRUPOLE AND ISOTOPE RATIO MASS SPECTROMETERS

This chapter was accepted for publication by the Journal of Forensic Sciences December, 2009.

4.1 Introduction

In 2007, more than 1.8 million drug-related arrests were carried out in the United States by federal, state, and local law enforcement agencies. Intelligence estimates indicate a vast majority of the cocaine available in U.S. drug markets is smuggled by Mexican drug trafficking organizations across the US/Mexican border. It is therefore crucial for law enforcement officials to monitor vessels or possible trafficking routes being used to bring drugs into the United States. According to US News and World Report, drug smugglers are using handmade submarines to ship up to 12 tons of cocaine into the USA per vessel. The Coast Guard speculated that 32% of all the cocaine being smuggled from Columbia into the USA in 2008 was being brought in using this type of vessel. When investigating vessels, the coast guard will often come across drugs hidden within containers on board. According to CBS station WFOR-TV in Miami, dozens of tightly wrapped bricks of cocaine were found carefully packed inside of hidden compartments in a fuel tank onboard a ship. As a commonly occurring Schedule I drug, cocaine is of major significance to a number of law enforcement agencies. The United States Drug Enforcement Administration (DEA) has created a cocaine signature program based on the abundance of coca extract impurities in the cocaine, in order to keep track of
the specific regions where large-scale crops of coca plants are grown. Some other specific signatures that can be used for identification and classification purposes are nitrogen isotope ratios \((^{15}\text{N}/^{14}\text{N})\) of the compound truxilline and carbon isotope ratios \((^{13}\text{C}/^{12}\text{C})\) of the compound trimethoxycocaine, both of which are found in relatively high abundance in coca plant matter.25

As an extension of the Locard exchange principle, forensic drug samples have the potential to be transported or transferred between the drug package and any person(s) or object(s) with which it comes into contact. Therefore, drugs found on a suspect’s clothing, a shipment container, or any surface of the crime scene could all be sources of trace residues of the drug. Samples of these trace amounts could be collected and tested as a possible source match or could provide exclusionary evidence. The steps that have occurred from the time the coca plant, (Erythroxylum coca and E. novogranatense), a C3 photosynthesis plant, was harvested to the time of possession by the suspect, are complex and varied. Therefore, the ability to trace a collected sample back to its source would prove very helpful. Stable isotope analyses provide an additional “isotopic fingerprint” that could further characterize forensic evidence, via a sample’s stable isotope signature.86 Chemical processing of materials can also result in distinctive stable isotopic ratios,5 as can impurities left behind during production.84 When cocaine is cut, mixed or converted to crack, the isotopic ratios remain largely preserved, thereby enabling source identification to a specific manufacturer.84, 87 Stable isotopes can provide sufficient insights that allow an investigator to trace the manufacturing origins of the material at hand or how many different ‘cooks’ or batches could have contributed to the samples that
were seized. Researchers have already shown that IRMS can be used to assign the geographical origins of illicit cocaine and the effects of fractionation during the illicit production of cocaine.

Eherlinger et al. showed that with the use of isotopic analysis, it is possible to pinpoint geographical locations of seized batches of cocaine and such results have already been put into a library. Eherlinger et al. have also showed the current major geographic origins of cocaine, and how the cocaine could be identified on the basis of natural variations in isotope ratios related to their variations in environmental habitat parameters. Janzen et al. used Euclidian distance as a tool to identify matching cocaine profiles to establish links between samples. Further research in these areas could, in time, result in a database that would allow the chemical tracking of selective drug routes.

The confirmatory identification of controlled substances is one of the most common and important tasks of a forensic drug analyst. The hyphenated technique of gas chromatography and mass spectrometry (GC/MS) has long been the gold standard in forensic science and serves as a category A, or confirmatory method, according to SWGDRUG guidelines. Single quadrupole mass spectrometers are a staple in most crime labs due to their ease of use, modest cost, robustness and uniform fragmentation patterns observed between vendors and models. Here, a single quadrupole mass spectrometer provides the retention time data and characteristic fragmentation patterns used to confirm the presence of cocaine and any impurities in the samples. Simultaneously, the IRMS determines the carbon isotopic abundance ratios of the cocaine or impurity peaks. To our knowledge, the simultaneous use of these two detectors has
only been demonstrated on a few occasions and never before for forensic applications. Single quadrupole mass spectrometers were coupled to GC-IRMS systems and tested for precision and accuracy of the coupled detectors using n-alkanes and polycyclic aromatic hydrocarbons both in soil,96 and to analyze sediments left by fire to determine the impact on climate biosphere interactions.97 In another application, a single quadrupole mass spectrometer coupled to a GC-IRMS was used to analyze a marine halogenated natural product within skua samples and whale blubber samples by Vetter et al.,98 and to take measurement of carbon isotopes of chloromethane emission flux in the atmosphere compared to that originating from various sources.99 A more extensive look at halomethanes was measured in the same manner by Kalin et al. for future hope in understanding the stratospheric ozone destruction.100 Ion trap mass spectrometers were coupled to GC-IRMS and tested for precision and accuracy of the coupled detectors,101 using a standard consisting of four alkanes of different concentrations,102 and specific positions in organic compounds separated from complex mixtures were analyzed to demonstrate the absence of rearrangement during activation and fragmentation.103 This approach eliminates the need for two separate sample injections into two different GC ovens with two different GC columns. Without this instrument modification, identification and compound specific $^{13}$C/$^{12}$C determination typically requires two independent GC systems.104 Very careful characterization is required to validate retention times between the two instruments to ensure the identification of each peak in the IRMS profile is correct. The benefits of this single injection method includes the lowering of sample preparation time and reduced sample amounts, a reduction in the risk of error due
to having to prepare two samples, along with the shear simplicity of using one autosampler and getting two results. Our main purpose in this technical note is to demonstrate that our modification does work and that the concept of coupling two detectors to get simultaneous results was achieved.

4.2 Materials and Methods

4.2.1 Chemicals

The samples of cocaine utilized in our research include two commercially available cocaine free base standards (A and B), a commercially available cocaine HCl standard (C), and three street crack cocaine samples (D, E, and F) details are listed in Table 4.1. Cocaine HCl and cocaine free base were both purchased from (Sigma Aldrich, St. Louis, Missouri) and all other cocaine samples were obtained through local law enforcement agencies.

Table 4.1

<table>
<thead>
<tr>
<th>Cocaine Sample</th>
<th>Company</th>
<th>Name</th>
<th>Lot #</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Sigma Aldrich, St. Louis, Missouri</td>
<td>Cocaine Free Base</td>
<td>97H1018</td>
</tr>
<tr>
<td>B</td>
<td>Sigma Aldrich, St. Louis, Missouri</td>
<td>Cocaine Free Base</td>
<td>44H0207</td>
</tr>
<tr>
<td>C</td>
<td>Sigma Aldrich, St. Louis, Missouri</td>
<td>Cocaine HCL</td>
<td>025K1024</td>
</tr>
<tr>
<td>D</td>
<td>Ohio Law Enforcement</td>
<td>Street Cocaine</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>Ohio Law Enforcement</td>
<td>Street Cocaine</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>Ohio Law Enforcement</td>
<td>Street Cocaine</td>
<td></td>
</tr>
</tbody>
</table>
All samples (A, B, C, D, E, and F) were prepared by dissolving 2 mg aliquots of each cocaine sample in 1 mL HPLC grade Methanol (Sigma Aldrich, St. Louis, Missouri). Three aliquots of each sample and a blank sample were analyzed 4 times each, in random order.

An IRMS standard (Chiron International Standards, Laramie, Wyoming) consisting of n-Undecane (C\textsubscript{11}) with a $\delta^{13}$C value of -26.11‰, n-Pentadecane (C\textsubscript{15}) with a $\delta^{13}$C value of -30.22‰, and n-Eicosane (C\textsubscript{20}) with a $\delta^{13}$C value of -33.06‰, each at 0.15 mg/mL in Cyclohexane was used to calibrate the CO\textsubscript{2} tank and was used as the reference gas throughout each run. 1 μL of the standard was injected into the GC and the value that was assigned as the user defined amount was -11.56‰, measured relative to universal standards and reported in the delta notation, $\delta$:

$$
\delta = 1000 \left( \frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \right)
$$

The value $R_{\text{sample}}$ is the abundance ratio of the minor, heavier isotope of the element to the major, lighter isotope (e.g. $^{13}$C/$^{12}$C). Our method was set up to run three standard gas peaks before the sample run and one at the end of the sample run to ensure instrument stability. The second reference peak, shown in Figure 4.3, was selected as the $R_{\text{standard}}$ value to be used for equation (1) when determining a $\delta$ value for the sample of cocaine.
4.2.2 Gas Chromatography – Mass Spectrometry / Isotope Ratio Mass Spectrometry

Gas chromatography mass spectrometry analyses were performed using a single quadrupole mass spectrometer (HP5970B, Hewlett Packard (now Agilent), Santa Clara, California) and an isotope ratio mass spectrometer (Delta Plus Advantage, Thermo Finnigan, Waltham, Massachusetts). These instruments were then used in combination with a gas chromatograph (Trace GC, Thermo Finnigan, Waltham, Massachusetts) equipped with an autosampler (AS3000, Thermo Finnigan, Waltham, Massachusetts). A typical cycle time for the GC-IRMS was 25-30 minutes and samples were placed on a 100 sample wheel, which allowed for unattended sample analysis. The GC column was a 5% Phenyl - methylpolysiloxane (DB-5) (60 m x 0.25 mm x 0.25 μm, J & W Scientific, Folsom California). The effluent was split using a low-dead-volume X-connector (Valco Instruments Co. Inc., Houston, Texas) so that ~10% of the effluent flowed to the single quadrupole mass spectrometer for structural elucidation and ~90% flowed to the IRMS for isotopic analysis. Because the IRMS and the single quadrupole mass analyzer differ in sensitivity, the flow rates to each were split in a 90:10 ratio, respectively, to obtain similar detection limits for both instruments. The transfer line to the HP single quadrupole instrument was a deactivated fused silica (1.0 m x 0.1 mm) capillary, (Agilent, Santa Clara, California). The transfer line from the oxidation tube to the GC Combustion III interface was a 1.0 m x 0.2 mm I.D. deactivated fused silica capillary (Thermo Finnigan, Waltham, Massachusetts). Split flows were measured manually,
outputted to 1 atm, using a universal flow meter (ADM2000, Agilent, Santa Clara, California).

The GC oven temperature was programmed as follows: initial temperature 170°C (hold 9.5 min); program rate, 16°C/min final temperature 280°C (hold 2 min). The injector temperature was set at 280°C and the helium carrier gas flow rate was 2.5 mL/min. A volume of 1 uL was injected in splitless mode using the aforementioned autosampler.

The temperature of the auxiliary transfer line to the single quadrupole was set at 280°C. The majority of the transfer line was heated using the heater and thermocouple that was originally supplied for a typical set up of a Trace GC and was controlled by the software provided, Isodat 2.0 (Thermo Finnigan, Waltham, Massachusetts). The transfer line that was attached to the single quadrupole mass spectrometer had to be installed on the right side, unlike typical left-sided setups. This required an extension to the original transfer line to allow for passage through the outer shell, circuit board, and oven wall to reach the inside of the oven, as shown in Figure 4.1a.
Figure 4.1 (a) Schematic of our GC-MS set up with a transfer line extension and heat tape configuration and (b) the schematic of GC-MS-IRMS wiring to control the auto-sampler signal to both PC’s indicating that the sample injection has been made and the run should begin.
The GC is configured to allow a transfer line through this wall, but needs to be drilled out. A 5 cm diameter hole was drilled in the outer wall on the right side of the GC oven containing the electrical circuit board and the outside panel. A 2 cm hole was drilled into the right side of the metal GC oven siding. A metal guide sleeve (7.5 cm x 0.12 cm I.D.) with a (3.2 cm) collar was placed into the 2 cm hole. The extension (10.0 cm x 0.75 mm I.D.) was added onto the end of the transfer line so that the transfer line extended into the inside of the oven. This portion of the transfer line was heated using a BIH heavy insulated heating tape (BriskHeat Corporation, Columbus, Ohio) that was controlled by a manual temperature controller (Staco Energy Products Co., Dayton, Ohio). An OMEGA DP20 (OMEGA Engineering, Inc., Stamford, Connecticut) external thermocouple was used to ensure that the heater was accurately heating the extended transfer line, thereby maintaining a specific temperature. Total ion chromatograms were recorded from m/z 50-550 using HP Chemstation B.02.05 (Hewlett Packard (now Agilent), Santa Clara, California). The trigger wire from the single quadrupole was connected to the generic handshake port in the back of the Trace GC so that the injection trigger from the autosampler would be recognized, as seen in Figure 4.1b. The data was exported as a text file for comparison with the NIST mass spectral library (US Secretary of Commerce, USA) to confirm the identity of the compounds of interest.

For isotope ratio measurements, the isotope ratio mass spectrometer (Delta Plus Advantage, Thermo Finnigan, Waltham, Massachusetts) was used via a combustion interface (GC Combustion III, Thermo Finnigan, Waltham, Massachusetts). The combustion oven temperature was held at 940°C and the reduction oven temperature was
held at 650°C. Data acquisition was carried out using Isodat 2.0 Software (Thermo Finnigan, Waltham, Massachusetts). For carbon GC-IRMS, ion chromatograms of three isotopic peaks of CO$_2$ were collected by three individual ion collectors with different specific sensitivities, i.e. ions at m/z 45 ($^{13}$C$^{16}$O$^{16}$O) and ($^{12}$C$^{16}$O$^{17}$O) were recorded with 100-fold sensitivity relative to m/z 44 and ions at m/z 46 ($^{12}$C$^{16}$O$^{18}$O) were recorded with 300-fold sensitivity relative to m/z 44. \textsuperscript{105,106} We began each run with three injections of the high purity (99.997\%) CO$_2$ reference gas (Airgas, Great Lakes, Independence, Ohio), of which the second CO$_2$ peak, seen in Figure 4.1a, is designated as the standard to be used for calculating the $\delta$ ($^{13}$C/$^{12}$C) values by the instrument acquisition software.

To improve the quality of the measurement and eliminate any error due to misbalance, each sample should ideally be adjusted so that the amplitude of the sample signal is as close to the CO$_2$ reference gas peak as possible. When standard and sample are run at significantly different signal sizes, the linearity of the instrument results is small, but significant, absolute differences in the measured $\delta^{13}$C values. \textsuperscript{107}

4.3 Results and Discussion

Cocaine was used as an example drug sample to demonstrate the quality and effectiveness of the single injection/dual detection system. A chromatogram and mass spectrum obtained on the single quadrupole mass spectrometer is shown in Figure 4.2. The retention index and fragmentation pattern of sample C both confirm the identity of the sample as cocaine. The spectrum shown in Figure 4.2 clearly displays the
fragmentation pattern peaks that are used to commonly identify cocaine by peaks 82, 182, and 303, providing a class A, confirmative analysis.

Figure 4.2 TIC chromatogram and mass spectrum of the peak at 26.52 minutes. The retention index and mass spectrum of this sample confirm the identity of the sample as cocaine. The NIST head to tail output confirm the presence of cocaine with a 77.1% probability score.

The National Institute of Standards and Technology (NIST) head to tail output confirming the presence of cocaine can also be seen in Figure 4.2, showing both the library and the actual spectrum. The probability score from the NIST library for all
samples ranged between 52.9% and 77.1%. The mass spectra from each cocaine sample look virtually identical due to the fact that they have the same chemical composition. This is true in cases where impurity peaks are not present. This further highlights the importance of a secondary analysis technique to establish the possibility, or exclusion, of a common source. Figure 4.3 shows the concurrent IRMS output acquisition for the same injection of cocaine sample C. The carbon isotopic ratios can be used to exclude the possibility that two samples of drugs are from the same source.

Figure 4.3  Example of a concurrent IRMS output acquisition for the single injection of cocaine sample C.
Our primary interest was to establish the sources of variation in δ^{13}C values for cocaine and to determine which samples could have come from the same source. To establish the variation of δ^{13}C values in each sample (sampling variance), three aliquots of each sample were analyzed in quadruplicate samplings.

Pooled standard deviations, for each sample in the measured carbon isotope ratios, varied between 0.17-0.31‰ (N=12). The plot shown in Figure 4.4 enables the reader to visualize the separation between the different batches of cocaine. The error bars show the 95% confidence interval. Using ANOVA, sampling error could be separated from the measurement error. Sampling standard deviations varied from 0.009‰ to 0.076‰ (average = 0.03‰) for the six samples, whereas measurement standard deviations varied from 0.17‰ to 0.30‰ (average = 0.23‰). The sampling standard deviations were on average eleven times smaller than the measurement variances, thereby demonstrating that the samples were all relatively homogenous and that most of the error in the results stem from the IRMS instrument itself.
Figure 4.4  Average $\delta^{13}$C values of six cocaine samples. Results represent three aliquots of each sample with quadruplicate sampling and error bars indicate 95% confidence interval.

Each cocaine sample was tested for unique $\delta^{13}$C values using monometric statistics. When comparing the F-calculated results in Table 4.2 with the F-critical values, our results show that the sample mean for sample A can be distinguished at the 95% confidence level (CL) from samples B, D, E, and F. Similarly, samples B, D, and E can all be distinguished at the 95% CL. The sample means of samples A and C are indistinguishable from one another, as are E and F.
Table 4.2

F-calculated values for six cocaine samples: cocaine free base standard Samples A and B, cocaine HCl standard Sample C, and three street crack cocaine Samples D, E, and F. Cells with a white background are significantly different.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>12.45</td>
<td>0.39</td>
<td>123.24</td>
<td>90.06</td>
<td>23.69</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>6.69</td>
<td>49.01</td>
<td>18.33</td>
<td>3.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>90.62</td>
<td>54.55</td>
<td>16.71</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>15.85</td>
<td>15.03</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.20</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Although pairs A and C, and E and F, cannot be excluded as having a potential common source from one another, B, D, E, and F can be excluded from all other samples. The known deviation in sample mean street cocaine is -25.3‰ to -35.2‰, a significantly broader range than the samples presented here. One can exclude two samples of cocaine as having a common origin by using the statistical probability values (P-values) provided in Table 4.3. If the P-values are less than 0.05, the results indicate that the difference in the two sample means is statistically significant at the 95% CL.
Table 4.3

*P*-values for six cocaine samples; cocaine free base standard Samples A and B, cocaine HCl standard Sample C, and three street crack cocaine Samples D, E, and F. Cells with a white background are significantly different.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.9 x 10^{-3}</td>
<td>0.54</td>
<td>1.8 x 10^{-10}</td>
<td>3.1 x 10^{-9}</td>
<td>7.3 x 10^{-5}</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>1.7 x 10^{-2}</td>
<td></td>
<td>5.0 x 10^{-7}</td>
<td>3.0 x 10^{-4}</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
<td>2.9 x 10^{-9}</td>
<td></td>
<td>2.2 x 10^{-7}</td>
<td>4.9 x 10^{-4}</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
<td></td>
<td>6.3 x 10^{-4}</td>
<td></td>
<td>8.2 x 10^{-4}</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Statistical validation and confidence reporting are two very important criteria recommended by the National Academy of Science Report to Congress in their recommendations for the forensic science community in the United States. Results involving samples with significantly different mean $\delta^{13}$C values clearly fall within the guidelines of statistical and confidence reporting. However, a significantly larger database of the frequency of $\delta^{13}$C values needs to be generated to determine the probability of two random samples having indistinguishable sample means.

4.4 Summary

IRMS has shown the ability to be a reliable and accurate method that can be used concurrently with a single quadrupole mass spectrometer to provide confirmatory identification of a scheduled drug (or impurities), with an additional level of discriminatory power with a simple, inexpensive modification. The use of IRMS for the
comparison of unknown samples of drugs has virtually unlimited applications. We believe this technique would be useful when comparing trace questioned samples found at a crime scene with bulk known samples that may be found at other locations. This type of modification would also be beneficial when dealing with extremely complicated samples matrices where peak identification is difficult. Through a second injection of each sample, it would be possible to obtain N, H, or O isotope analysis, which could add a greater degree of discriminating power in establishing or excluding potential common sources of drugs. In the future, when combining more than one isotope ratio, IRMS analyses will be an asset in providing additional information as to the geographic locations of crops being grown and harvested, and for providing statistical measures of differences between samples as already been shown by Ehleringer et al.25
CHAPTER 5: SIMULTANEOUS IDENTIFICATION AND $\delta^{13}$C CLASSIFICATION OF CANNABINOL IN UNKNOWN MARIJUANA SAMPLES USING GC WITH CONCURRENT SINGLE QUADRUPOLE AND ISOTOPE RATIO MASS SPECTROMETERS

5.1 Introduction

According to the World Drug Report 2008, marijuana or Cannabis sativa L is the most frequently abused illicit drug in the world. 111 166 million people, or approximately 3.9% of the world population, are estimated to abuse the drug. 112 In 2006, 10% of the 8th graders in the United States abused marijuana as well as 25% of the 10th graders and 32% of the 12th graders. 113 Some teenagers justify their marijuana consumption by claiming relief from different health problems, such as depression, sleeping difficulties, and physical pain. 114 It is reported in a variety of different publications that adolescents tend to have difficulties in concentration, attention and learning after repeated drug intake. 115

The main active constituents in Cannabis sativa L are cannabinoids such as cannabinol (CBN), cannabidiol (CBD), and $\Delta^9$-tetrahydrocannabinol (THC), cannabigerol (CBG) and cannabichrome (CBC). 116 Their structures are shown in Figure 5.1. Cannabis sativa L also contains different terpenes (e.g. mycrene), sesquiterpenes (e.g. Caryophyllene) and terpenoid-like compounds, 117 although these are not thought to be psychoactive.
Figure 5.1  Cannabinoid structures of the main active constituents in the Cannabis sativa L.
More than 60 cannabinoids have now been identified in *Cannabis sativa* L., however the major psychoactive effects are attributable to THC. THC content varies for different plant parts in the following order: bracts > flowers > leaves > smaller stems > larger stems > roots > seeds. The average THC content in seized samples ranges from 1-14%. For Sinsemilla, or plants cultured in the Netherlands, Switzerland, and British Columbia, the THC content can be up to 20%. THC is capable of relieving anorexia, nausea, and vomiting. The effects are well documented in the treatment of painful conditions such as, asthma, glaucoma, and spasms. Hallucinogenic effects are experienced when a human absorbs 10 µg/kg of THC into their total body mass. According to Grotenhermen et al., when a fetus is exposed to cannabis in the uterus, negative results can occur during development of the brain and subtle impairment of cognitive abilities are possible later in life. The potential of the active ingredients to induce a mental and physical addiction is discussed by Hyman et al. and Brajevic-Gizdic et al.

Cannabinoids can be detected in the bodily fluids of excessive users up to several weeks after stopping the intake of THC. This is due to the accumulation of THC in fatty tissue facilitated by its lipophilic properties. Jung *et al.* showed the detection of 9-tetrahydrocannabinolic acid and THC in human blood serum and urine using LC/MS/MS. THC can also be detected in fingernails and hair. Recent drug use can be detected in the saliva of suspected users through the use of liquid/liquid extractions and GC/MS.
In criminal cases, the forensic analysis of marijuana-related cases typically involves the confirmation of drug identity for the prosecution of crimes such as possession, possession with intent to distribute and trafficking. Microscopic and macroscopic botanical features and the Duquenois-Levine color test are most commonly used to identify exhibits as originating from *cannabis sativa*, and GC/MS is used to confirm the presence of THC when botanical features are not present. In civil cases, the identification of drug and drug metabolites in samples from suspected users are commonly used to establish drug use and abuse. Federal agencies are often interested in the drugs’ origin, and in determining large-scale trafficking routes for potential legal/enforcement solutions. Determination of the drugs origin is possible by matching the DNA of the plant or by comparing the natural isotopic abundance ratios to other reported seized plants with known geographical origins, as described below.

Because *Cannabis sativa* L. is a C₃ plant like corn, cotton and soy, its photosynthesis activities can be recorded in the isotope ratio of carbon. The net rate assimilation of carbon dioxide is determined by biophysical processes, such as CO₂ transport through the leaf and stomata, and biochemical processes in the organelles of the plant cells. Environmental factors such as climate, water availability, temperature and light intensity have a huge impact on the assimilation as well. To date, the high numbers of variables for these processes impede a complete detailed understanding. By approximation, the background δ¹³C value for atmospheric CO₂ is -8 ± 0.2‰. Plant tissues are depleted in respect to the atmospheric source because photosynthesis and enzymatic fixation are discriminating against the heavier isotope. *Cannabis sativa* L.
follows the Benson-Calvin photosynthetic pathway and therefore shows δ\(^{13}\)C values typically in the range of -24 to -35‰, depending upon the growth conditions.\(^{133}\) The ratio of the CO\(_2\) concentration in the stomata to the concentration in the air is controlled by the stomata conductance relative to the photosynthetic activity. Interpretation of this relationship indicates the breeding conditions (indoor or outdoor growth).\(^{134}\) IRMS can therefore be used as a forensic tool for establishing the common origin of seizures, and in tracking the geographic origin of seized samples.

Depending on the nature of the sample, there are two common methods to introduce the sample into the IRMS system: an elemental analyzer (EA) for solid or bulk samples and a GC for complex mixtures. EAs require the combustion of a solid or liquid sample and provides gross or bulk isotope ratio values of the entire physical sample.\(^1\) The advantage of the GC, as compared to the EA, is that there is a pre-separation of the individual components according to their retention times. The measured isotope ratios, measured after chromatographic separation, are therefore compound specific, and can provide significantly more detail about a complex sample than can a single weighted average value. To date, EA/IRMS has been used by several researchers\(^{133-136}\) to provide bulk analyses of cannabis samples. However, to the best of our knowledge, no results using GC/IRMS of individual cannabinoids can be found in the extant literature. Here, we use a GC to separate the cannabinoid extracts of \textit{cannabis sativa L.} plant material, and simultaneous EI/MS and combustion/IRMS to identify and measure the isotope ratios of each separated component, respectively. Sampling procedures and ANOVA analysis was
used to establish the relative contribution of isotope variance between sampling error and measurement error, the former of which was negligibly small.

5.2 Materials and Methods

5.2.1 Chemicals

The samples of marijuana utilized in our research include four street samples obtained through local law enforcement agencies. Each sample was more than ten years old and sometimes contained trace amounts of THC. These samples were known to contain significantly more THC until recent years. CBD and CBN are known to be degradation products of THC.137 The cannabinoids CBN and CBD were shown to be dominant components in each sample and are still specific to cannabis plants. Cannabis plant matter which has not undergone aging would contain quantities of THC which would enable an additional or alternative discrimination tool.

Samples were re-labeled A-D for this study. Original sample sizes for samples A-D varied from ~1 g to ~20 g. Replicate aliquots were taken at random locations of each sample for subsequent analysis. From each aliquot, approximately 300 mg of leaf matter was pulverized by adding 6-8 stainless steel ball bearings and rapidly agitating the vial for 5 minutes using a Mini Beadbeater (Biospec Products Inc., Bartlesville, OK). Approximately 200 mg of each powdered marijuana sample was then put in a 1.5 mL glass vial and extracted with 1 mL of acetone (Sigma Aldrich, St. Louis, Missouri). The sample was sonicated for 15 minutes before centrifuging for 2 minutes to pelletize the
solid plant matter. The liquid supernatant was then filtered to remove any particulates. 150 μL of the remaining sample was then transferred to a 1.5 mL autosampler vial with 200 μL microwell inserts and placed into the autosampler carousel to await injection.

5.2.2 Gas Chromatography – Mass Spectrometry / Isotope Ratio Mass Spectrometry

Separations were performed on a gas chromatograph (Trace GC, Thermo Finnigan, Waltham, Massachusetts) equipped with an autosampler (AS3000, Thermo Finnigan, Waltham Massachusetts). Detection was accomplished using dual mass spectrometer detectors, as described previously.138 Approximately 10% of the column effluent was split to a single quadrupole mass spectrometer (HP5970B, Hewlett Packard (now Agilent), Santa Clara, California) for EI fragmentation analysis and the remaining 90% was split to an isotope ratio mass spectrometer (Delta Plus Advantage, Thermo Finnigan, Waltham Massachusetts) for isotopic analysis. The GC column was a 5% Phenylpolydimethylsiloxane (DB-5) 60 m x 0.25 mm x 0.25 μm column (J & W Scientific, Folsom California). The GC effluent was split using a low-dead-volume X-connector (Valco Instruments Co. Inc., Houston, Texas).

The GC oven was held at 100°C for 5.0 min, and then ramped at 20°C/min to a final temperature 300°C for 8.0 min. The total separation time was 23 minutes. The injector temperature was set at 280°C and the helium carrier gas flow rate was 2.5 mL/min. A volume of 1 μL was injected in splitless mode using the aforementioned autosampler. The temperature of the auxiliary transfer line to the single quadrupole was set at 300°C.
EI mass spectra were recorded between the ranges of m/z 50-550 using HP Chemstation B.02.05 (Hewlett Packard (now Agilent), Santa Clara, California). Post-acquisition, the data was exported as a text file for comparison with the NIST mass spectral library (US Secretary of Commerce, USA) to confirm identity of the cannabinoids of interest. For the IRMS measurements, the combustion oven temperature was held at 940°C and the reduction oven temperature was held at 650°C. Data acquisition was carried out using Isodat 2.0 Software (Thermo Finnigan, Waltham, Massachusetts).

5.3 Results and Discussion

Our system allows for a single sample injection that can be analyzed by two separate detection systems simultaneously. Results from the single quadrupole mass spectrometer are in the form of a total ion chromatogram and mass spectrum, and the chromatogram of carbon isotopic analyses from the IRMS. All four samples contained CBN as the major or second most abundant cannabinoid. CBD was the most abundant cannabinoid in samples C and D, but was only a trace component in samples A and B. Because of the age of our samples, THC was only detected at trace levels in a few aliquots. Example TICs from the single quadrupole MS and an example of a head to tail comparison for CBN is shown in Figure 5.2. The head to tail comparison of CBD for samples C and D can also be seen in Figure 5.2. The retention index and fragmentation pattern of all four samples clearly identify CBN with probability scores from the NIST library ranging from 70.9 – 84.5% and CBD from samples C and D of 57.4% and 70.0%
respectively. These results were presented at Midwestern Universities Analytical Chemistry Conference (MUACC) hosted by the Michigan State University, Dec. 2009 the poster of which can be found in the appendices as Appendix C.1.
Figure 5.2  TIC chromatogram and mass spectra with the NIST head to tail library output of samples A, B, (top row) C, and D (bottom row). Samples A and B containing the cannibinoid CBN and samples C and D having both CBD and CBN.
The fragment ions that are most commonly used to identify CBN are m/z 238, 295, 310. For CBD, the major fragments are m/z 174, 231, 314. The results from the single quadrupole mass spectrometer provide a category A, or confirmatory method of analysis according to SWGDRUG guidelines. The IRMS can then be used to further discriminate the unknown sample by assigning isotope ratios to the individual components within the sample. An even greater degree of discriminating power in establishing or excluding potential common sources of drugs would be possible by acquiring the N, H, or O isotope ratios, by chemometric analysis, or by elemental analysis of the metal impurities. The pooled average isotopic ratios for CBN each sample were; A = -29.33±0.05‰, B = -30.01±0.10‰, C = -30.31±0.14‰, and D = -29.90±0.17‰. The 95% confidence intervals were; A = 0.05‰, B = 0.09‰, C = 0.11‰, and D = 0.09‰. According to West et al. our results indicate that the marijuana samples used for our study were probably indoor or shade grown. Indoor grown marijuana samples have a range between -32‰ to -29‰ and total ranges for marijuana samples with known origin in the USA range from -51.8‰ to -20.3‰.

The simultaneous IRMS output acquisition for single sample injections of marijuana A, B, C, and D are displayed in Figures 5.3 and 5.4. The carbon isotopic ratios can be used to exclude the possibility that two samples of drugs are from the same origin. Our primary interest was to establish the sources of variation in $\delta^{13}C$ values for marijuana cannabinoid CBN for possible source matches. Three aliquots of each sample were analyzed, four times each, in order to establish the variation of $\delta^{13}C$ values in each marijuana sample (sampling variance).
Figure 5.3  Example of a concurrent IRMS output acquisition for the single injection of marijuana samples A, and B.
Figure 5.4  Example of a concurrent IRMS output acquisition for the single injection of marijuana samples C, and D.
The replicate levels of each aliquot were tested using a Grubbs test to determine whether any outliers existed. Based on the 95% confidence level hypothesis test, six outliers were removed. These six outliers happened to be the first six of the approximately forty-eight chromatograms that were collected in the analytical sequence. The significantly different results of the first six injections of the day indicate an unknown source of bias in the results. A similar result was observed when repeating the entire sequence on a separate day. Because the isotope ratios of the reference gas (CO₂) was not significantly different in the first few runs of the day, we assume that the freshly regenerated oxidation tube causes bias for the first 6-8 runs of the day before displaying more stable behavior. Fewer outliers have been noted in more recent studies when standards are run multiple times through the regenerated oxidation tube before initiating an analytical sequence.

The bar graph in Figure 5.5 shows the isotope ratio results for CBN in each of the aliquots from the four samples. The separation between the different samples of marijuana can be visualized with error bars showing the 95% confidence intervals for each aliquot that range from 0.03 to 0.17. ANOVA was employed to separate the sampling error from the measurement error. Sampling standard deviations varied from 0.006‰ to 0.049‰ (average = 0.03‰) for the four samples, whereas the measurement standard deviations varied from 0.11‰ to 0.22‰ (average = 0.14‰). Based on these standard deviations, the measurement error is approximately eight times larger than the sampling error. These results indicate that the marijuana samples were relatively homogeneous and that most of the error in the results was due to the IRMS
measurements. Our errors for replicate analyses of a sample are consistent with the literature and specifications for the instrument.

**Figure 5.5** Average $\delta^{13}C$ values of CBN of four marijuana samples. Results represent three aliquots of each sample with quadruplicate sampling and error bars indicate 95% confidence interval.

When comparing the F-calculated results in Table 5.1 with the F-Critical values, our results show that the sample mean for sample A can be distinguished at the 95% confidence level (CL) from samples B, C, and D. Samples C and D can be similarly
distinguished at the 95% CL. The samples means of samples B and D are not significantly different, therefore cannot be excluded as having a potential common source. Because the true origin of these samples is not known, the samples could in fact be from a common source.

Table 5.1

*F*-calculated values for $\delta^{13}C$ values of CBN in four marijuana samples; A, B, C, and D. Cells with a white background are significantly different.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>X</td>
<td>59.30</td>
<td>117.46</td>
<td>30.69</td>
</tr>
<tr>
<td>B</td>
<td>X</td>
<td>X</td>
<td>33.58</td>
<td>3.03</td>
</tr>
<tr>
<td>C</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>37.03</td>
</tr>
<tr>
<td>D</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

One can exclude two samples of marijuana as having a common origin by using the statistical probability values (P-values provided in Table 5.2. If the P-values are less then 0.05, the results indicate that the difference in the two sample means is statistically significant at the 95% CL.

Table 5.2

*P*-values for $\delta^{13}C$ values of CBN in four marijuana samples; A, B, C, and D. Cells with a white background are significantly different.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>X</td>
<td>2.9 x 10^{-7}</td>
<td>1.4 x 10^{-9}</td>
<td>2.4 x 10^{-5}</td>
</tr>
<tr>
<td>B</td>
<td>X</td>
<td>X</td>
<td>1.1 x 10^{-5}</td>
<td>0.1</td>
</tr>
<tr>
<td>C</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>6.0 x 10^{-6}</td>
</tr>
<tr>
<td>D</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>
All of the aliquot data was pooled and a t-test was conducted to further show the significance of the difference between each sample. When comparing the t calculated values with the t table values, provided in table 5.3, all of the samples were significantly different except for samples B and D which was established previously by calculating both the f-values and p-values.

Table 5.3

*T - values for $\delta^{13}C$ values of CBN in four marijuana samples; A, B, C, and D. Cells with a white background are significantly different.*

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>X</td>
<td>47.42</td>
<td>28.39</td>
<td>10.10</td>
</tr>
<tr>
<td>B</td>
<td>X</td>
<td>X</td>
<td>9.88</td>
<td>1.65</td>
</tr>
<tr>
<td>C</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>6.52</td>
</tr>
<tr>
<td>D</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

The National Academy of Science has reported to Congress that they recommend forensic science communities in the United States to use statistical validation and confidence reporting due to the significant importance these factors present. Results involving samples with significantly different mean $\delta^{13}C$ values means clearly fall within the guidelines of statistical and confidence reporting. However, a significantly larger database of frequency of $\delta^{13}C$ values needs to be generated to determine the probability of two random samples having indistinguishable sample means.
5.4 Summary

The use of IRMS for the comparison of unknown samples of drugs has unlimited applications. The additional discriminatory factor that IRMS adds is unique in that the source of a drug sample can be matched or excluded as probable by looking at the differences in the number of neutrons. In addition, sample matching or exclusion, through the measurement and comparison of the samples $\delta^{13}C$ is possible based upon the strong correspondence between cannabinoids within the marijuana plant. The creation of a universal chemical fingerprint system for all the illicit drugs would prove to be invaluable to forensic investigators and law enforcement officials. The geographical origins of crops have already been studied and the use of this information has already proven its worth for the use of isotopic information that is available by using IRMS. The identification and tracking of illicit drug distribution routes should become far more focused and cost efficient.
6.1 Introduction

The current increase in home foreclosures in the United States, combined with the plunging real estate market, has lead to a significant rise in insurance fraud. According to Allstate Insurance Company spokesman Mike Siemienas, in California alone the state’s insurance division reports that the number of questionable residential fires in 2007 increased by 76 percent over 2006.\textsuperscript{142} According to Alabama Fire Inspector Chip Curreri, “We’ve seen a 400 percent increase up north involving houses that are just about to be foreclosed and people are collecting insurance on it.”\textsuperscript{143}

These troubling trends have engendered insurance industry concerns that homeowners will view arson as an escape from their financial troubles. A recent report by the industry-funded Coalition Against Insurance Fraud notes that with “untold thousands of homeowners struggling with ballooning subprime mortgage payments, fraud fighters are watching closely for a spike in arson by desperate homeowners who can no longer afford their home payments.”\textsuperscript{142} A myriad of improvements and enhancements to the instrumentation used for the identification of accelerants have occurred over the years. Indeed, the rapid identification of ignitable liquids in suspected arson fires is crucial to the timely apprehension of suspected arsonists. According to the National Fire Protection Association, 20% of all fires are caused by arsonists.\textsuperscript{144} Arson statistics between 1998 and 2007 are presented in Table 6.1.
Table 6.1

The number of fires, deaths, and dollar loss due to intentionally set structure fires that occurred from 1999 to 2008.

<table>
<thead>
<tr>
<th>Year</th>
<th>Fires</th>
<th>Deaths</th>
<th>Injuries</th>
<th>Direct Dollar Loss (In Millions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1999</td>
<td>523,000</td>
<td>3,040</td>
<td>18,525</td>
<td>$8,490</td>
</tr>
<tr>
<td>2000</td>
<td>505,500</td>
<td>3,535</td>
<td>19,600</td>
<td>$8,501</td>
</tr>
<tr>
<td>2001¹</td>
<td>521,500</td>
<td>3,220</td>
<td>17,225</td>
<td>$8,874</td>
</tr>
<tr>
<td>2002</td>
<td>519,000</td>
<td>2,775</td>
<td>15,600</td>
<td>$8,742</td>
</tr>
<tr>
<td>2003²</td>
<td>519,500</td>
<td>3,385</td>
<td>15,600</td>
<td>$8,678</td>
</tr>
<tr>
<td>2004</td>
<td>526,000</td>
<td>3,305</td>
<td>15,525</td>
<td>$8,314</td>
</tr>
<tr>
<td>2005</td>
<td>511,000</td>
<td>3,105</td>
<td>15,325</td>
<td>$9,193</td>
</tr>
<tr>
<td>2006</td>
<td>524,000</td>
<td>2,705</td>
<td>14,350</td>
<td>$9,636</td>
</tr>
<tr>
<td>2007</td>
<td>530,500</td>
<td>3,000</td>
<td>15,350</td>
<td>$10,638</td>
</tr>
<tr>
<td>2008</td>
<td>515,000</td>
<td>2,900</td>
<td>14,960</td>
<td>$12,360</td>
</tr>
</tbody>
</table>

Unfortunately, homes are not the only targets for arsonists as vehicular arson cases are also regrettably common. A recent case report by Service et al.¹⁴⁶ of a fire damaged Porsche proved that the owner set fire to the vehicle himself and later claimed that the heater had caused flames to ignite the car from the vents. When gas prices were climbing, an alarming percentage of vehicle fires were being reported when many were cases involving arson. According to John Eager, senior director of claims for the Property Casualty Insurers Association of America, “Incidents of arson, like other crimes, are frequently linked to an economic downturn. The reasons are many: not only do auto thieves try to destroy evidence but financially-strapped people try to get out of costly auto leases. Even farmers torch expensive farm equipment in an attempt to collect on their insurance policy.”¹⁴⁴

In cases where traditional analytical techniques fail, new analytical techniques and sample methods are needed to provide complementary information. One such
technique which we believe can assist in distinguishing between sources of accelerants is isotope ratio mass spectrometry (IRMS). IRMS is a specialized technique that is used to provide information about the geographic, chemical, and biological origins of substances.

Forensic arson investigators have long-recognized the need to distinguish between different sources of arson evidentiary material. Such determinations were formerly difficult or impossible in cases where two samples had chemically identical molecules. However, isotopic analysis now provides a means to look beyond the chemical composition of matter to the level of the nucleus. Naturally occurring stable carbon isotopic composition consists of $^{12}\text{C}$ that makes up 98.89% and $^{13}\text{C}$ making up the remaining 1.11% Thus, an analysis of the isotopic ratios of a substance’s carbon content reveals a unique isotopic profile which serves as a chemical fingerprint. Jasper et al. used an analogy of isotopic analysis to the combination on a combination lock which corresponded to a 1 in 1.4 billion chance that a random accelerant sample would be identical to that associated with a given arson event.76

Forensic analysis frequently extends well beyond the boundaries of the crime scene itself. Forensic evidence is often times, either knowingly or unwittingly, transported from and to the crime scene. The traces of ignitable liquids on a suspect’s clothing, in a vehicle, at a suspect’s home or work, in a transport container, or at a crime scene, could all be compared, thereby allowing investigators to form a possible source match. Unbeknownst to most individuals, the accelerants used as catalysts to start fires have distinctive isotopic profiles, thereby allowing investigators to match trace samples found within fire debris. The presence of pyrolytic products resulting from the
combustion of synthetic materials can cause complications in data interpretation. For example; new newspaper, newly lacquered furniture, paint spray, and paraffins in shoe polish can all be confused with mineral turpentine.\textsuperscript{148} According to a study by Fernandes et al.,\textsuperscript{148} volatile residues in burnt household items could not be mistaken for commonly used fire accelerants such as printed materials, adhesives, finishes, flooring, etc. In a study by Whyte et al.,\textsuperscript{149} it was clear that the viscosity of the accelerant, the porosity of the substrate, and the interaction between these two were key factors in deciding the resultant volatile organic compound profile after burning, as was the temperature the materials reached upon burning. It was also shown that the samples do not need to be fresh in order to be useful for analysis.\textsuperscript{149} Another concern addressed by Ren and Bertsch\textsuperscript{150} was the influence of water on the recovery of accelerants. Their results showed that water caused a slight shift toward larger molecular mass components. However, the overall influence was only moderate. A complete study on the influence of factors such as type of accelerant used, type of burned material, time between starting and extinguishing of the fire and availability of air on the possibility of detection of accelerant traces was completed by Borusiewicz et al.\textsuperscript{151} Their results showed that among the investigated factors, the kind of burned material was the most important factor influencing the recovery of ignitable liquid residues. A review was also written by Pert et al.\textsuperscript{152} on analytical techniques for arson residues, and a complete review of fire investigation and ignitable liquid residue analysis was conducted by Sandercock.\textsuperscript{153} Two and three dimensional GC and GC-MS were the most commonly used instrument for the detection of trace residues.
In the analysis presented here, the single quadrupole mass spectrometer provides the retention time data and characteristic fragmentation patterns used to confirm the identity of each compound in samples, while the IRMS simultaneously determines the carbon isotopic abundance ratios of each compound in the same sample. The ability to inject one sample and obtain results that simultaneously confirm the identity of the sample and provide the isotope ratio has quality assurance, cost- and time-saving benefits, but is not absolutely necessary; Laboratories frequently perform compound identification and compound-specific isotope ratio analysis on two different GC instruments.

The hyphenated technique of gas chromatography and mass spectrometry (GC/MS) has long been a gold standard in forensic science and serves as a category A, or confirmatory method according to SWGDRUG guidelines.95 The effectiveness of baseline resolution and peak identification decreases when fire debris pyrolysates have ions with the same mass as the target class.154 Here, we report the first forensic application of GC-MS-IRMS for weathered and unweathered arson accelerants and for identification of those ignitable liquid residues associated fire debris by use of a GC-MS coupled to an IRMS. For examples of products and their respective characteristic mass fragmental ions, a list of accelerant classifications can be seen in Figure 6.1.155
Figure 6.1 Accelerant Classification System Table

6.2 Materials and Methods

6.2.1 Chemicals

Samples of common household chemicals (other than class 2-3 distillates) which can be used as accelerants include: Goof Off (A & B), WD-40 (A & B), lighter fluid (A & B), and turpentine (A & B). Goof Off A, 125 ml (Lilly Industries, Inc., Grand Rapids, Michigan, Lot# E202E34904) was purchased at a local carpet store, Carpet One Floor and Moore, and Goof Off B, 474 ml (Lilly Industries, Inc., Grand Rapids, Michigan, Lot#A116E12215) was purchased at the local home improvement store, Lowes. WD-40, sample A, 56 g (WD-40 Company, San Diego, California, Item No. 10002, Lot# 4040M)
was purchased at a local home improvement store, Home Depot, and WD-40, sample B, 226 g (WD-40 Company, San Diego, California, Item No. 10008, Lot# 2343G) was purchased at a local home improvement store, Lowes. Turpentine A, 946 mL (WM Barr & Co., Inc., Memphis, Tennessee, Product No. QGT69, Lot# 804-166) was purchased at a local home improvement store, Lowes, and Turpentine B, 946 mL (PSC, Pearland, Texas, Lot# 804-01B) was purchased at a local home improvement store, Home Depot. Charcoal lighter fluid A, 946 mL (The Kingsford Products Co., Oakland, California, Lot# M28185A1313) was purchased at a local Wal-Mart and charcoal lighter fluid B, 946 mL (The Kingsford Products Co., Oakland, California, Lot# M25325B0834) was purchased at a local home improvement store, Lowes.

Four standard liquid samples were prepared by mixing 1 mL of the liquid samples with 9 mL Hexane (Sigma Aldrich, St. Louis, Missouri). Both the Goof Off and the WD-40 samples were further diluted to 1:1000 hexane.

An IRMS standard containing (C₁₁,C₁₅,C₂₀) 0.15 mg in 1 mL cyclohexane 0.15 mg/mL n-Undecane C₁₁, n-Pentadecane C₁₅, and n-Eicosane C₂₀ was used prior to each sequence run (Chiron International Standards, Laramie, Wyoming). CO₂ was referenced relative to the C₁₁ peak, as described in Chapter 4 (pg 74).

6.2.2 Simulated fire debris

Carpet samples were prepared by cutting ten – 5 cm x 5 cm squares and placing them into individual glass jars. The carpet samples were then doused into each of the individual accelerants. The excess accelerant was drained and the carpet sample was
ignited by a Bunsen burner in a Pyrex container in a fume hood. The carpet was allowed to burn until the samples self-extinguished. Most of the accelerants ignited relatively easily, but goof off generally required several attempts to start burning. All of the accelerants produced very sooty flames and left a large soot deposit on the glass jars used for the test burning. The backing of the carpet warped substantially owing to the heat, and the fibers of the carpet were either consumed entirely or melted onto the backing. Approximately 2 cm x 2 cm pieces of the burned material were cut from the 5 cm square carpet and placed into a glass vial. The 2 cm x 2 cm charred remains were extracted with 1.5 mL of hexane under sonication for 10 minutes. After sonication, the samples were vortexed for 5 minutes. After settling, the liquid was decanted from the top of the vial and placed in autosampler vials.

6.2.3 Gas Chromatography – Mass Spectrometry / Isotope Ratio Mass Spectrometry

Gas chromatography mass spectrometry analyses were performed using a single quadrupole mass spectrometer (HP5970B, Hewlett Packard (now Agilent Technologies), Santa Clara, California) and an isotope ratio mass spectrometer (Delta Plus Advantage, Thermo Finnigan, Waltham, Massachusetts) were used in combination with a gas chromatograph (Trace GC, Thermo Finnigan, Waltham, Massachusetts) equipped with an autosampler (AS3000, Thermo Finnigan, Waltham, Massachusetts). The GC column used was a DB-5 (30 m x 0.25 mm x 0.25 μm, J & W Scientific, Folsom California), the effluent was split using a low-dead-volume X-connector so that ~10% of the effluent flows to the single quadrupole mass spectrometer for structural elucidation and ~90%
flows to the IRMS for isotopic analysis. The column used from the X-connector and the transfer line of the single quadrupole mass spectrometer was a Fused Silica Deactivated column (0.100 mm x 1 m, Agilent Technologies, Santa Clara, California).

6.2.4 Operating Conditions

For Goof Off and WD-40, the GC oven temperature was programmed as follows: initial temperature 35°C (hold 5 min), program rate, 3°C/min final temperature 250°C (hold 5 min).

For Turpentine and Charcoal Lighter Fluid: The GC oven temperature was programmed as follows: initial temperature 35°C (hold 5 min), program rate, 3°C/min to a temperature 90°C (hold 0 min), 20°C/min final temperature 270°C (hold 1 min).

The injector temperature was set at 270°C and the helium carrier gas flow rate was 1.5 mL/min. A volume of 1 uL was injected in split mode (10:1) using the aforementioned autosampler. The auxiliary transfer line temperature was set at 280°C. In the full-scan mode, spectra were recorded over the range m/z 30-500, using the standard software for data analysis, ChemStation software (Hewlett Packard (now Agilent Technologies), Santa Clara, California). Individual components in the MS chromatograms were determined from searching in the NIST library and their identities were reported when the matching scores were satisfactory. For isotope ratio measurements, the isotope ratio mass spectrometer (Delta Plus Advantage, Thermo Finnigan, Waltham, Massachusetts) was used via a combustion interface (GC Combustion III, Thermo Finnigan, Waltham, Massachusetts). The combustion oven temperature was held at 940°C and the reduction oven temperature was held at 650°C.
Data acquisition was carried out using the standard software of the instrument, Isodat 2.0 Software (Thermo Finnigan, Waltham, Massachusetts).

In carbon GC-IRMS, ion chromatograms of three isotopic peaks are collected by three individual ion collectors with different sensitivities, i.e. m/z 44 (\(^{12}\text{C}^{16}\text{O}^{16}\text{O}\)), the isobaric ions m/z 45 (\(^{13}\text{C}^{16}\text{O}^{16}\text{O}\)) and (\(^{12}\text{C}^{16}\text{O}^{17}\text{O}\)), recorded with 100-fold sensitivity relative to m/z 44, and m/z 46 (\(^{12}\text{C}^{16}\text{O}^{18}\text{O}\)); recorded with 300-fold sensitivity relative to m/z 44.\(^{105,106}\) At the beginning of each run, three pulses of CO\(_2\) reference gas were admitted into the inlet system for 20 s. The run begins with three injections of the CO\(_2\) reference gas standard, of which the second CO\(_2\) peak, shown in Figure 6.2, is designated as the standard in the instrument acquisition software. The ratios of these isotopes are always measured relative to an isotopic standard so as to eliminate any bias or systematic error in the measurements. These standards are linked to internationally recognized standards such as Vienna Pee Dee Belemnite (VPDB) for carbon, Vienna Canyon Diablo Troilite meteorite (V-CDT) for sulfur, Vienna Standard Mean Ocean Water (VSMOW) for oxygen and hydrogen, and laboratory air for nitrogen.\(^2\)
Figure 6.2 Example of an IRMS chromatogram for one injection of an accelerant sample, Turpentine (B). The second CO$_2$ peak is designated as the internal standard in the instrument acquisition software.

To improve the quality of the measurement and eliminate any error due to misbalance, each sample was adjusted so that the signal amplitude was as close to the CO$_2$ reference gas peak as possible. When the standard and sample are run at significantly different intensities, the linearity of the instrument results is small but significant absolute differences in the measured $\delta^{13}$C values\textsuperscript{107}

6.3 Results and Discussion

6.3.1 Standard Accelerants

Goof Off samples had three distinctive peaks that were analyzed in order to establish the likelihood of a common origin. The single quadrupole confirmed the
identity of these three peaks as ethylbenzene (peak 1), p-Xylene (peak 2), and o-Xylene (peak 3) using the NIST database shown in Figure 6.3.

Figure 6.3  TIC chromatogram and NIST head to tail library output of Goof Off (A).

A typical chromatogram obtained on the IRMS is shown in Figure 6.4. Five aliquots of each sample were each analyzed four times. The δ^{13}C values and error showing the 95% CI for each peak in sample A were -31.39‰ ±0.98 (peak 1), -33.94‰ ±1.21 (peak 2), and -37.39‰ ±0.85 (peak 3). The δ^{13}C values and 95% CI for each peak in sample B were –31.22‰ ±0.84 (peak1), -34.13‰ ±0.98 (peak2), and -38.62‰ ±0.72 (peak 3).
Figure 6.4  Example of an IRMS chromatogram for one injection of an accelerant sample, Goof Off (A).

ANOVA tests were then performed on all three peaks of the two samples of Goof Off to determine any significant difference between the two sample means. $F_{\text{calc}}$ values for each peak were 0.06, 0.06, and 3.80. Because the $F_{\text{crit}}$ value at a 95% CI for all three peaks was 4.10, the samples could not be distinguished at 95% C.L. Figure 6.5 displays the results of the three peaks analyzed.
Figure 6.5  Bar graph showing $\delta^{13}$C for 3 major peaks from Goof Off samples A & B with error bars representing a 95% C.I.

WD-40 samples A and B had three major peaks eluting at 1180 s, 1450 s, and 1719 s, and several minor peaks. Peaks that were either not well resolved or that had an intensity less than 1000 mV were not used for comparisons. The single quadrupole confirmed the identity of these three peaks as Nonane (peak 1), Decane (peak 2), and Undecane (peak 3) using the NIST database shown in Figure 6.6.
Figure 6.6  TIC chromatogram and NIST head to tail library output of WD-40 (B).

The $\delta^{13}$C values and 95% CI for each peak in sample C were $-40.08\%\pm1.33$ (peak 1), $-29.64\%\pm0.49$ (peak 2), and $-31.03\%\pm0.32$ (peak 3), respectively. The $\delta^{13}$C values and 95% CI for each peak in sample D were $-35.29\%\pm1.14$ (peak 1), $-29.07\%\pm0.53$ (peak 2), and $-28.40\%\pm0.18$ (peak 3), respectively, as seen in Figure 6.7.
Figure 6.7  Example of an IRMS chromatogram for one injection of an accelerant sample, WD-40 (B). Peak 1 (902 s) is Nonane, peak 2 (1181 s) is Decane and peak 3 (1455 s) is Undecane.

ANOVA tests were then performed on all three peaks of the two samples of WD-40 to establish the probability that the mean values were significantly different. Figure 6.8 shows the results of all three peaks analyzed.

F_{calc} values for each peak were 28.60, 2.35, and 199.36. Because the F_{crit} value at a 95% C.L. for all three peaks was 4.10, peaks 1 and 3 establish that the samples are significantly different at the 95% C.L. and therefore not likely to be from a common origin.
Figure 6.8  Bar graph showing $\delta^{13}\text{C}$ for 3 major peaks from WD-40 samples A & B with error bars representing a 95% C.I.

Turpentine samples also had three major constituents. The three main components of turpentine are ethylbenzene (peak 1), $\alpha$-pinene (peak 2), and camphene (peak 3) seen in Figure 6.9. The identity was established from NIST database and from EI-MS spectra on the single quadrupole in real time.
Figure 6.9  TIC chromatogram and NIST head to tail library output of Turpentine (B).

The δ^{13}C values and 95% CI for each peak in sample A was -35.33‰ ±0.42 (peak 1), -30.69‰ ±0.17 (peak 2), and -32.42‰ ±0.34 (peak 3). The δ^{13}C values and 95% CI for each peak in sample B was -34.75‰ ±0.14 (peak 1), -28.91‰ ±0.11 (peak 2), and -31.10‰ ±0.17 (peak 3), as seen in Figure 9.10.
**Figure 6.10** Example of an IRMS chromatogram for one injection of an accelerant sample, Turpentine (B). Peak 1 (795 s) is Ethylbenzene, Peak 2 (842 s) is α-pinene and peak 3 (883 s) is Camphene.

ANOVA tests were performed on all three peaks of the two samples of Turpentine to establish the sources of error and any significant differences between the sample means. F\(_{\text{calc}}\) values for each peak were 6.25, 292.73, and 43.31. Because the F\(_{\text{crit}}\) value, at a 95% C.L. for all three peaks was 4.12, the samples were most likely not from the same source according to all three of the peaks analyzed, as displayed in Figure 6.5.
Charcoal lighter fluid samples A and B also had three major peaks as well as numerous minor peaks. We did not evaluate peaks with an intensity less than 1000 mV on the IRMS. The results were similar to the identity of WD-40 with the identity of the three peaks as Nonane (peak 1), Decane (peak 2), and Undecane (peak 3) using the NIST database shown in Figure 6.12. The $\delta^{13}$C values and 95% CI for each peak in sample A were $-27.35\% \pm 0.90$ (peak 1), $-25.48\% \pm 0.26$ (peak 2), and $-26.91\% \pm 0.38$ (peak 3). The $\delta^{13}$C values and 95% CI for each peak in sample B were $-28.39\% \pm 1.21$ (peak 1), $-24.93\% \pm 0.31$ (peak 2), and $-25.54\% \pm 0.45$ (peak 3).
Figure 6.12  Example of an IRMS chromatogram for one injection of an accelerant sample, Charcoal lighter fluid (B). Peak 1 (592 s) is Nonane, Peak 2 (856 s) is Decane and peak 3 (1134 s) is Undecane.

ANOVA tests were performed on all three peaks of the two samples of charcoal lighter fluid. $F_{\text{calc}}$ values for each peak were 1.60, 6.43, and 17.65. The $F_{\text{crit}}$ values were 4.54, 4.38, and 4.97 respectively at 95% C.L. for all three peaks. Comparison of peaks 2 and 3 establish that the samples are significantly different at the 95% C.L. and therefore not likely from a common origin, as displayed in Figure 6.13. These results were presented at the Ohio University Research and Creative Activity Fair, May 2009 the poster of which can be found in the appendices as Appendix D.1.
Figure 6.13 Bar graph showing $\delta^{13}$C for 3 peaks from Charcoal Lighter Fluid samples A & B with error bars representing a 95% C.I.

6.3.2 Scorched Samples

When accelerants are found on a suspect, it is important to have a means to compare a known non-scorched sample to a scorched sample that may have been found at the scene. Here, we simulated arson by scorching a piece of carpet as described in detail in the experimental section.

To compare the results of the turpentine simulated fire debris samples A(scorched) and B(scorched) and the liquid samples A and B, F_{calc} values were compared to F_{crit} values. In Table 6.2, cells with white backgrounds are significantly different and
the cells with gray backgrounds are not. Results show that there is no significant
difference between non-scorched and partially scorched samples even after partial
combustion. Residues of samples A and B can be distinguished from each other as having
significantly different $\delta^{13}C$ values for $\alpha$-pinene.

Table 6.2

*F*-calculated values for four accelerant samples; A, B, A(scorched), and B(scorched). Cells with a white background are significantly different at the 95% C.L.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>A (scorched)</th>
<th>B (scorched)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>X</td>
<td>227.39</td>
<td>5.51</td>
<td>77.24</td>
</tr>
<tr>
<td>B</td>
<td>X</td>
<td>X</td>
<td>1916.54</td>
<td>7.40</td>
</tr>
<tr>
<td>A (scorched)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>72.47</td>
</tr>
<tr>
<td>B (scorched)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

To reinforce the results and exclude two samples of turpentine as having a
common origin or source, the probability values (P-values) are provided in Table 6.3. If
the P-values are less than 0.05, the results indicate that the difference in the two sample
means is statistically significant at the 95% CL.

Table 6.3

*P*-values for four accelerant samples; A, B, A(scorched), and B(scorched). Cells with a white background are significantly different

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>A (scorched)</th>
<th>B (scorched)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>X</td>
<td>$1.1 \times 10^{-4}$</td>
<td>0.08</td>
<td>$9.3 \times 10^{-4}$</td>
</tr>
<tr>
<td>B</td>
<td>X</td>
<td>X</td>
<td>$1.6 \times 10^{-6}$</td>
<td>0.05</td>
</tr>
<tr>
<td>A (scorched)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>$1.0 \times 10^{-3}$</td>
</tr>
<tr>
<td>B (scorched)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>
The bar graph in Figure 6.7 of the average of A and B compared to the scorched samples A and B are displayed so that the reader can visualize the differences or similarities in the samples, with error bars spanning the 95% confidence level (N=3).

*Figure 6.14* Average $\delta^{13}C$ values of turpentine aliquots; ($\alpha$-pinene sample A and scorched sample A (scorched)) and ($\alpha$-pinene sample B, and scorched sample B (scorched)) with error bars showing 95% C.I.

6.4 Summary

IRMS has shown the ability to be a reliable and accurate method that can be used concurrently with a single quadrupole mass spectrometer to provide both a class A confirmatory analysis and a comparison analysis. In the absence of the IRMS detector, the samples shown herein likely could not be distinguishable as being from different sources. The IRMS provides a very straightforward and easily-comprehensible
difference between two samples as well as a probability scores for the comparison of sample means.

We believe this technique would be useful when comparing trace or questioned samples found at crime scenes with bulk or known samples. Fire investigators, insurance fraud agents, homeland security, FBI, DEA, and many other law enforcement agencies routinely require rapid confirmation as to whether the scene of a fire was indeed arson and who the arsonist may be. This technique offers a way to determine if an accelerant was used, which accelerant it was, and to establish potential differences between samples that are chemically and physically indistinguishable by any other means. The additional information gained by analyzing and comparing trace samples with bulk samples will ultimately enable law enforcement officials to better focus their investigations during cases of suspected arson.
CHAPTER 7: MEASUREMENT OF $^{13}$C/$^{12}$C ISOTOPIC COMPOSITION OF INDIVIDUAL AMINO ACIDS IN HUMAN HAIR FOR FORENSIC COMPARISONS

7.1 Introduction

Human hair is a very important class of forensic evidential material collected in relation to crimes. An average human scalp has between 175-300 terminal hairs per square centimeter and the typical person loses between 50-100 hairs naturally per day. During a struggle or altercation, additional hair is often lost through forcible removal. For comparison of questions (Q) and known (K) hair samples, DNA analysis is by far the method of choice. For chromosomal DNA analysis, a hair sample must either be plucked or shed so that the DNA can be extracted from the root or the root sheath material.

In the absence of preferred chromosomal DNA, mtDNA may also be extracted from the hair shaft for maternal heredity comparisons. In the absence of DNA, microscopy can help discriminate between crude classes of hair types (blonde vs. black, afro-Caribbean vs. Caucasian, etc.) but may not discriminate within a sub-class of hair type. The absence of DNA samples in hair occurs more frequently than one would probably expect and, in a case such as this, there is a need to find other means of testing. Among attempts to discriminate hair samples based on chemical techniques, the most common approach is to analyze trace metal impurities in the hair shaft, such as with SS-MS, LA-ICP-MS, ICP-OES, and AAS.

Isotope ratio mass spectrometry is a very sensitive method that can be used to discriminate between samples that have identical chemical composition by looking into the nucleus of each sample and detecting the differences in their neutrons. The isotopic
carbon content of hair has previously been studied many times using EA-IRMS on the contemporary man,\textsuperscript{162,163} mummies,\textsuperscript{164} and even the ice man.\textsuperscript{165} EA-IRMS uses the total average of the whole hair sample and does not separate the various components of hair prior to analysis. Human hair consists of approximately 65-95\% proteins.\textsuperscript{166} The intercellular layer consists of \textasciitilde 12\% basic amino acids and \textasciitilde 17\% acidic amino acids.\textsuperscript{166} Other publications have looked at how diet affects the carbon and nitrogen content in individual amino acids in hair.\textsuperscript{163} Petzke et al.\textsuperscript{167-169} found that it was possible to distinguish between vegetarians and omnivores by looking at the $^{13}\text{C}$ and $^{15}\text{N}$ abundances in hair protein. Mekota et al. studied the effect on stable nitrogen and carbon isotopes in hair of patient’s starvation and recovery phases of patients suffering from anorexia nervosa.\textsuperscript{170} Once produced, hair, nail, and bone proteins are remarkably stable; they therefore preserve the significance of dietary habits like no other soft-tissue proteins.

Of the most abundant hair proteins, the most common N-terminal amino acids consist of valine, threonine, glycine, alanine, serine, glutamic acid, and aspartic acid. C-terminal amino acids consist of threonine, glycine, alanine, serine, glutamic acid, and aspartic acid. There are various factors that can create differences in amino acid analysis, such as genetics, weathering (exposure to sunlight), cosmetic treatments, experimental procedures, and diet. Clarence Robins wrote a detailed book discussing such factors and their influence on hair.\textsuperscript{166} The goal of this project is to develop a method for derivatizing and analyzing individual amino acids to enable comparison of Q and K hair samples.
7.2 Materials and Methods

7.2.1 Subjects

The collection of hair samples for the following experiments was approved by the Ohio University Institutional Review Board (IRB # 09X194); the approval form can be found in the appendices as Appendix E.1. All hair samples were donated by volunteers for use in our laboratory for forensic studies who were given a consent form to sign and a complete detailed explanation of the research study which can be found in the appendices as Appendix E.2.

7.2.2 Reagents and Standards

The six amino acids that were used for a standard were L-alanine, Glycine, L-Histidine, L-(+)-Lysine, L-Proline, and L-Tyrosine all purchased from Sigma-Aldrich (St. Louis, MO). The derivatizing agent was N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + trimethylchlorosilane (TMCS), 99:1 purchased from Supelco Analytical (Bellefonte, PA). The solvent used was Acetonitrile HPLC Grade purchased from EMD Chemicals (Gibbstown, NJ). The acid hydrolysis agent was Hydrochloric acid, 37% reagent (ACS) grade purchased from GPS Chemicals (Columbus, OH).
7.2.3 Derivatization of Standards

Approximately 80 mg of each of the six amino acid standards were individually weighed into 2 mL silanized amber vials. 1 mL of BSTFA + TMCS was added to each of the samples with an additional 1 mL of acetonitrile. The samples were then heated for 30 minutes on a hot plate at 70°C. 1 uL of the solution was then injected into the injection port using an AS 3000 autosampler (Thermo Scientific, Waltham, MA).

All the previously published articles on derivatization of amino acids derivatize hair to N-pivaloyl-i-propyl esters and this process is relatively complicated and time-consuming. We are the first to use a trimethylsilyl donor derivative, N,O-bis(trimethylsilyl)trifluoroacetamide plus trimethylchlorosilane (BSTFA + TMCS) for compounds specific δ13C analysis of amino acids in human hair.

7.2.4 Derivatization and Pulverization of Hair

Each hair sample was washed two times with water and then once with methanol. The individual hair samples were vortexed for approximately 1 minute in each solvent to remove surface residues/contaminants and then dried under a stream of nitrogen to ensure that no water remained before derivatization.171 Hair samples, approximately 100 mg, were pulverized using a pulverizing method used in our laboratory.172 The hair was placed into a capped plastic vial, 6-8 stainless steel ball bearings were added, and the vial was violently agitated back and forth for 5 minutes using a Mini Beadbeater (Biospec
Products Inc., Bartlesville, OK) at 4800 rpm. The ball bearings were then removed and the fine powder was transferred into a 10 mL glass vial.

Hair sample free amino acids are obtained from proteins by using strong acid hydrolysis. Each hair sample was weighed out in 80 mg samples and 5 mL of Concentrated HCl were added to the hair. The solution was then put into an oven for 16 hours at 110°C. After hydrolysis, the liquid sample was filtered to remove any particulates that may have been remaining. The filtered sample was dried under a constant stream of Nitrogen. 2 mL of BSTFA + TMCS and 1 mL of acetonitrile were then added to the sample and heated for 30 minutes at 70°C. 150 μL of the sample was transferred into a glass autosampler vial using a pipette and placed into the autosampler carousel to await injection.

7.2.5 Gas Chromatography – Mass Spectrometry / Isotope Ratio Mass Spectrometry

The gas chromatography mass spectrometry analyses were preformed using a single quadrupole mass spectrometer (HP5970B, Hewlett Packard (now Agilent), Santa Clara, California) and an isotope ratio mass spectrometer (Delta Plus Advantage, Thermo Finnigan, Waltham Massachusetts). These instruments were then used in combination with a gas chromatograph (Trace GC, Thermo Finnigan, Waltham, Massachusetts) equipped with an autosampler (AS3000, Thermo Finnigan, Waltham Massachusetts). A typical cycle time for the GC-IRMS was 65-70 minutes and samples were placed on a 100 sample wheel which allowed for unattended sample analysis. The GC column was a TRACE™ TR-5MS 5% Phenyl Polysilphenylene-siloxane (60 m x 0.25 mm x 1.0 μm,
Thermo Finnigan, Waltham Massachusetts). The effluent was split using a low-dead-volume X-connector (Valco Instruments Co. Inc., Houston, Texas) so that approximately 10% of the effluent flowed to the single quadrupole mass spectrometer for structural elucidation and approximately 90% flowed to the IRMS for isotopic analysis.

The GC oven temperature was programmed as follows: initial temperature 70°C (hold 9.0 min); program rate, 3°C/min final temperature 300°C (hold 8.0 min). The injector temperature was set at 280°C and the helium carrier gas flow rate was 1.0 mL/min. A volume of 1 μL was injected in splitless mode using the aforementioned autosampler. The temperature of the auxiliary transfer line to the single quadrupole was set at 280°C.

Total ion chromatograms were recorded between the ranges of m/z 50-550 using HP Chemstation B.02.05 (Hewlett Packard (now Agilent), Santa Clara, California). The data was exported as a text file for comparison with the NIST mass spectral library (US Secretary of Commerce, USA) to confirm identify of the individual amino acids of interest.

Isotope ratio measurements were conducted on an isotope ratio mass spectrometer (Delta Plus Advantage, Thermo Finnigan, Waltham, Massachusetts) via a combustion interface (GC Combustion III, Thermo Finnigan, Waltham, Massachusetts). The combustion oven temperature was held at 940°C and the reduction oven temperature was held at 650°C. Data acquisition was carried out using Isodat 2.0 Software (Thermo Finnigan, Waltham, Massachusetts).
7.3 Preliminary Results

Compound specific isotope measurements of individual amino acids in hair are a relatively new and uncommon development. Through the use of BSTFA + TMCS 90:1 as a derivatizing agent we were able to separate nine individual amino acids; alanine, valine, leucine, isoleucine, serine, aspartic acid, proline, tyrosine, and cystine. In addition to establishing the analysis method, we performed preliminary comparison of six different hair samples to validate that the method works on a variety of hair types and that the error in analysis is small enough to permit discrimination between subjects. Glycine could not be resolved isotopically due to the presence of an interference peak, L-Proline. The standard deviation of each individual amino acid within the derivatized samples of individual amino acids in hair ranged between 0.173‰ and 3.950‰. The average carbon isotopic values of six samples with nine individual amino acids are listed in Table 7.1. The error at the 95% C.I. for the nine amino acids using BSTFA + TMCS as the derivatizing agent for the replicate GC-IRMS analyses can be found in Table 7.2.
Table 7.1

Carbon isotope ratios of individual amino acids as established by derivatization GC-C-IRMS (N=3)

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>Alanine $\delta^{13}$C (%)</th>
<th>Valine $\delta^{13}$C (%)</th>
<th>Leucine $\delta^{13}$C (%)</th>
<th>Isoleucine $\delta^{13}$C (%)</th>
<th>Serine $\delta^{13}$C (%)</th>
<th>Aspartic Acid $\delta^{13}$C (%)</th>
<th>Proline $\delta^{13}$C (%)</th>
<th>Tyrosine $\delta^{13}$C (%)</th>
<th>Cystine $\delta^{13}$C (%)</th>
</tr>
</thead>
</table>

Table 7.2

Error at 95% CI of individual amino acids as established by derivatization GC-C-IRMS (N=3)

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>Alanine $\delta^{13}$C (%)</th>
<th>Valine $\delta^{13}$C (%)</th>
<th>Leucine $\delta^{13}$C (%)</th>
<th>Isoleucine $\delta^{13}$C (%)</th>
<th>Serine $\delta^{13}$C (%)</th>
<th>Aspartic Acid $\delta^{13}$C (%)</th>
<th>Proline $\delta^{13}$C (%)</th>
<th>Tyrosine $\delta^{13}$C (%)</th>
<th>Cystine $\delta^{13}$C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol</td>
<td>0.468</td>
<td>0.661</td>
<td>0.972</td>
<td>0.732</td>
<td>0.634</td>
<td>0.477</td>
<td>0.180</td>
<td>0.675</td>
<td>0.240</td>
</tr>
<tr>
<td>A0009</td>
<td>0.374</td>
<td>0.325</td>
<td>0.604</td>
<td>0.721</td>
<td>0.381</td>
<td>0.528</td>
<td>0.232</td>
<td>0.377</td>
<td>2.973</td>
</tr>
<tr>
<td>A0016</td>
<td>2.297</td>
<td>1.079</td>
<td>0.823</td>
<td>0.573</td>
<td>0.380</td>
<td>1.144</td>
<td>1.009</td>
<td>0.882</td>
<td>1.278</td>
</tr>
<tr>
<td>A0018</td>
<td>2.926</td>
<td>1.214</td>
<td>0.339</td>
<td>0.801</td>
<td>0.898</td>
<td>0.881</td>
<td>0.453</td>
<td>0.445</td>
<td>3.898</td>
</tr>
<tr>
<td>A0019</td>
<td>0.447</td>
<td>0.458</td>
<td>0.420</td>
<td>0.726</td>
<td>0.436</td>
<td>0.393</td>
<td>0.323</td>
<td>0.368</td>
<td>1.638</td>
</tr>
<tr>
<td>A0021</td>
<td>0.621</td>
<td>1.295</td>
<td>0.199</td>
<td>0.000</td>
<td>0.292</td>
<td>0.455</td>
<td>0.123</td>
<td>0.000</td>
<td>0.428</td>
</tr>
</tbody>
</table>
The identity of each amino acid was confirmed in real-time using the concurrent EI single quadrupole mass spectrometer. The single quadrupole also helped to identify co-eluting peaks not identifiable on the IRMS instrument. A typical EI-MS chromatogram is shown in Figure 7.1 and the concurrent IRMS profile is shown in Figure 7.2.

*Figure 7.1* TIC chromatogram and mass spectra for a single injection of hair sample A0009 displaying individual amino acids.
Figure 7.2 Concurrent IRMS output acquisition for the simultaneous injection of hair sample A0009 displaying individual amino acids.

These preliminary results suggest that it would be possible to separate individual amino acids within hair by using a one-step derivatization method. The nine amino acids that were separated had good chromatographic resolution as can be seen in Figure 7.2. To visualize the difference between the samples, bar graphs of the amino acids from each hair sample can be seen in Figures 7.3-7.5.
Figure 7.3  Comparison of $\delta^{13}C$ values for six hair samples showing the results of Alanine, Valine, and Leucine with 95% C.I. (N=3).
Figure 7.4  Comparison of $\delta^{13}C$ values for six hair samples showing the results of Isoleucine, Serine, and Aspartic Acid with 95% C.I. (N=3).
In our future studies hair samples will be segmented so that month to month hair growth analysis of the carbon isotopes of individual amino acids will be analyzed. The hair samples will not be as large and determination of an individual’s hair can be documented so that it is possible to compare questioned hair samples with known samples. Eventually, by comparing the month to month hair segments, a time frame could also be established by looking at the hair growth. For example Figure 7.6 shows a hypothetical hair collected at a crime scene that was segmented from month to month by centimeters from the root. The individual amino acid Valine was used as an example of how amino acid δ^{13}C values could be matched to a suspected hair sample three months
later. By looking at the individual amino acids within the individual hair samples months’ growth it would be possible to determine whether or not the suspected hair could have come from the same source by looking at the carbon isotopes.

![Graph showing δ13C values for different amino acids](image)

**Figure 7.6** Hypothetical example of how segmented analysis of δ13C of amino acids in hair could be used to establish potential common origins and time since shedding. Sample Q1 came from the same donor as K1, but with a three-month offset. K2 came from a different donor. Other amino acids could be used to confirm or relate the positive comparison.

### 7.4 Summary

We have shown that it is possible to determine the individual amino acids within hair samples using a single step derivatization method. With this type of information, it would be possible to link Q hair samples with known samples. The obvious downfall to
the way in which we collected samples is that the quantity was very large. In the future, we would like to study single strands of hair and the possibilities of segmenting the hair into sections of monthly growth, as previously described. After determining the section that relates for a specific time period, we could then study the individual sections of the hair to determine how the amino acids change over time during the growth of the hair. Because the amino acids are known to change with variations in diet and health, the comparison of segmented $\delta^{13}C$ amino acid samples could even provide a determination of time period between when a hair was shed to the present day. (Assuming hair from present day is a continuation of hair from Q time period).
CHAPTER 8: CONCLUSION AND FUTURE WORK

The benefits of having a dual detector system for analyzing forensic samples has been shown successfully in our studies with several different applications. The ability to distinguish between samples by looking at the number of neutrons within the nucleus adds tremendous discriminatory power. This discriminatory power enhances the class A confirmatory analysis of gas chromatography - mass spectrometry by providing an additional isotope ratio mass spectrometric analysis. Because IRMS is well documented in the scientific literature, is commonly used in other scientific fields of study, and has precedent for admission in civil and criminal cases, it clearly exceeds Daubert requirements for admissibility in court.

The most influential part of our instrument modification is the ability to inject one sample, one time and get two simultaneous results. The benefits of this single injection method includes the lowering of sample preparation time and reduced sample amounts, a reduction in the risk of error due to having to prepare two samples, along with the shear simplicity of using one autosampler and getting two results. We believe this technique would be useful when comparing trace questioned samples found at crime scenes or on suspects and victims with bulk samples from known sources. Through a second injection of each sample, it would be possible to obtain N, H, or O isotope analysis, which could add a greater degree of discriminating power in establishing or excluding potential common sources of drugs or amino acids.

Future benefits of IRMS in the realm of drug control includes the ability to link trace amounts of controlled or illegal drugs to bulk sources, leading eventually to the
determination of trafficking routes. Also in the future, IRMS will be an asset in providing additional information as to the geographic locations of crops being grown and harvested. The geographical origins of crops have already been studied and of this information has already proven its worth for the use of isotopic information that is available by using IRMS, the identification and tracking of illicit drug distribution routes should become far more focused and cost efficient.

The creation of a universal chemical fingerprint system for all illicit drugs would prove to be invaluable to forensic investigators and law enforcement officials. If databases could be developed for frequency distribution of isotope ratios of interest for high-frequency or high-profile applications such as burglary, homicide, and drug cases, and if instrument vendors could improve reliability and robustness, then regional, state, and local crime labs should be able to make a strong case for implementation of IRMS in their labs.

Carbon isotopic analysis has become an important forensic tool that can be used to analyze various forensic samples. Here we have used IRMS to uniquely identify isotopes of individual amino acids within hair samples. We were able to use a one step derivatization method to separate nine individual amino acids to be used for both isotopic analysis and mass spectral identification through the NIST database. With this information it could be possible to analyze the month-to-month growth of hair to determine monthly carbon isotopic change, for use in identifying and comparing hair samples. With this type of analysis, if DNA was not attainable, it would be possible to link hair from a crime scene to the suspect, even after an extended period of time has
passed. We plan to further study individual amino acids in hair and expand on the differences between derivatizing agents to determine the best separation and resolution.

We have shown how versatile and valuable a GC coupled to two different detectors can be for fire investigators, insurance fraud agents, homeland security, FBI, DEA, and many other law enforcement agencies. The possibilities of IRMS are virtually unlimited as far as forensic sample analysis is concerned. There is unlimited potential offered by the use of IRMS with equally unlimited applications for both non-volatile and volatile compounds.
Appendix A.1 GC-IRMS Standard (C\textsubscript{11}, C\textsubscript{15}, C\textsubscript{20})

**GRAVIMETRIC CERTIFICATE**

**General Product Data:**
- **Product Name:** GC-IRMS Standard (C\textsubscript{11}, C\textsubscript{15}, C\textsubscript{20})
- **Product No.:** 0451.3-150-CY

**Batch Specific Data:**
- **Batch No.:** 1222

**Product description:**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Prod no.</th>
<th>CAS no.</th>
<th>Batch no.</th>
<th>$\delta^{13}$C</th>
<th>Purity</th>
<th>Analyte conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Undecane C\textsubscript{11}</td>
<td>0414.11</td>
<td>1120-21-4</td>
<td>0876</td>
<td>-26.11</td>
<td>99.1%</td>
<td>0.15 mg/mL</td>
</tr>
<tr>
<td>n-Pentadecane C\textsubscript{15}</td>
<td>0418.15</td>
<td>629-62-9</td>
<td>0880</td>
<td>-30.22</td>
<td>99.9%</td>
<td>0.15 mg/mL</td>
</tr>
<tr>
<td>n-Elcorane C\textsubscript{20}</td>
<td>0423.20</td>
<td>112-95-8</td>
<td>0885</td>
<td>-33.06</td>
<td>99.5%</td>
<td>0.15 mg/mL</td>
</tr>
</tbody>
</table>

Solvent: Cyclohexane, p.a., >99.5\%

Tolerance: The uncertainty in the preparation of this standard is less than ± 1%

Quantity: 1 mL

Storage: Dark and cool

Expiry date: Guaranteed 1 year from date of issue

Trondheim, 06 July 2007

Issued by: Kine Nielsen

Approved by: Inge Fenstad
# Appendix A.2 GC-IRMS Standard (C\textsubscript{25}, C\textsubscript{30}, C\textsubscript{36})

## GRAVIMETRIC CERTIFICATE

### General Product Data:
- **Product Name:** GC-IRMS Standard (C\textsubscript{25}, C\textsubscript{30}, C\textsubscript{36})
- **Product No.:** 0453.3-150-CY

### Batch Specific Data:
- **Batch No.:** 5408

### Product description:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Prod. no.</th>
<th>CAS no.</th>
<th>Batch no.</th>
<th>δ\textsubscript{13}C</th>
<th>Purity</th>
<th>Analyte conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Pentacosane C\textsubscript{25}</td>
<td>0401.25</td>
<td>629-89-2</td>
<td>0886</td>
<td>-28.21</td>
<td>99.7 %</td>
<td>0.15 mg/mL</td>
</tr>
<tr>
<td>n-Triacontane C\textsubscript{30}</td>
<td>0408.30</td>
<td>638-68-6</td>
<td>0889</td>
<td>-28.60</td>
<td>98.4 %</td>
<td>0.15 mg/mL</td>
</tr>
<tr>
<td>n-Hexatriacontane C\textsubscript{36}</td>
<td>0410.36</td>
<td>630-66-8</td>
<td>0874</td>
<td>-26.15</td>
<td>97.2 %</td>
<td>0.15 mg/mL</td>
</tr>
</tbody>
</table>

- **Solvent:** Cyclohexane, p.a. >99.5 %
- **Tolerance:** The uncertainty in the preparation of this standard is less than ±1 %
- **Quantity:** 1 mL
- **Storage:** Dark and cool
- **Expiry date:** Guaranteed 1 year from date of issue

Trondheim, 06 July 2007

**Issued by:** Kine Nielsen

**Approved by:** Inge Fenstad
Appendix B.1 Identification and Individualization of Cocaine Samples Using Gas Chromatography Coupled Simultaneously to a Quadrupole Mass Spectrometer and an Isotope Ratio Mass Spectrometer

IDENTIFICATION AND INDIVIDUALIZATION OF COCAINE SAMPLES USING GAS CHROMATOGRAPHY COUPLED SIMULTANEOUSLY TO A QUADRUPOLE MASS SPECTROMETER AND AN ISOTOPE RATIO MASS SPECTROMETER

B.1 Introduction

The confirmatory identification of controlled substances is one of the most common and important tasks of a forensic drug analyst. It is therefore important that undergraduate students receive education on, and experience with, the instrumentation and scientific principles with which the separation and identification of substances are performed. The hyphenated technique of gas chromatography and mass spectrometry (GC/MS) has long been a gold standard in forensic science and serves as a category A, or confirmatory, method according to SWGDRUG guidelines.

This laboratory exercise was introduced for the first time in the forensic chemistry laboratory class (CHEM 487B) at Ohio University in the Spring quarter, 2008, a capstone course for our forensic chemistry majors. The lab exercise is applicable to other upper-level chemistry or forensic science majors and teaches students how to a) handle forensic evidence in a manner consistent with that expected in a crime laboratory setting, b) perform a confirmatory test for the presence of cocaine, and c) simultaneously with step b, perform an isotope ratio comparison of the $^{13}\text{C}/^{12}\text{C}$ ratios of cocaine to establish individualization or potential common sources. This experiment requires very specialized instrumentation (an isotope ratio mass spectrometer with a combustion interface) which,
although not commonly present in most chemistry departments, is often accessible through mass spectrometry facilities or in departments such as the geological or biological sciences.

In our laboratory, the effluent from the GC is split via a low-dead-volume T-connector so that approximately 10% of the effluent flows to the single quadrupole mass spectrometer for structural elucidation and ~90% flows to the IRMS for isotopic analysis. The ability to singly inject only one sample and have results that simultaneously confirm the identity of the sample and provide the isotope ratio has many benefits, but is not absolutely necessary. Most laboratories perform compound identification and compound-specific isotope ratio analysis on two different GC instruments, which would be a simple modification to the experiment.

Researchers have shown that IRMS can be used to assign the geographical origins of illicit cocaine and the effects of fractionation during the illicit production of cocaine. Isotope ratios of elements such as carbon, hydrogen, oxygen, and nitrogen can become locally enriched or depleted by a variety of different kinetic and thermodynamic factors, making their isotope ratios even more unique. Here, only the ratios for carbon are measured and, as always, they are measured relative to an isotope standard to eliminate any bias or systematic error in the measurements. These standards are, or can be linked to, a universally-accessible standard such as a Vienna Pee Dee Belemnite (V-PDB). In our case, we used a carbon isotope standard from Chiron (details below). Because the ratios are measured relative to standards, the ratios are also reported relative to universal standards in the delta notation, $\delta$:
\[ \delta = 1000(\frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} ) \]  

where \( R_{\text{sample}} \) is the abundance ratio for the minor, heavier isotope of the element of interest to the major, lighter isotope (e.g. \( ^{13}\text{C}/^{12}\text{C} \)). The \( R_{\text{standard}} \) values are usually selected because they represent a stable material which is highly enriched in the heavy (minor) isotopes. Most analyzed substances are depleted in the heavy isotope relative to the standard and will therefore, have negative delta values.

Through this experiment, students will gain hands-on experience in operating this highly-advanced analytical instrument and in determining the possibility of common sources, such as linking hypothetical evidence from a crime scene and a suspect. We present this laboratory experiment to introduce this novel, rapidly developing forensic analytical technique and to provide students with the opportunity to handle samples as if they were handling real evidence. The statistical tests used in this experiment are consistent with those used in other publications in this journal.\(^ {173-176} \) Students were allowed to work in groups of three in our trial run of the experiment.

### B.2 Experimental

#### B.2.1 Materials and Equipment

- Auto sampler (AS3000, Thermo Finnigan, Waltham, Massachusetts)
- GC (Trace GC, Thermo Finnigan, Waltham, Massachusetts)
- DB5 Column (30 m x 0.25 mm x 0.25 \( \mu \)m, J & W Scientific, Folsom California)
Single Quadrupole Mass Spectrometer (HP5970B, Hewlett Packard (now Agilent), Santa Clara, California)

IRMS (Delta Plus Advantage, Thermo Finnigan, Waltham, Massachusetts)

Isodat 2.0 Software (Thermo Finnigan, Waltham, Massachusetts)

100 uL – 1000 uL auto pipettes

Volumetric glassware

Brown glass autosampler vials and caps

Cocaine samples (Sigma Aldrich, St. Louis, Missouri). Cocaine is a schedule II drug and requires a DEA and state pharmacy license to legally handle the drug.

Methanol HPLC grade (Sigma Aldrich, St. Louis, Missouri)

IRMS standards (C_{11}, C_{15}, C_{20}) 0.15 mg in 1 mL cyclohexane (Chiron International Standards, Laramie, Wyoming)

Evidence Tape (Arrowhead Forensics, Lenexa, Kansas)

B.2.2 Hazards

Methanol is highly toxic when inhaled, swallowed, or touched, and any waste should be disposed of appropriately. Methanol is highly flammable and should not be handled near an open flame. Students should wear safety goggles and gloves when handling chemicals.
B.2.3 Procedure

- Provide the students with two (or more) sealed evidence bags (ziplock bags with real evidence tape and an artificial evidence tag) containing “suspected cocaine” (evidence should be labeled “suspected” until the identity is confirmed in the laboratory). The students should read and sign the request for analysis form and the transfer of evidence forms. The cocaine samples are labeled A and B henceforth.

- Calibrate IRMS using standards. This is performed the morning of the experiment, or at the same time the samples are run. Add the IRMS standard \((C_{11}, C_{15}, C_{20}) 0.15 \text{ mg in } 1 \text{ mL cyclohexane in a labeled glass autosampler vial with a septum cap and place in the autosampler carousel.}\)

- Prepare at least 2 replicate solutions for each cocaine sample. This will help establish the variance in the cocaine isotope ratios in each sample. Each replicate is prepared as follows: Add a 2 mg aliquot of cocaine to a labeled 2 mL glass autosampler vial and dissolve to 1 mL in methanol. Seal with a septum cap, vortex and place vial into carousel.

- Prepare a labeled vial containing blank methanol.

- Perform 4 replicate injections of each aliquot of each sample and blank for each group of students. All the groups’ samples, standards and blanks should be injected in random order, to help establish the true variance of the measurements.

- Return any unused samples to the instructor using the evidence bags, evidence tape and transfer of evidence forms.

- Analyze and interpret data using the Q-test, F-test (ANOVA), and t-tests.
write a scientific lab report and/or an “official” crime lab report.

B.3 Results and Discussion

In this laboratory experiment, students work in groups of three. Each group of students places their samples into the autosampler while the laboratory instructor points out the main parts of the instrument and the functions thereof. The students help set up the sequence table for injection in random order, and also help check the chromatographic and mass spectrometric running conditions. The instructor oversees the students, answers questions, and stimulates discussion about the instrument and its operation. While the instrument is running, the students have to re-acquaint themselves with the various statistical tests that will be used to interpret the data.

The students either download the data themselves or are provided their results by the instructor. Two data files are saved for each sample: a mass spectrum stored on the single quadrupole mass spectrometer (a conventional electron ionization full-mass-scanning chromatogram) and an IRMS chromatogram saved on the IRMS. The students use the literature and/or a NIST library to confirm that the retention time and mass spectrum of their sample matches that of cocaine. The experimentally-determined retention index for each cocaine sample can be calculated from the retention time of cocaine relative to the retention time of the same n-alkane standards used to calibrate the IRMS (see experimental section for details). An example of the chromatogram and mass spectrum collected with the single quadrupole instrument is provided in Figure B.1. The
mass spectrum of cocaine has well-known characteristic ion peaks observed at m/z 82, 182, 198, 272 and 303.94.

Figure B.1 TIC chromatogram and mass spectrum of the peak at 8.6 minutes. The retention index and mass spectrum of this sample QE61 confirm the identity of the sample as cocaine.

The corresponding IRMS results can be seen in Figure B.2, which shows a $\delta^{13}C/^{12}C$ value of -35.488 for the same cocaine sample as seen in Figure B.1. Students record the signals
obtained from each chromatogram and tabulate their results. They then determine any outliers using the Q-test and establish whether or not the original cocaine samples are significantly different at the 95% C.L.

Figure B.1 Example of an IRMS output file for one injection of cocaine sample QE61. The isotope ratio is automatically adjusted to the second reference peak.

Students write a lab report that includes copies of all the spectra, a completed report worksheet with tables that list the peaks and their assignments, and a detailed explanation of the reasoning they used to determine whether or not the cocaine samples could originate from the same source. The report reflects the students understanding of
the material covered in the lectures and reinforces the advantages and limitations of IRMS.

The following equations are used by the students to calculate statistical tests:  

\[
Q_{\text{calculated}} = \frac{\text{gap}}{\text{range}} \tag{2}
\]

\[
F_{\text{calculated}} = \frac{s^2_{\text{between}}}{s^2_{\text{within}}} \tag{3}
\]

\[
S_{\text{pooled}} = \sqrt{\frac{s^2_1 (n_1 - 1) + s^2_2 (n_2 - 1)}{n_1 + n_2 - 2}} \tag{4}
\]

\[
\chi_{\text{calculated}} = \frac{|\bar{X}_1 - \bar{X}_2|}{s_{\text{pooled}}} \sqrt{\frac{n_1 n_2}{n_1 + n_2}} \tag{5}
\]

\[
\text{if } s^2_{\text{analysis}} = s^2_{\text{within}}
\]

\[
s^2_{\text{between}} = s^2_{\text{analysis}} + n s^2_{\text{sampling}} \Rightarrow s^2_{\text{sampling}} = \frac{1}{n} \left( s^2_{\text{between}} - s^2_{\text{analysis}} \right) \tag{6}
\]

\[
s^2_{\text{overall}} = s^2_{\text{analysis}} + s^2_{\text{sampling}} \tag{7}
\]

An example of the post-lab calculations is given below utilizing the $\delta^{13}$C/$\delta^{12}$C isotope ratios in Table 1, obtained from a randomly selected student (RSS). The same data is presented in the scatter plot in Figure B.3.
The students perform a Q-test at the 95% confidence limit to determine if any questionable data points should be excluded. Since the $Q_{\text{critical}}$ value at $n=4$ is 0.829, none of the data points in the RSS data set are considered outliers.\textsuperscript{177} After rejecting any outliers (and adding an explanation for the occurrence of any outliers in the report), the students calculate an average, standard deviation, and variance of each aliquot as seen in Table B.1.
Table B.1

$^{13}C/^{12}C$ Ratios for Two Randomly Selected Cocaine Samples Prepared as Described in the Procedure: 2 Aliquots Were Taken From Each Sample and Run 4 Times Each in Random Order

<table>
<thead>
<tr>
<th>Sample</th>
<th>δ%o Value</th>
<th>Q_calc values</th>
<th>Average δ%o</th>
<th>Std. Dev.</th>
<th>Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>QE61 A</td>
<td>-35.717</td>
<td>0.14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE61 A</td>
<td>-35.684</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE61 A</td>
<td>-35.661</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE61 A</td>
<td>-35.488</td>
<td>0.76</td>
<td>-35.638</td>
<td>0.1023</td>
<td>0.0105</td>
</tr>
<tr>
<td>QE61 B</td>
<td>-35.285</td>
<td>0.57</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE61 B</td>
<td>-36.044</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE61 B</td>
<td>-36.230</td>
<td>0.20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE61 B</td>
<td>-35.823</td>
<td>-35.846</td>
<td>0.4090</td>
<td>0.1673</td>
<td></td>
</tr>
<tr>
<td>QE10 A</td>
<td>-35.425</td>
<td>0.49</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE10 A</td>
<td>-35.177</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE10 A</td>
<td>-35.139</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE10 A</td>
<td>-34.923</td>
<td>0.43</td>
<td>-35.166</td>
<td>0.2057</td>
<td>0.0423</td>
</tr>
<tr>
<td>QE10 B</td>
<td>-35.162</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE10 B</td>
<td>-35.173</td>
<td>0.06</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE10 B</td>
<td>-35.011</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE10 B</td>
<td>-35.003</td>
<td>0.05</td>
<td>-35.087</td>
<td>0.0928</td>
<td>0.0086</td>
</tr>
</tbody>
</table>

Sources of variance in analysis are analyzed by the students using a one-way ANOVA performed by Microsoft Excel’s statistical macro ANOVA: Single Factor. The F-test establishes whether the variance of measurements of the samples are significantly different, and also whether different aliquot data can be pooled. ANOVA is used to assess the sources of error in the measurements. The average variance within samples is the average of the individual variances of each cocaine sample due to the analysis. The average variance between samples is the average of the mean variances of all cocaine samples’ replicated injections due to sampling. In this case, for n=4 and h=4, the $F_{\text{calculated}}$ value is 9.39, which exceeds the $F_{\text{critical}}$ value of 3.49 as seen in table 5.2. Because
$F_{\text{calculated}} > F_{\text{critical}}$ in this example, the difference between $s^2_{\text{between}}$ and $s^2_{\text{analysis}}$ or $s^2_{\text{analysis}}$ is significant at the 95% confidence level. These results indicate that the sampling procedure contains significantly more error than the analysis, or isotope ratio determination, step of the procedure. According to the results from this analysis, the students find that the contribution to the overall variance from sampling is more than twice the contribution from the analytical measurement, so any attempts to improve (minimize) the error should focus on the sampling aspect of the procedure; e.g. ensuring the sample is well-mixed and homogenized before sampling and taking more than two aliquots.

Table B.2

*ANOVA Excel Results of Two Cocaine Samples, Each Split into 2 Aliquots Run 4 Times in Random Order*

<table>
<thead>
<tr>
<th>Groups</th>
<th>Count</th>
<th>Sum</th>
<th>Average</th>
<th>Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column 1 (QE61 A)</td>
<td>4</td>
<td>142.550</td>
<td>35.638</td>
<td>0.0105</td>
</tr>
<tr>
<td>Column 2 (QE61 B)</td>
<td>4</td>
<td>143.382</td>
<td>35.846</td>
<td>0.1673</td>
</tr>
<tr>
<td>Column 3 (QE10 A)</td>
<td>4</td>
<td>140.664</td>
<td>35.166</td>
<td>0.0423</td>
</tr>
<tr>
<td>Column 4 (QE10 B)</td>
<td>4</td>
<td>140.349</td>
<td>35.087</td>
<td>0.0086</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F crit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
<td>1.6112</td>
<td>3</td>
<td>0.537</td>
<td>9.393</td>
<td>0.0018</td>
<td>3.490</td>
</tr>
<tr>
<td>Within Groups</td>
<td>0.6861</td>
<td>12</td>
<td>0.057</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>2.2973</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A t-test is performed to determine whether the two aliquots of each sample are significantly different at the 95% confidence level. The subsequent $t_{\text{calculated}}$ values for aliquot comparisons of the QE61 and QE10 samples are 0.987 and 0.698, respectively.
The $t_{\text{critical}}$ value at the 95\% C.L. for 6 degrees of freedom ($N_1+N_2-2$) is 2.447, which indicates that there is no significant difference between the sample aliquots of either sample QE61 or QE10 at the 95\% confidence level. Data from the two aliquots of each sample are then pooled before comparing the two samples. For this RSS, the student obtains values of $\bar{X} = 35.742$ and $s = 0.298$ for sample QE61, and $\bar{X} = 35.127$ and $s = 0.154$ for sample QE10. The $s_{\text{pooled}}$ value for the comparison of these two samples is 0.237, which provides a $t_{\text{calculated}}$ value of 5.19. This number is compared to a $t_{\text{critical}}$ value of 2.145 for DOF=14 at the 95\% C.L. Thus, the students determine that the mean $^{13}\text{C}/^{12}\text{C}$ isotope ratio values for the two cocaine samples are significantly different at the 95\% confidence level, and are not likely to have originated from the same source. This conclusion is in agreement with the samples provided (one from a commercial supplier, and one seized during an arrest). The students are then required to report this conclusion, and their reasoning behind it, in a formal lab report.

B.4 Conclusion

The goal of this laboratory is to provide students with a hands-on learning experience using forensic techniques, procedures, forms, and the highly specialized analytical technique of IRMS. It is crucial for students to understand the sources of error in their analyses, and how these sources of error can be minimized. In this case, improving the analytical measurement is futile because most of the error is in the sampling. To reduce the error, and improve the individualization, it would be more effective to analyze additional aliquots of each sample, or to better homogenize the
sample prior to sampling. Students may or may not reach similar conclusions on different instruments with different samples.

This experiment was performed in a senior forensic chemistry laboratory and encountered no apparent difficulties. Students were able to prepare samples and start the analysis within the time allotted for one laboratory period (three hours). In principle, most of the lab time should be used to run the analysis, as there is not an extensive amount of sample preparation required for this experiment. The foremost challenge of the post lab report entails the calculations needed to determine if the sources of cocaine could be the same (see Appendices). The students are required to program these calculations in an Excel spreadsheet, which is subsequently included in the formal report.

**Supporting JCE Online Material**
Student handouts and notes for the instructor are attached as appendices.

Appendix B.1.1
Ohio University Crime Laboratory Isotope Ratio – Mass Spectrometry Lab

Appendix B.1.2
Request for evidence examination form

Appendix B.1.3
Crime lab receipt and tracking of physical evidence form

Appendix B.1.4
Official crime laboratory report

Appendix B.1.5
Example of an actual RSS lab report
Appendix B.1.1 Isotope Ratio Mass Spectrometry Laboratory (pages 1-5)

Ohio University Crime Laboratory
Gas Chromatography-Isotope Ratio Mass Spectrometry Lab

Scenario – Detective Warren of the OUPD is trying to establish if a recent series of cocaine seizures has a common origin or source. If so, he might be able to build a case against a major supplier or dealer. You are provided with two cocaine samples, and asked to determine whether or not they could be from a common origin. To make this determination, your advisor has recommended using an Isotope Ratio Mass Spectrometer to measure the natural isotopic abundance of carbon in the cocaine samples.

Important Notes:
1) You should work in groups for this lab
2) You will each need to submit a CLR and a SLR for this lab
3) As always, be sure the instruments have been tuned or calibrated within 24 hrs of running your questioned samples. Be sure to run blanks, too
4) As always, containers/vials should be labelled with Name, date and an identifier. E.g. “GPJ 04/25/08 QD23"

Method

Zealand Muccio, a graduate student in Dr. Jackson’s group, will be present to supervise/assist with running the instrument. In our system, the GC effluent from this GC is split so that a certain percentage goes to a single quadrupole (HP5970) for compound identification, and the remainder is sent to the combustion chamber/IRMS for isotopic analysis.

The instrument details are provided in appendix 1.

To be performed in CLIP 193
1) Split each sample (A and B) into 3 equal aliquots (1-3). From each aliquot, dissolve ~2 mg of each cocaine sample in ~1 mL of methanol.

To be performed in CLIP 174
2) Run four replicate injections (i-v) of each aliquot (in alternating order) on the Thermo Scientific Trace GC in Dr. Jackson’s Lab (CLIP 174). This should give a total of 24 injections for each group. This may take overnight to run. An excel spreadsheet will be emailed to one member of each group at the earliest possible time. The spreadsheet will also include the average and s.d. of other groups’ samples. These values should aid your data interpretation.

Homework:
3) Look at all the A-sample data and all the B-sample data separately. Do you have any suspected outliers? If so, perform a Q-test to see whether or not you can reject the outliers.
4) Calculate the mean and standard deviation of the carbon isotope ratio for each aliquot (e.g. A1, A2, A3, B1, B2 and B3).
5) Perform a t-test at the 95% C.L. on each of the aliquots within a sample (i.e. A1 with A2, A1 with A3, A2 with A3. Do not compare As with Bs yet!). Would you expect any differences in a homogeneous sample? Are any of your aliquots significantly different? If so, explain why/how.
6) Pool the mean and standard deviation of each sample to give an estimate of the ‘true’ mean and standard deviation for each sample.
7) Perform a t-test between sample A and B. Is there a significant difference between the two data sets at the 95% C.L. or 99% C.L.?
GC-IRMS Lab

8) Use the results of the t-test to interpret your group’s results, and report on the possibility that the two cocaine samples could come from the same common source. Do you have any other supporting data (such as impurity profiles in the HP5970) to support your claims?

Because this lab will only take a small amount of lab time, use you spare time to answer the following questions. The answers should be included as an appendix to your SLR.

1. Draw a labeled schematic of the GC-C-IRMS. Provide a brief (2-3 sentences) description of what happens to the sample from sample injection on the GC to ion collection. (5 pts)

2. What are ALL the different combinations of isotopes of carbon and oxygen being measured at m/z 44, 45, and 46? (5 pts)

3. Why and how is the water removed from the sample? (5 pts)

4. What is the purpose of the oxidation reactor and the reduction reactor? (5 pts)

5. What other interfaces could be coupled to the IRMS besides the GC? (5pts)

6. What is back flush, and why is it used on this instrument? (5 pts)
GC-IRMS Lab

GC Method
- cocaine.gcm
  - Oven initial temperature 170°C held for 2 minutes
  - Ramp at 16°C per minute up to 280°C and held for 2 minutes
  - Right inlet 300°C
  - Split 10:1
  - Constant flow rate 1.0 mL/min
  - Auxiliary temp (to HP5970) 280°C

Autoinjector Method
- autoinjector.asm
  - Sample volume (1 μL)
  - Plunger strokes (1)
  - Viscous sample (No)
  - Sample depth in vial (Bottom)
  - Pre-injection solvent (A)
  - Cycles (1)
  - Injection depth (Standard)
  - Pre-inj dwell time (s) 0
  - Post-inj dwell time (s) 0
  - Post injection Solvent (A)
  - Cycles (1)

IRMS Method
- autoinjectorCOCAINEmet

Instrument
  - Experiment (Continuous flow)
  - Configuration (GC III AS3000)
  - Gas Configuration (CO2)
  - Acquisition Script (Acquisition.isl)
  - Integration Time (0.200) seconds
  - Peak Center Cup (Cup 2)
  - Peak Center Predelay (15) seconds
  - Peak Center Posdelay (0) seconds
  - Reference Port (Reference)

Time Events
  - 1 Backflush on
  - 2 Split off
  - 20 Reference on
  - 40 Reference off
  - 60 Reference on
  - 80 Reference off
  - 100 Reference on
  - 120 Reference off
  - 300 Backflush off and Split on
GC-IRMS Lab
- 750 Reference on
- 770 Reference off
- Acquisition start (immediately)
- Acquisition end time (790)

Evaluation @ CO2
- Evaluation type (CO2_SSH)
- Reference time (80.00)
- Reference name (User Defined)
- d 13C/12C
- vs (VPDB)
- d 18O/16O (0.000)
- vs (VSMOW)

Peak Detection @ CO2
- Peak Detection (checked)
- Background Detection (checked)
- Detection on mass (44)
- Spile filter (not checked)
- Start slope (0.2) mVs
- End slope (0.4) mVs
- Peak min height 50 mV
- Peak Resolution % (50)
- Max Peak Width (180)
- Enable (not checked)
- Factor (0.55) rArea/Pk Width / Pk Height
- Background Type (Individual BGD)
- History (5) seconds
- Perform timeshift (checked)
- Extended timeshift (unchecked)
- Max timeshift (0.5) seconds

Sequence Method
-autoinjector.seq
- Line 1
- GC Method – cocaine.gcm
- AS Sample – 1
- AS Method – autoinjector.asn
- Method – autoinjectorCOCAINE.met

Mass Spectrometer Method
-IBMS2.d

MS SIM/Scan Parameters
- Runtime (20) minutes
- EM Voltage (1600 Abs)
- Solvent Delay (4) minutes
- Acq Mode (scan)

Real Time Plot
- Time Window (20) minutes
- MS Window 1 Plot Type (Total)
GC-IRMS Lab
- Y-scale (0) to (32112640)

MS Window 2
- Plot type (extracted ion)
- Y-scale (0) to 782

MS Scan Parameters
- Start time (4) minutes
- Mass Range Low (50) High (500)
- Threshold Sampling (150) (2)
- MS window 1 mass range (50) (550)
- MS window 2 mass range (190) (800)

Starting the analysis:
- Put the sample vial in the auto injector 1 slot
- Click on Sample Name with big green arrow on the mass spectrometer
- Fill in information
- Click on Start Run
- Come over to IRMS
- Click on the Sequence
- Fill in the columns with the appropriate methods
- Highlight row 1 it will turn blue
- Press start with green arrow
Appendix B.1.2 Crime Lab Request for Evidence Examination Form

<table>
<thead>
<tr>
<th>Field</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Investigating Officer</td>
<td></td>
</tr>
<tr>
<td>Agency:</td>
<td></td>
</tr>
<tr>
<td>Address:</td>
<td></td>
</tr>
<tr>
<td>City:</td>
<td></td>
</tr>
<tr>
<td>Phone:</td>
<td></td>
</tr>
<tr>
<td>Email:</td>
<td></td>
</tr>
<tr>
<td>Victim(s):</td>
<td></td>
</tr>
<tr>
<td>Suspect/Accused(s):</td>
<td></td>
</tr>
<tr>
<td>Laboratory #:</td>
<td></td>
</tr>
<tr>
<td>Case #:</td>
<td></td>
</tr>
<tr>
<td>Zip:</td>
<td></td>
</tr>
<tr>
<td>Citation #:</td>
<td></td>
</tr>
<tr>
<td>ORI #:</td>
<td></td>
</tr>
<tr>
<td>Offense:</td>
<td></td>
</tr>
<tr>
<td>Offense Date:</td>
<td></td>
</tr>
<tr>
<td>Offense City/County:</td>
<td></td>
</tr>
<tr>
<td>Exhibits: (transmit evidence received)</td>
<td></td>
</tr>
<tr>
<td>Case History:</td>
<td></td>
</tr>
<tr>
<td>Examinations Requested:</td>
<td></td>
</tr>
<tr>
<td>Forward to A.F.I.S.</td>
<td></td>
</tr>
<tr>
<td>Forward for additional analysis (specify)</td>
<td></td>
</tr>
<tr>
<td>Submitting Officer Signature:</td>
<td></td>
</tr>
<tr>
<td>Date:</td>
<td></td>
</tr>
<tr>
<td>Received from:</td>
<td></td>
</tr>
<tr>
<td>Received By:</td>
<td></td>
</tr>
<tr>
<td>Date/Time:</td>
<td></td>
</tr>
</tbody>
</table>
Appendix B.1.3 Receipt and Tracking of Physical Evidence Form

<table>
<thead>
<tr>
<th>Item Designation</th>
<th>Relinquished by</th>
<th>Date</th>
<th>Time</th>
<th>Relinquished to</th>
<th>Date</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

If relinquished to outside agency, provide the details:

RECEIVED BY: (Print) ___________________________ Signature ___________________________ Date: __________ Time: __________

TITLE: ___________________________ AGENCY: ___________________________

This section is for submission of the crime lab report:

RECEIVED BY: (Print) ___________________________ Signature ___________________________ Date: __________ Time: __________

TITLE: ___________________________ AGENCY: ___________________________

This section is for submission of the scientific lab report:

RECEIVED BY: (Print) ___________________________ Signature ___________________________ Date: __________ Time: __________

TITLE: ___________________________ AGENCY: ___________________________
Appendix B.1.4 Official Crime Laboratory Report

Ohio University Crime Laboratory
Department of Chemistry and Biochemistry
Ohio University
136 Clippinger Laboratories, 100 University Terrace
Athens, OH 45701-2579
Tel: 740-593-1737

Official Crime Laboratory Report

To ___________________________  Date ___________________________

Suspect/Accused ___________________________

SSN ___________________________

Requestor ___________________________

Examination Requested ___________________________

Agency ___________________________

Case No. ___________________________

The following is an official report from the Ohio University Crime Laboratory giving the results of examinations conducted on specimens received from your office. This examination has been made with the understanding that the specimen is connected with an official investigation of a criminal matter and that the laboratory report will be used for official purposes only, related to the investigation of subsequent criminal prosecution. This report is not authorized for use with a civil proceeding.

Specimens:

List exhibit numbers with and physical descriptions here (replace this sentence)

Based on observations made and results of test performed by me on aforesaid specimens, the following statement of findings is made:

This is just an example below provided for a drug identification

<table>
<thead>
<tr>
<th>EXHIBIT</th>
<th>WEIGHT</th>
<th>SUBSTANCE</th>
<th>SCHEDULE</th>
<th>No. UNIT DOSES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q-1</td>
<td>23.93g</td>
<td>Cocaine (base)</td>
<td>II</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Q1 is defined as “crack” under Ohio Revised Code

Faithfully Submitted ___________________________

Glen P. Jackson (replace with your name)

TO THE ACCUSED

You have the right to demand the testimony of the person making this report; except when the report is used as evidence in a preliminary hearing or a Grand Jury Hearing, upon giving notice prior to the trial to the prosecuting attorney in accordance with the Rules of Criminal Procedure.

CHEM 417B, Spring 2008
Appendix B.1.5 Example of an Actual RSS Lab Report (pages 1-11)

Ohio University Crime Laboratory
Department of Chemistry and Biochemistry
Ohio University
136 Clappinger Laboratories, 100 University Terrace
Athens, OH 45701-2979
Tel: 740-593-1737

Official Crime Laboratory Report

To__Robert C. Warren__________________________
Name
Ohio University Police Department
Agency
Suspect/Accused__Mark and David Gawman
SSN__N/A
Examination Requested__Drug I.D. and
possible determination of a
common source

Case
No.__SK1472

The following is an official report from the Ohio University Crime Laboratory giving the results of examinations conducted on specimens received from your office. This examination has been made with the understanding that the specimen is connected with an official investigation of a criminal matter and that the laboratory report will be used for official purposes only, related to the investigation of subsequent criminal prosecution. This report is not authorized for use with a civil proceeding.

Specimens:

EXHIBIT DESCRIPTION
QE02 Vial with white powder
QE47 Vial with white powder

Based on observations made and results of test performed by me on aforesaid specimens, the following statement of findings is made: The two known samples of cocaine may have come from a common origin because there is no statistical difference between the two samples.

Faithfully Submitted ____________________________________________

TO THE ACCUSED
You have the right to demand the testimony of the person making this report; except when the report is used as evidence in a preliminary hearing or a Grand Jury Hearing, upon giving notice prior to the trial to the prosecuting attorney in accordance with the Rules of Criminal Procedure.
Gas Chromatography – Isotope Ratio Mass Spectrometry Lab

Received before deadline Yes ☐ No ☐ To be filled out by TA only

Introduction
The purpose of this lab is to determine if two samples of cocaine seized from a drug raid came from a common origin using a GC-IRMS. Gas chromatography coupled with isotope ratio mass spectrometry (GC-IRMS) utilizes a gas chromatogram first. The gas chromatogram separates the compounds with a gas mobile phase. Specific compounds have specific retention times, or how long a sample takes to separate and come off the column. These retention times can be used as an identifier for a sample. Cocaine typically has a single peak retention time around 8.4 – 9.1 minutes. The next step is combustion of the substance where the products are water and carbon dioxide. The mass spectrum of carbon dioxide is taken and the relative amounts of $^{13}$C and $^{12}$C isotopes are compared. The IRMS component is used to determine the variation of stable naturally occurring isotopes in a compound. A natural variation in isotope ratios is due to fractionation which can occur biologically, physically, or chemically. Depending on the mass of isotope from the same element, reactions will occur at different rates. A negative delta value, or isotope ratio, indicates that the isotope is depleted compared to the heavy isotope standard. A positive delta value indicates that the isotope is heavier richer compared to the heavy isotope standard$^{1}$. The mass spectrometer is used to detect the sample when an electron hits the sample and ionizes it into characteristic fragments that can be used for identification. The characteristic m/z ratio peaks from Clark’s for cocaine are: 82, 182, 83, 105, 303, 77, 94, and 96$^{2}$.

Combustion of Cocaine

\[
C_{21}H_{23}NO_{4} + O_{2} \rightarrow CO_{2} + H_{2}O
\]

Experimental
Two white powder samples, QE02 and QE47, in a glass vial were analyzed by a Thermo Scientific Trace GC-IRMS instrument. The GC-IRMS was a Delta Plus Advantage with serial number 07738D. The software used to analyze the data was Isodat Acquisition 2.0 on a Dell Desktop GX260 computer. The GC column was a DB5 column manufactured by J & W Scientific. The film thickness was 0.25µm on a 30m column. The diameter of the column was 0.25mm. The GC had an initial oven temperature of 170°C that was held for 2 minutes and it was ramped at 16°C per minute up to 280°C where it was held there for 2 minutes. The right inlet was at 300°C. The split was at a 10 to 1 ratio with a constant flow rate of 1.0 mL/min. The IRMS has a continuous flow experiment and a GC III AS3000 configuration. The integration time was 0.200 seconds. The peak center cup was cup 2, the peak center predelay was 15 seconds, and the postdelay was 0 seconds. The MS had a runtime of 20 minutes with an EM voltage of 1600 Vals. The solvent delay was 4 minutes and the Acq mode was in scan. The two
individual samples were split into 3 – 2mg aliquots and dissolved in 1mL of methanol. Four replicate injections were made of each aliquot for a total of 24 runs. All samples were dissolved in methanol made by Pharmco-aaper, lot number 0710246, and it was opened in December of 2007. The samples were placed in the auto injector. The Sample Name button was clicked on the computer to fill out the information. Next the start run button was pushed and on the IRMS instrument the sequence button was pushed where the columns were filled with the appropriate methods. Analysis began by pressing the green arrow.

**Results and discussion**

Two known samples of cocaine, QE02 and QE47, were analyzed using GC-IRMS to determine if the samples were significantly different and if they came from a common origin. Appendix 1 is the request for examination form and Appendix 2 is the chain of custody form for the samples. Appendix 7 is the set of questions. Appendix 3 is the complete set of data from the two samples. Each sample was split into 3 aliquots and 4 runs were done on each aliquot for a total of 12 runs for each sample. All of the calculations completed are also found in Appendix 3. The T-test value for a comparison of aliquot 1 and 2 for QE02 is 1.052. The T-test value for a comparison of aliquot 1 and 3 for QE02 is 1.578. The T-test value for a comparison of aliquot 2 and 3 for QE02 is 0.814. The T value at a 95% confidence interval from a T-table for 4 samples is 4.176.

Since all the comparisons in QE02 are below this value there is not a significant difference between any of the aliquots. The T-test value for a comparison of aliquot 1 and 2 for QE47 is 0.0027. The T-test value for a comparison of aliquot 1 and 3 for QE47 is 1.291. The T-test value for a comparison of aliquot 2 and 3 for QE47 is 1.607. The same T value from a T-table was used for comparison. Since all the comparisons in QE47 are below this value, there is not a significant difference between any of the aliquots. In a homogeneous sample there could be several sources of differences. One difference could be due to impurities in the sample. A small piece of dirt or dust from a different carbon source may have entered the sample when it was being made. There is a very small chance that the instrument may have picked up this small impurity during analysis causing a thought to be pure sample to have impurities. The homogenous sample could also have differences due to a random flux in the measurement of the machine.

When the data pooled, the mean for QE02 was 35.9537 with a standard deviation of 0.5082. The mean for QE47 was 36.0403 with a standard deviation of 0.6596. The T-test value for comparison of QE02 and QE47 is 0.2081. The T value at a 95% confidence interval from a T-table for 11 samples is 2.593. The T value at a 99% confidence interval from a T-table for 11 samples is 3.495. Since the comparison between sample QE02 and QE47 are below both of the above T values, there is not a significant difference between the 2 questioned samples. Appendix 4 is a chromatogram of the different m/c ratios of CO2 in the two samples. Appendices 5 and 6 are the standard gas chromatograph, mass spectrum, and isotope-ratio analysis for cocaine that can be used for comparison to the two questioned samples.

**Conclusion**

A gas chromatography coupled with isotope ratio mass spectrometry analysis was done on two samples of cocaine, QE02 and QE47, to determine if they came from a
common origin. The natural abundance of carbon and oxygen ratios was measured in each of the samples for comparison. After a T-test analysis, the samples were found to have no significant difference. This means that the samples could have very well come from a common source.

I think a more thorough background on the instrument and how to analyze the results would help to improve this lab. When I received the chromatogram I had no idea how to interpret it or what it meant. The only thing I based my conclusion on was the actual numbers.

References
Ohio University Crime Lab
Request for Evidence Examination
(Attach additional pages as needed)

Investigating Officer: Robert C. Warren
Agency: OUPD
Address: 435 Billabong Drive
City, State: Athens, OH Zip: 45701
Phone: (740) 593-0797 Fax: (740) 593-0148
Email: R.C.Warren@law.com

Laboratory #: OUCL 2008
Case #: 
Citation #: 
ORI #: 

Offense: Possession, dealing
Offense City/County: Athens, OH

Victim(s): State
Suspect/Accused(s): Mark Gawman, David Gawman

Exhibits: (Initial exhibits received)

GE1

Test

GE47

Test

Case History:
Suspected drugs seized from suspects' homes are thought to be connected to a larger distribution network.

Examinations Requested:
Drug I.D. and possible determination of a common source.

Forward to A.F.I.S.  Forward for additional analysis (specify)

Submitting Officer Signature: Date: 4/29/08
Print Submitting Officer: R.C. Warren

Received from: Received By: Date/Time:

9:21 am 4/29/08
<table>
<thead>
<tr>
<th>Item Designation</th>
<th>Relinquished by</th>
<th>Date</th>
<th>Time</th>
<th>Relinquished to</th>
<th>Date</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q602</td>
<td></td>
<td>03/2003</td>
<td>9:22 am</td>
<td></td>
<td>04/2003</td>
<td>9:22 am</td>
</tr>
<tr>
<td>Q647</td>
<td></td>
<td>04/2003</td>
<td>9:22 am</td>
<td></td>
<td>04/2003</td>
<td>9:22 am</td>
</tr>
<tr>
<td>Q603</td>
<td></td>
<td>04/0903</td>
<td>9:51 am</td>
<td></td>
<td>04/0903</td>
<td>9:51 am</td>
</tr>
<tr>
<td>Q647</td>
<td></td>
<td>04/0903</td>
<td>9:51 am</td>
<td></td>
<td>04/0903</td>
<td>9:51 am</td>
</tr>
</tbody>
</table>

If relinquished to outside agency, provide the details:

RECEIVED BY: (Print) ___________________________ Signature ___________________________ Date: _____________ Time: _____________

TITLE: ___________________________ AGENCY: ___________________________

This section is for submission of the crime lab report:

RECEIVED BY: (Print) ___________________________ Signature ___________________________ Date: _____________ Time: _____________

TITLE: ___________________________ AGENCY: ___________________________

This section is for submission of the scientific lab report:

RECEIVED BY: (Print) ___________________________ Signature ___________________________ Date: _____________ Time: _____________

TITLE: ___________________________ AGENCY: ___________________________
<table>
<thead>
<tr>
<th>Sample</th>
<th>Vial</th>
<th>File</th>
<th>δ Value</th>
<th>Average</th>
<th>Std Dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>QE02</td>
<td>13</td>
<td>58</td>
<td>-36.201</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE02</td>
<td>13</td>
<td>47</td>
<td>-36.81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE02</td>
<td>13</td>
<td>22</td>
<td>-35.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE02</td>
<td>13</td>
<td>17</td>
<td>-36.457</td>
<td>-36.282</td>
<td>0.575</td>
</tr>
<tr>
<td>QE02</td>
<td>14</td>
<td>60</td>
<td>-35.455</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE02</td>
<td>14</td>
<td>56</td>
<td>-35.672</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE02</td>
<td>14</td>
<td>27</td>
<td>-36.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE02</td>
<td>14</td>
<td>20</td>
<td>-36.347</td>
<td>-35.9185</td>
<td>0.383</td>
</tr>
<tr>
<td>QE02</td>
<td>15</td>
<td>54</td>
<td>-36.242</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE02</td>
<td>15</td>
<td>43</td>
<td>-35.188</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE02</td>
<td>15</td>
<td>41</td>
<td>-35.259</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE02</td>
<td>15</td>
<td>6</td>
<td>-35.84</td>
<td>-35.6323</td>
<td>0.589</td>
</tr>
<tr>
<td>QE47</td>
<td>16</td>
<td>60</td>
<td>-36.928</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE47</td>
<td>16</td>
<td>62</td>
<td>-36.889</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE47</td>
<td>16</td>
<td>32</td>
<td>-35.426</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE47</td>
<td>16</td>
<td>32</td>
<td>-35.667</td>
<td>-36.2275</td>
<td>0.856</td>
</tr>
<tr>
<td>QE47</td>
<td>17</td>
<td>51</td>
<td>-35.871</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE47</td>
<td>17</td>
<td>44</td>
<td>-36.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE47</td>
<td>17</td>
<td>2</td>
<td>-37.143</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE47</td>
<td>17</td>
<td>1</td>
<td>-35.822</td>
<td>-36.229</td>
<td>0.682</td>
</tr>
<tr>
<td>QE47</td>
<td>18</td>
<td>53</td>
<td>-35.668</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE47</td>
<td>18</td>
<td>38</td>
<td>-35.397</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE47</td>
<td>18</td>
<td>37</td>
<td>-35.232</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QE47</td>
<td>18</td>
<td>13</td>
<td>-36.461</td>
<td>-35.6645</td>
<td>0.168</td>
</tr>
</tbody>
</table>

\[
A_1 - A_2 = (-36.282) - (-35.9185) = 0.6745 \\
0.575^2 + 0.383^2 = 0.8124 \\
1.052 \\
\]

\[
A_1 - A_3 = (-36.282) - (-35.6323) = 0.6547 \\
0.575^2 + 0.682^2 = 0.8652 \\
1.517 \\
\]

\[
A_2 - A_3 = (-35.9185) - (-35.6323) = 0.2862 \\
0.383^2 + 0.682^2 = 0.8652 \\
0.514 \\
\]

\[
A_1 - B_1 = (-36.279) - (-35.9645) = 0.3145 \\
0.575^2 + 0.169^2 = 0.6306 \\
1.291 \\
\]

\[
B_1 - B_3 = (-36.279) - (-35.9646) = 0.3144 \\
0.575^2 + 0.169^2 = 0.6306 \\
1.291 \\
\]

\[
B_2 - B_3 = (-36.279) - (-35.9646) = 0.3144 \\
0.575^2 + 0.169^2 = 0.6306 \\
1.291 \\
\]

\[
A - B = (-36.282) - (-35.9185) = 0.3635 \\
0.575^2 + 0.383^2 = 0.8124 \\
1.052 \\
\]
1. The sample is injected into the GC, where it gets vaporized and goes onto the column and carried through the column for separation by a carrier gas. The sample then passes through a combustion reactor where it becomes oxidized and then it is reduced to remove excess oxygen and reduce nitrogen oxides to nitrogen. Excess water is removed and the sample is introduced into the MS where the sample is ionized by electron ionization. The ions are separated according to their momentum where the ions are then detected by Faraday cups to compute the isotope ratios.

2. 

<table>
<thead>
<tr>
<th>m/z</th>
<th>Isotope C and O ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>44</td>
<td>$^{13}C^{16}O_2$</td>
</tr>
<tr>
<td>45</td>
<td>$^{12}C^{16}O_2$</td>
</tr>
<tr>
<td>46</td>
<td>$^{13}C^{16}O_2$</td>
</tr>
</tbody>
</table>

3. Water is removed from the sample by passing a stream of gas through it.

4. The oxidation reactor combusts organic samples into pure gases and the reduction reactor reduces NO to N$_2$ and removes excess O$_2$.

5. Other then gas chromatography (GC), Liquid chromatography (LC), Elemental Analyzer (EA), Dual Inlet (DI), and Continuous Flow (CF), are other types of interfaces coupled with IRMS.

6. Back flush is used to reoxidize the sample and send it to the oxidation chamber.
Appendix C.1 Poster Presentation at MUACC hosted by the Michigan State University.

Simultaneous Identification and δ¹³C Classification of Cannabinol in Unknown Marijuana samples using GC with Concurrent Single Quadrupole and Isotope Ratio Mass Spectrometers

ZELAND MUCcio, CLAUDIA WÖCKEL and CLAREN P. JACKSON*

Ohio University, Center for Intelligent Chemical Instrumentation, Athens, OH 45701-2979
E-mail: jackson@ohio.edu

Introduction
Marijuana is the most frequently abused illicit drug in the world used by both adults and minors. 1,2 Tetrahydrocannabinol (THC), the major psychoactive ingredient in marijuana, varies in concentration for different plant parts in the following order: buds > flowers > leaves > smaller sizes > larger sizes > buds > seeds. A single-inlet multivial detection system was used here to provide simultaneous results of both the total ion chromatogram and mass spectrum from the single quadrupole mass spectrometer and isotope carbon analysis of the cannabinoids from the HRMS. 3 The cannabinoid of interest in our research was CBN, known to be a degradation product of THC. The carbon isotope ratios can be used to elucidate the possibility that two samples of marijuana were from the same source by sample matching, either to each other or to a sample from a crop. Our primary intent was to establish the sources of variation in δ¹³C values from four different marijuana samples and to determine plausible sources matches.

Instrumentation and Methods
Sample Preparation:

- 300 mg of leaf material was pulverized by adding 50 mL of 1:1 chloroform/methanol to a 1:5 glass vial
- 200 mg of each powdered marijuana sample was then placed in a 1:5 glass vial

- 1 mL of acetone added
- Sample was sonicated for 15 minutes before centrifuging for 2 minutes to pelletize the solid matrix
- Liquid supernatant was then filtered to remove any particulates
- 150 mL of the remaining sample was then transferred to a 1 mL autosampler vial with 200 mL of methanol and placed into the autosampler carousel for analysis

Instrumentation:
- Trace GC
- DB-5 column (40 m x 0.25 mm x 0.25 μm)
- Heated at 300°C for 8 min
- Ramped 20°C/min to 300°C
- Heated 300°C for 8 min

- DQ Mass spectrometer detector
- Isotope Ratio Mass Spectrometer

- 10% of effluent
- Single Quadrupole Mass Spectrometer
- 10% of effluent

- All four samples contained CBN as the major or second most abundant cannabinoid. CBN was the least abundant cannabinoid in samples C and D, but only a trace component in samples A and B. Figures 3-5 show simultaneous TIC and IRMS chromatograms of samples B and D respectively.

- The bar graph in Figure 5 shows the Isotope ratio values for CBN in each of the aliquots from the four samples. ANOVA was performed to separate the sampling error from the measurement error. Sampling standard deviations varied from 0.009 to 0.045 (average = 0.017) for the four samples, whereas the measurement standard deviations varied from 0.11% to 0.22% (average = 0.14%). Based on these standard deviations, the measurement error is approximately eight to ten times larger than the sampling error.

- When comparing the F-Statistics results in Table 1 with the F-Critical values, our results show that the same mean for sample A can be distinguished at the 95% confidence level (CL), from samples B, C, and D. Samples C and D can be similarly distinguished at the 95% CL. The sample means of samples B and D are not significantly different, so cannot be excluded as having a potential common source.

- One can conclude two samples of marijuana as having a common origin by using the statistical probability values provided in Table 2. If the P-Values are less than 0.05, the results indicate that the difference in the two sample means is statistically significant at the 95% CL.

Conclusion
The use of IRMS for the comparison of unknown samples of drugs has unlimited applications. The additional discriminatory factor that IRMS adds is unique in that the source of a drug sample can be matched or excluded as probable by looking at the differences in the number of isotopes. In addition, sample matching or exclusion through the measurement and comparison of the samples δ¹³C is possible based upon the strong correspondence between cannabinoids within the marijuana plant.

Future Work
The creation of a Universal chemical fingerprint system for all the illicit drugs would prove to be invaluable for forensic investigations and law enforcement officials. The geographical origins of crops have already been studied and the use of this information has already proven its worth for the use of our information that is available by using IRMS, the identification and tracking of illicit drug distribution routes should become far more focused and cost efficient.

References

Acknowledgements
The authors want to thank Roger Hustedt from Thermionics for technical support. We also thank NSF for funding this project through grant number 0755665.
Isotope Ratio Mass Spectrometry – A Rapidly Developing Analytical Technique Applied to Forensic Samples

Zeland Muccio and Glen P. Jackson*

Center for Intelligent Chemical Instrumentation, Ohio University, Athens, OH 45701-2979

E-mail: jackson@ohio.edu

Appendix D.1 Poster Presentation at Ohio University Research and Creative Activity Fair

Objective

To determine, using isotope ratio mass spectrometry for isotopic analysis, the differences in the number of neutrons between questioned and known samples to assist or establish potential criminal cases.

Introduction

Forensic science methodologies have long recoginized the need to distinguish between different sources of evidence material. Such determinations were formerly difficult or impossible in cases where two samples had identical physical or chemical properties. One of the most interesting and potentially revealing techniques is Isotope Ratio Mass Spectrometry (IRMS). In the analysis presented here, the IRMS is used to compare the carbon isotope abundance ratios of various compounds in the sample. The results of these comparisons help identify and classify complex mixtures.

Gridded variations in isotopic abundances

The average isotope ratio of each elemental tracer was fixed around the time of the event’s formation. However, isolated variations can occur:

1. Presumably, the different elements in each sample were carbonized at different times, which could lead to variations in the isotope ratios.

2. The isotope ratios of different elements in each sample were influenced by the environment, which could lead to variations in the isotope ratios.

3. The isotope ratios of different elements in each sample were influenced by the environment, which could lead to variations in the isotope ratios.

4. The isotope ratios of different elements in each sample were influenced by the environment, which could lead to variations in the isotope ratios.

Future Work

IRMS offers the potential of unrivaled applications for non-volatile and volatile compounds while achieving higher accuracy and precision via automated isotopic measurements. We plan to use the C15 and C18 isotopes to refine the analysis and provide a more accurate representation of the isotopic variations observed in the samples.

Acknowledgements

Mark and Nancy Ewashko for equipment assistance. We are grateful to the donors and sponsors for their unwavering support. This work was conducted with support from the National Institute of Justice (NIJ), grant number 2018-DN-BA0012, for which we are very grateful.
Appendix E.1 Institutional Review Board Approval Form (09X194).

The following research study has been approved by the Institutional Review Board at Ohio University for the period listed below. This review was conducted through an expedited review procedure as defined in the federal regulations as Category(ies):

**Project Title:** Measurement of 13C/12C Isotopic Composition of Individual Amino Acids After N-(trimethylsilyl) ester Derivatization of Human Hair

**Primary Investigator:** Zeland Muccio

**Co-Investigator(s):** Yan An

**Faculty Advisor:** Glen Jackson

**Department:** Chemistry

![Signature](signature)

Robin Stack, CIP
Office of Research Compliance

**Approval Date:** 11/17/2009

**Expiration Date:** 11/16/2010

This approval is valid until expiration date listed above. If you wish to continue beyond expiration date, you must submit a periodic review application and obtain approval prior to continuation.

Adverse events must be reported to the IRB promptly, within 5 working days of the occurrence.

The approval remains in effect provided the study is conducted exactly as described in your application for review. Any additions or modifications to the project must be approved by the IRB (as an amendment) prior to implementation.
Appendix E.2 Institutional Review Board Consent Form (09X194).
Ohio University Consent Form

Title of Research: **Measurement of $^{13}\text{C}/^{12}\text{C}$ Isotopic Composition of Individual Amino Acids in Human Hair for Forensic Comparisons**

Researchers: Dr. Glen P. Jackson, Zeland Muccio, Christine Fisher, Yan An.

You are being asked to participate in research. For you to be able to decide whether you want to participate in this project, you should understand what the project is about, as well as the possible risks and benefits in order to make an informed decision. This process is known as informed consent. This form describes the purpose, procedures, possible benefits, and risks. It also explains how your personal information will be used and protected. Once you have read this form and your questions about the study are answered, you will be asked to sign it. This will allow your participation in this study. You should receive a copy of this document to take with you.

**Explanation of Study**

The primary outcome of the research will be a new tool by which forensic professionals can establish a link between questioned hair samples (such as found at a crime scene or on a suspect’s clothing) and known hair samples (such as collected from a suspect’s scalp or victim). Current methods rely on microscopy, which is not very discriminating, or DNA analysis, which is not always possible. Our proposed approach is to measure the isotope ratios of different carbon atoms (e.g. the ratio of carbon atoms with seven neutrons to carbon atoms with six neutrons) in different components of the hair shaft. The hair shaft is the major part of hair that we see and brush. By comparing hair in this way so, we hope to create a new tool for comparing questioned hair samples—such as from a crime scene or found on a suspect—to known samples, such as from a victim.

The isotopes of carbon found in hair are known to be dependant on our diets. Because our diets are very different from person to person, so too should be the isotopes of carbon in different components of our hair. This project will test the hypothesis that the carbon isotope ratios of different components in hair can be used to establish or disprove that the hair could have come from a potential common source. In this case, a common source would imply that the questioned hair would have to have been grown for an overlap in time on the same scalp with the known sample.

Office of Research Compliance

Rev. 05/2008
Risks and Discomforts

There should not be any physical risks or discomforts to you. The hair collection involves collecting your hair as if you were going to the barber shop/hair salon to get a normal hair-cut. A small amount of hair (~80 mg) will be cut with scissors at the root, and should not cause a noticeable loss, if rolled into a ball this amount of hair would be approximately the size of a pea. You will be shown an example of an 80 mg hair sample before consent. You may choose the desired location on your scalp from which the sample will be collected. Alternatively, you may choose to collect your own hair sample during your regular visit to the hair salon.

Benefits

While there are no anticipated benefits to you as an individual, the primary outcome of the research will be a new tool by which forensic professionals can establish a link between questioned hair samples (such as found at a crime scene or on a suspect’s clothing) and known hair samples (such as collected from a suspect’s scalp or victim).

Confidentiality and Records

You will not be required to complete a questionnaire. No personal information will be collected.

Your hair sample will be assigned a number so that your confidentiality is protected. The number will be written on your consent form, which will be stored in a different location to your hair sample. The sample bags will not contain any personal information. The hair sample will be stored until the research project is completed and then the hair samples will be destroyed after the results are published and no longer required within the 3 year limit. In the event that the results are published, only the sample number will be used to describe each sample. No personal information about you will be collected from you or released.

Additionally, while every effort will be made to keep your study-related information confidential, there may be circumstances where this information must be shared with:

* Federal agencies, for example the Office of Human Research Protections, whose responsibility is to protect human subjects in research;
* Representatives of Ohio University (OU), including the Institutional Review Board, a committee that oversees the research at OU;

Compensation

There is no financial compensation for your valuable contribution to this study.

Office of Research Compliance  Rev. 05/2008
Contact Information
If you have any questions regarding this study, please contact Dr. Glen P. Jackson at jacksong@ohio.edu. Tel: 740-593-0797

If you have any questions regarding your rights as a research participant, please contact Jo Ellen Sherow, Director of Research Compliance, Ohio University, (740)593-0664.

By signing below, you are agreeing that:
• You have read this consent form (or it has been read to you) and have been given the opportunity to ask questions.
• Known risks to you have been explained to your satisfaction.
• You understand Ohio University has no policy or plan to pay for any injuries you might receive as a result of participating in this research protocol.
• You are 18 years of age or older.
• Your participation in this research is given voluntarily.
• You may change your mind and stop participation at any time without penalty or loss of any benefits to which you may otherwise be entitled.

Signature_________________________________________ Date____________________

Printed Name________________________________________
Supplement S.1 Supplemental Material Submitted with Publication to the Journal of Chemical Education with “Isotope Ratio Mass Spectrometry – A New Tool for the Forensic Chemist” (slides 1-19)

Forensic applications of isotope ratio mass spectrometry—A review

Sarah Benson\textsuperscript{a,b,*}, Chris Lennard\textsuperscript{a}, Philip Maynard\textsuperscript{b}, Claude Roux\textsuperscript{b}


- Good source of information for the different fields:
  - FIRMS (Forensic IRMS group in the UK since 2002)
  - NITECRIME EU: Natural isotopes and trace elements analysis in Criminalistics and environmental forensics
- Mostly at the research stage right now
- Paint and varnish
  - Carbon and Nitrogen : Finnigan Mat, Application flash report No. 15 12/1995 PL 0/1177
- GC/IRMS has been accepted by the International Olympic Committee (IOC) Medical Commission (1997) as a viable technique for distinguishing between exogenous and endogenous steroid metabolites (Fourel, as cited by Phillips et al. [26])
Mass analyzers: sector instruments

- Can be scanning or multi-collector (MC)
- MC provides better isotope ratios
  - Self-corrects for fluctuations/drift in continuous ion sources (ICP/TIMS/EI)
- Need to carefully calibrate instruments for isotope analysis
- Classically used for accurate mass measurements or isotope ratios
- Can now operate on time scale (and quantity) of GC separations
  - GC-IRMS or compound-specific isotope ratios (CSIR)
- Need to convert organics to CO$_2$, N$_2$ and H$_2$O

Isotope ratio monitoring with multiple collectors

![Diagram of mass analyzers and isotope ratio monitoring system]

Della Plus Advantage brochure, Thermo Finnigan, San Jose, CA
Compound-specific isotope ratio monitoring (GC/C/IRMS)

\[ \delta^{13}C = \frac{R_{\text{sample}}}{R_{\text{reference}}} - 1 \times 1000 \]

or

\[ \delta^{13}C = \frac{R_{\text{sample}} - R_{\text{reference}}}{R_{\text{reference}}} \times 1000 \]

\( R_{\text{reference}} = [^{13}\text{C}] / [^{12}\text{C}] = 0.0112372 \) for Pee Dee Belemnite (a form of calcium carbonate found in a mountain in North Carolina). Europeans often use Vienna PDB as a reference.

PDB provides one of the largest \(^{13}C\) ratios known: this makes most delta values negative w.r.t. PDB

\(^{13}C/^{12}C\) isotope ratios

- Convert organic to CO\(_2\)
  \( ^{12}\text{C}^{16}\text{O}_2 = m/z 44 \)
  \( ^{13}\text{C}^{16}\text{O}_2 = m/z 45 \)

but

\( ^{12}\text{C}^{17}\text{O}^{16}\text{O} \) also appears at \( m/z 45 \) Th, too, so need to correct for this extra signal at \( m/z 45 \)

Therefore, Measure \( m/z 46 \) as well, and make use of known \( ^{18}\text{O}^{17}\text{O} \) and \( ^{18}\text{O}^{16}\text{O} \) ratios to make correction

<table>
<thead>
<tr>
<th>Element</th>
<th>Atomic weight</th>
<th>Natural relative abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^1\text{H})</td>
<td>1.0078</td>
<td>99.985</td>
</tr>
<tr>
<td>(^2\text{H})</td>
<td>2.0141</td>
<td>0.015</td>
</tr>
<tr>
<td>(^{13}\text{C})</td>
<td>12</td>
<td>98.99</td>
</tr>
<tr>
<td>(^{12}\text{C})</td>
<td>13.003</td>
<td>1.11</td>
</tr>
<tr>
<td>(^{14}\text{N})</td>
<td>14.003</td>
<td>99.63</td>
</tr>
<tr>
<td>(^{15}\text{N})</td>
<td>15.000</td>
<td>0.37</td>
</tr>
<tr>
<td>(^{16}\text{O})</td>
<td>15.994</td>
<td>99.74</td>
</tr>
<tr>
<td>(^{17}\text{O})</td>
<td>16.999</td>
<td>0.037</td>
</tr>
<tr>
<td>(^{18}\text{O})</td>
<td>17.999</td>
<td>0.204</td>
</tr>
<tr>
<td>(^{31}\text{P})</td>
<td>31.072</td>
<td>95.95</td>
</tr>
<tr>
<td>(^{32}\text{P})</td>
<td>32.065</td>
<td>4.22</td>
</tr>
</tbody>
</table>
Carbon isotope ratios in plants and animals

- C3 plants (most), $\delta^{13}C = -22$ to $-35 \text{%}_0$
- C4 plants (corn and cane), $\delta^{13}C = -8$ to $-20 \text{%}_0$
- Terrestrial animals, $\delta^{13}C = -15$ to $-25 \text{%}_0$
- Petroleum products, $\delta^{13}C = -20$ to $-50 \text{%}_0$
Forensic isotope ratio mass spectrometry: FIRMS

http://www.forensic-isotopes.rdg.ac.uk/

Inter-laboratory testing of GC-IRMS

Although promising, improvements needed in some labs
Tracing the geographical origin of cocaine

Cocaine carries a chemical fingerprint from the region where the coca was grown.

James R. Ehleringer*, John E. Gasco*, Michael J. Lott†, Valerie L. Ford†

Individualization of drugs

Different cocaine seizures can be differentiated (excluded as common in origin)
**GC-IRMS for comparison of safety matches**

- Initial study to determine $^{13}$C variation within one box and between different brands
- Within one box variation is between 1.5‰ to 2.5‰
- $^2$H isotope abundance gives indication of harvest region

![Graph](image)

*Figure 2. Comparison of matches seized from a suspect with those found at a scene of crime and control samples based on $^{13}$C and $^2$H isotope composition. Data points are the mean of triplicate analysis per isotope; error bars are ±1σ.*

---

**Bulk IRMS of packaging tapes**

- P1-P5 Rolls bought in store from different brands
- P6-P10 Tape taken from packages delivered to the lab

![Graph](image)

**Table 1. Summary of overall analyzed protocol for the comparison of PSA tapes.** If at any step the data are divergent then it is possible to conclude that two samples do not have a common origin. If the protocol yields parallel results at all stages this provides strong evidence that the two tapes share a common origin.

<table>
<thead>
<tr>
<th>Step</th>
<th>Sample intact tape</th>
<th>Remove adhesive layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$^{13}$C determination</td>
<td>$^{13}$C determination</td>
</tr>
<tr>
<td>2</td>
<td>$^2$H determination</td>
<td>$^{13}$C determination</td>
</tr>
<tr>
<td>3</td>
<td>$^2$H determination</td>
<td>$^2$H determination</td>
</tr>
<tr>
<td>4</td>
<td>$^{18}$O determination</td>
<td>$^{18}$O determination</td>
</tr>
</tbody>
</table>

---


**GC-IRMS for ecstasy synthesis**

![Diagram of chemical pathways]

Table 2. 95% confidence limits for δ13C, δ15N and δ34S based on 20 replicate analyses of the extract of each IS.

<table>
<thead>
<tr>
<th>Compound</th>
<th>δ13C (‰)</th>
<th>δ15N (‰)</th>
<th>δ34S (‰)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS1</td>
<td>-22.5 to -20.0</td>
<td>2.0 ± 0.5</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>IS2</td>
<td>-22.0 to -19.5</td>
<td>1.5 ± 0.3</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>IS3</td>
<td>-21.5 to -19.0</td>
<td>1.0 ± 0.3</td>
<td>0.0 ± 0.1</td>
</tr>
</tbody>
</table>


---

**Determination of the origin of urinary norandrosterone traces by gas chromatography combustion isotope ratio mass spectrometry**

Moritz Hebestreit, Ulrich Henker, Gregor Fühöllner, Hans Geyer, Ute Gänzler, Ute Mareck, Thomas Piper, Mario Theis, Christiane Ayotte and Wilhelm Schänzer

Received 16th March 2006, Accepted 4th July 2006
First published as an Advance Article on the web 28th July 2006

*Analyst* 2006, 131, 1021–1026

NA: 19-norandrosterone
AND: Androsterone

NA is metabolite of Nandrolone and 19-nortestosterone (2 anabolic steroids)
AND is produced naturally in the body and also as a metabolite of NA
Fig. 8 $\delta^{13}C_{\text{VPDB}}$-values of NA vs. AND of 25 doping control samples; 12 samples reanalysed under varying conditions; the ellipses indicate the presumable classification into endogenous or exogenous origin of NA.

Detection of Epitestosterone Doping by Isotope Ratio Mass Spectrometry

RODRIGO ACUÑEIRA,† CAROLINE K. HATTON,† and DON H. CATLIN‡

- Epitestosterone (E) is a masking agent that changes the ratio of Testosterone (T) to Epitestosterone (T/E).
- T/E > 6 is suspect

- Test on 456 Healthy males
  - $\delta^{13}C$ average athlete -23.8‰
  - $\delta^{13}C$ 4 different synthetic -30.3‰

<table>
<thead>
<tr>
<th>Table 2. Mean $\delta^{13}C$, SD, and CV for synthetic epistosterone obtained from four chemical vendors.</th>
<th>Vendor</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Mean, ‰</td>
<td>-30.3</td>
</tr>
<tr>
<td>SD, ‰</td>
<td>0.27</td>
</tr>
<tr>
<td>CV, ‰</td>
<td>0.90</td>
</tr>
</tbody>
</table>

*Ratio of the number of each epistosterone.

Table 4. Urinary epistosterone $\delta^{13}C$ values for 43 healthy male controls.

<table>
<thead>
<tr>
<th>Vendor</th>
<th>All</th>
<th>Hispanic</th>
<th>Caucasian</th>
<th>Black</th>
<th>Asian</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean, ‰</td>
<td>-23.8</td>
<td>-23.6</td>
<td>-24.2</td>
<td>-23.2</td>
<td>-23.8</td>
</tr>
<tr>
<td>SD, ‰</td>
<td>0.93</td>
<td>0.90</td>
<td>0.74</td>
<td>0.68</td>
<td>1.43</td>
</tr>
<tr>
<td>CV, ‰</td>
<td>3.9</td>
<td>4.1</td>
<td>3.1</td>
<td>2.9</td>
<td>6.0</td>
</tr>
<tr>
<td>n</td>
<td>43</td>
<td>16</td>
<td>15</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Minimum</td>
<td>-25.6</td>
<td>-25.6</td>
<td>-25.2</td>
<td>-24.2</td>
<td>-25.3</td>
</tr>
<tr>
<td>Maximum</td>
<td>-21.8</td>
<td>-22.3</td>
<td>-22.4</td>
<td>-22.6</td>
<td>-21.8</td>
</tr>
<tr>
<td>Range</td>
<td>3.8</td>
<td>3.4</td>
<td>2.8</td>
<td>1.6</td>
<td>3.6</td>
</tr>
</tbody>
</table>

* The ethnicity of three individuals was not declared.
Benchtop gas chromatography–mass spectrometry (GC–MS) was first employed in Olympic doping control during the 1984 Los Angeles Olympic Games.

Responsibility for doping control is held by the World Anti-Doping Agency (WADA), which, amongst its many responsibilities, administers the list of prohibited substances.

In the case of steroids, synthetic copies of endogenous steroids are derived from plant sources, such as soy, that have a low $^{13}$C content. This isotopic signature is carried forward to urinary steroid metabolites that are $^{13}$C depleted in relation to their endogenous analogues that are derived from dietary sources.

Stable carbon isotope ratios – you are what you eat!

### Review of IRMS in sports doping

- IRMS is used for many different types of doping and masking agents
  - All studies found look only at $^{13}$C/$^{12}$C
    - Cheaper, easier commercial access
- Time frame to see change is dependent on metabolites
- Differentiation between endogenous and exogenous is usually straight forward and unquestionable
DIFFERENTIATION BETWEEN ORIGINS OF EXTRA VIRGIN OLIVE OILS BY GC/C/IRMS USING PRINCIPAL COMPONENT ANALYSIS, LINEAR DISCRIMINANT ANALYSIS AND HIERARCHICAL CLUSTER ANALYSIS

ANDREAS BAUM, YAO LU, ZELAND MUCCIO, GLEN P. JACKSON, AND PETER B. HARRINGTON

Center for Intelligent Chemical Instrumentation, Department of Chemistry and Biochemistry, 175 Clippinger Laboratories, Ohio University, Athens, OH 45701, USA

1. Introduction

1.1 Purpose

Rapid and accurate chemical methods of food quality assurance are important for validation and the integrity of the products sold to consumers [1, 2, 3], Food quality standards vary internationally, thus products shipped across international borders are particularly in need of a standardized method of quality assurance [4, 5]. One method to determine the origin of food products is isotope ratio mass spectrometry [IRMS.pdf]. Due to isotopic fractionation, plants from different geographic regions exhibit different
metabolic pathways and rates thus they will accumulate different isotopes ratios of common elements [6]. Plants have three different metabolic pathways to fix carbon during photosynthesis. We may distinguish between C3 plants, C4 plants, and CAM plants (Crassulacean Acid Metabolism). Plants that survive solely on C3 fixation (C3 plants) tend to thrive in areas where both sunlight intensity and temperatures are moderate, carbon dioxide concentrations are around 200 ppm or higher, and ground water is plentiful. The C3 plants, which originated during Mesozoic and Paleozoic eras, predate the C4 plants and still represent approximately 95% of the Earth's plant biomass, including olive trees. C3 plants lose 97% of the water taken up through their roots to transpiration [7]. The isotopic signature of C3 plants shows higher degree of $^{13}$C depletion than the C4 plants [8]. CAM plants, such as Cacti, can grow in dry environments and can resist little and irregular rainfall. The isotope ratio of an olive is not only influenced by metabolic pathways, but also by regional factors such as water-use efficiency.

1.2 Extra Virgin Olive Oils

The word **virgin** implies that the oil was produced by the use of physical, and not chemical, means. Extra-virgin olive oil (EVOO) comes from cold pressing of the olives, contains no more than 0.8% acidity (IOOC Standard), and is judged to have a superior taste. The expectation of extra-virgin and virgin olive oil is that they do not contain refined oil. [9]
1.3 Chemical Composition of Olive Oils

Olive oils contain about 98-99% fatty acids which are bonded in mono-, di- and triesters of glycerol. Natural oils contain triglycerides as the major component (e.g., 98%); trace amounts of diglycerides; and phytonutrients such as vitamins and antioxidants at 1-2% concentrations.

The most dominant fatty acids in olive oil are palmitic (16:0), palmitoleic acid (16:1), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3). These structures are given in Fig. 1.
Figure 1. Structures of the fatty acids detected in olive oil.

Because the polarity of the carboxyl group causes tailing under gas chromatographic separations with polar stationary phases, the acids usually are chemically transformed to fatty methyl esters (FAMEs). Therefore, the polarity and tailing is reduced and the separation can be achieved in an appropriate time window.
1.4 GC/C/IRMS

The differences in carbon isotopic abundances result from the different growing locales and seasons. The analyzed samples representing the different olive oils cannot be distinguished to the point of geographical origin by GC analysis itself. According to the results, the FAME profiles of the two oils were not distinguishable by their chromatograms. Therefore, IRMS was used to distinguish between the olive oils by measuring isotope ratio differences that arise from different regional influences for each FAME (e.g., different atmospheric carbon dioxide concentrations and water-use efficiency). The FAMEs from the oils were evaluated with respect to their isotope ratios, and then statistically compared using two-way analysis of variance (ANOVA).

The isotope ratio $\delta$ is usually measured relative to a standard reference material. For this study, values were normalized to the carbon from carbonate in the shells obtained from Vienna Pee Dee Belemnite (VPDB). The $\delta$ is calculated as

$$
\delta = \frac{1000(R_{\text{Sample}} - R_{\text{Standard}})}{R_{\text{Standard}}} 
$$

(1)

for which $R_{\text{Sample}}$ is the abundance ratio of the heavier isotope to the lighter isotope (e.g., $^{13}\text{C}/^{12}\text{C}$). The $R_{\text{Standard}}$ values are usually selected because they represent a stable material which is highly enriched in the heavier isotope. Most substances are depleted
with respect to the heavy isotope in comparison to the standard, and therefore δ values less than zero are expected.[10]

2. Experimental

2.1 Materials

Six bottles of commercial extra virgin olive oils (New Market, Athens, OH) were purchased: 3 bottles of “Azeite de Oliveira Virgem Extra” (origin: Portugal) and 3 bottles of “Sultan 100 % Extra Virgin” (origin: Turkey). The color of the Portuguese oils varied noticeably. The first bottle (A1) was yellow-clear while the other bottles (A2 and A3) could be described as yellow-green. Oil from each bottle was analyzed as a separate sample. A Triglyceride C16-C22 Standard Mix (LA83308, Supelco, Bellfonte, PA, USA) was used to help identify the fatty acids.

2.2 Sample Preparation

For the preparation of the FAMEs, the AOAC method [11] was modified as follows: 0.5 g of the oil sample was weighed into a 30-mL vial; 5 mL of 0.5 M methanolic sodium hydroxide solution was added for saponification and heated to 60-70 °C for 10 min. Then 4 mL of 14% BF₃ in methanol was carefully added to the vials
(attention: strong bubbling occurs!) and heated at the same temperature for another 10 min.

The solutions were periodically stirred with a glass rod. After cooling to room temperature the oily drops in the solution disappeared. The fatty acid methyl esters were extracted three days later. The samples were stored in glass screw cap vials and secured with Parafilm.

For the extraction 10 mL of saturated sodium chloride solution and 5 mL of n-hexane (reagent grade) were added to the sample vials. The organic phase was separated from the aqueous and transferred to another 5-mL vial. A spatula of anhydrous sodium sulfate was added to the vial to remove any residual water. The organic phase was then decanted into a new 5-mL vial, sealed with a screw-cap, wrapped with Parafilm, and stored in a freezer.

2.3 GC/C/IRMS

Gas chromatography/mass spectrometry (GC/MS) analyses were performed using a GC (Trace GC, Thermo Finnigan, Waltham, Massachusetts) with an IRMS detector (Delta plus Advantage, Thermo Finnigan, Waltham, Massachusetts). The GC effluent was split using a low-dead-volume X-connector so that approximately 10% of the effluent flowed to the single quadrupole mass spectrometer for structural elucidation and 90% to the IRMS for isotopic analysis. The GC effluent was directed into a combustion oven to convert the organic materials to carbon dioxide prior to ionization.
Figure 2. Schematic of the GC/C/IRMS used in this work.

Note the use of a single quadrupole mass spectrometer to identify compounds based on their fragmentation patterns prior to combustion and isotope ratio analysis.

In GC/C/IRMS, ion chromatograms of three isotopic peaks are collected by three individual ion collectors with different sensitivities, i.e. $m/z$ 44 ($^{12}\text{C}^{16}\text{O}_2$), the isobaric ions $m/z$ 45 ($^{13}\text{C}^{16}\text{O}_2$ and $^{12}\text{C}^{16}\text{O}^{17}\text{O}$), and $m/z$ 46 ($^{12}\text{C}^{16}\text{O}^{18}\text{O}$). Samples were injected using an autosampler (AS3000, Thermo Finnigan, Waltham, Massachusetts). The data acquisition was accomplished with the standard software of the instrument Isodat 2.0 (Thermo Finnigan, Waltham, Massachusetts).
Different temperature programs were used to achieve the best separation results.

While the chromatographic peaks of methyl palmitoleate and methyl palmitate could be measured with good resolution ($Rs = 1.9$) by applying temperature program 1 and splitless injection, methyl linoleate, methyl oleate, and methyl stearate could not be resolved at these conditions. To achieve separation for those substances, temperature program 2 and a split ratio of 1:10 was used.

Due to a high concentration of methyl oleate the combustion chamber was flushed with He for 830 s after the injection while using temperature program 1 to reduce the
concentration of CO₂ entering the reduction oven. The flush was turned off after 1820 s for the remaining duration of the run. When applying temperature program 2 the combustion chamber did not need to be flushed in order to limit the CO₂ from entering into the reduction oven because the injection was split with a 1:10 ratio.

3. Results

3.1 Quantitative determination of major components

The contents of the olive oils were quantified relatively using the peak areas. These results are reported in Table 1.

Table 1.

Average Relative Quantities of Olive Oil Components with 95% Confidence Intervals

<table>
<thead>
<tr>
<th></th>
<th>A – Portuguese [%]</th>
<th>B – Turkish [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl Palmitoleate C16:1</td>
<td>0.5 ± 0.3</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>Methyl Palmitate C16:0</td>
<td>15 ± 2</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>Methyl Linoleate C18:2</td>
<td>2.1 ± 0.4</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>Methyl Oleate C18:1</td>
<td>79 ± 5</td>
<td>75 ± 5</td>
</tr>
<tr>
<td>Methyl Stearate C18:0</td>
<td>2.0 ± 0.4</td>
<td>3.5 ± 0.3</td>
</tr>
</tbody>
</table>
3.2 Two-way analysis of variance

A total of four FAME Peaks were observed. Unfortunately, only three peaks gave reproducible isotope ratios. The three peaks correspond to the FAMEs methyl palmitoleate, methyl palmitate, and methyl oleate.

The first evaluation compared the isotope ratios of the three FAME peaks. If the peaks do not vary significantly they would not be useful for multivariate methods like PCA or LDA.

The other factor that was studied, was whether the oils from two different origins had different or similar isotope ratios. Two-way ANOVA was applied to investigate the differences among the three FAME peaks and two geographical sources. The results calculated by MS Excel and are given in Table 2.

Table 2.

Two-way ANOVA for Peaks, Geographic Origin (Oils), and Interaction

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F crit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peaks</td>
<td>12.6</td>
<td>2</td>
<td>6.29</td>
<td>18.4</td>
<td>2×10⁻⁷</td>
<td>3.10</td>
</tr>
<tr>
<td>Oils</td>
<td>12.1</td>
<td>1</td>
<td>12.1</td>
<td>35.4</td>
<td>5×10⁻⁸</td>
<td>3.95</td>
</tr>
<tr>
<td>Interaction</td>
<td>0.541</td>
<td>2</td>
<td>0.270</td>
<td>0.791</td>
<td>0.457</td>
<td>3.10</td>
</tr>
<tr>
<td>Within</td>
<td>30.8</td>
<td>90</td>
<td>0.342</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>56.0</td>
<td>95</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The factors of peaks and origins were significantly different at a 95% level of significance. Interaction between these two factors was insignificant.

3.3 Principal component analysis

Figure 3 gives the distribution of olive oil samples with respect to the isotope ratios of the three FAMES. One can see from this figure that by using only a single isotope ratio that the two geographic regions cannot be resolved.

\[ \delta^{13}C/\delta^{12}C \] values (\(x=\)Portuguese, \(o=\)Turkish).

Figure 4 is a plot of the principal component scores. The scores can be understood as a linear combination of the pretreated isotope ratios while the loadings express the
relative weighting of the isotope ratio. Before PCA, the data were normalized to unit vector and mean-centered.

\[ \text{PC1 [‰] (76.1 %)} \]
\[ \text{PC2 [‰] (21.8 %)} \]

Figure 4. Principal component scores of the normalized, mean-centered isotope ratios of the three FAME peaks

The scores of the Portuguese oils formed a larger cluster than the Turkish oils. The Portuguese oils varied with respect to color as well and their isotope ratios were less precise, as given in Table 3.

Table 2

Average $\delta$ Values of Methyl Ester Peaks and 95% Confidence Intervals
Additionally, the Turkish oil isotope ratios clustered by bottle, whereas the Portuguese oils did not. ANOVA was used with one factor as the geographic region and the other factor was the three different GC peaks. In Table 2, the results indicate that each GC peak and corresponding FAME gave a different isotope ratio. Interaction was not significant, so the isotope ratios for each peak and oil combination did not vary significantly.

### 3.4 Hierarchical Cluster Analysis

The measured isotope ratios were now used to calculate the Euclidean distance matrix. A MATLAB script was written to draw a dendrogram using an average linkage algorithm. The dendrogram is given in Figure 5.
Figure 5. Hierarchal clustering using a dendrogram (Portuguese, 1-9, Turkish, 10-18).

Bottles are consecutive groups of 3.

Good clustering is achieved from the isotope ratios among the two olive oils. The isotope ratios were not distinct for each bottle.

3.5 Linear Discriminant Analysis

Linear discriminant analysis calculates a linear function of the variables, $D$, which maximizes the ratio of the between-group variance to the within-group variance.

Geometrically, the discriminant is a line through the clusters of points, such that the
projections of the points of the two groups are separated as much as possible (Fig. 6).

[13]

Figure 6. Situation where principal component (PC) and linear discriminant function (DF) are essentially the same (a) and orthogonal (b).[13]

LDA [14] was used to establish collected classifier for the olive oils. The following model [15] was applied to discriminate between the two geographic origins of the oils
or using matrices

\[ D = w_0 + w_1 x_1 + w_2 x_2 + \cdots + w_n x_n \]  

(2)

\[ d_i = w_0 + w^T x_i \]  

(3)

\[ w_0 = \frac{1}{2} (x_{1i} - x_{2i})^T S^{-1} (x_{1i} - x_{2i}) \]  

(4)

\[ w^T = (x_{1i} - x_{2i})^T S^{-1} \]  

(5)

\[ S = \frac{A^T A + B^T B}{n_1 + n_2 - k} \]  

(6)

\( X \) – Training set matrix (Isotope Ratios of FAMEs)

\( w \) – Weights of the variables

\( w_0 \) – Bias value (should equal zero for standardized data)

\( \bar{x} \) – Sample mean vectors, that describe the location of the centroids in \( m \)-dimensional space of each class

\( S \) – Pooled variance-covariance matrix

\( n_1 \) – Number of objects in class A

\( n_2 \) – Number of objects in class B

\( A \) – Data matrix of A isotope ratios

\( B \) – Data matrix of B isotope ratios

\( k \) – Number of classes
This leads to the following linear discriminant function (7), where \( x_1, x_2 \) and \( x_3 \) represent the standardized \( ^{13}\text{C} / ^{12}\text{C} \) isotope ratios from each FAME peak (\( w_0 \) equals Zero). If \( d_i \) is greater than zero the sample belongs to the class A, while a smaller linear discriminant than zero indicates membership of class B (Figure 7).

![Linear Discriminant D](image)

\[ d_i = 2992x_1 + 1713x_2 + 699.3x_3 \]

(7)
4. Conclusion

The results show that classification between extra virgin olive oils of different origins is possible and applicable by isotope ratio mass spectrometry of the FAMEs. It was shown that by modeling three different FAME peaks, enhanced resolution of the geographic origin was obtained relative to using the total isotope ratio of the oil.

The major component, oleic acid, was so large relative to the other fatty acids, that it caused sometimes caused problems with the reduction oven. Selective extraction methods of the FAME headspace might prevent this FAME from saturating the reduction oven.

5. Acknowledgement

The study was supported by Ohio University, The Center for Intelligent Chemical Instrumentation and NSF grant number 0745590 (GPJ). AB is thankful for the ISAP exchange program (DAAD, Germany) between Leipzig University, Germany and Ohio University, USA which together funded a travel abroad program.
6. References

REFERENCES


15. Clough, R.; Evans, P.; Catterick, T.; Evans, E., $\delta^{34}$S measurements of sulfur by multicollector inductively coupled plasma mass spectrometry. *Analytical Chemistry* 2006, 78, 6126-6132.


51. Harrison S; Monahan, F. Z., A; Bahar, B; Moloney, A; Scrimgeour, C; Schmidt, O, Three-dimensional growth of bovine hoof as recorded by carbon stable


70. Bergslien, E., Teaching to avoid the "CSI Effect" keeping the science in forensic science. *Journal of Chemical Education* **2006**, 83, (5), 690-691.


90. Sewenig, S.; Fichtner, S.; Holdermann, T.; Fritschi, G.; Neumann, H., Determination of $\delta^{13}$C$_{PDB}$ and $\delta^{15}$N$_{AIR}$ values of cocaine from a big seizure in Germany by stable isotope ratio mass spectrometry. *Isotopes in Environmental and Health Studies* **2007**, *43*, (4), 275-280.


98. Vetter, W.; Gleixner, G., Compound-specific stable carbon isotope ratios ($\delta^{13}$C values) of the halogenated natural product 2,3,3',4,4',5,5' - heptachloro -1'-methyl-1,2'-bipyroole (Q1). Rapid Communications in Mass Spectrometry 2006, 20, 3018-3022.


102. Hall, J.; Barth, J.; Kalin, R., Routine analysis by high precision gas chromatography/mass selective detector/isotope ratio mass spectrometry to 0.1 parts per mil. Rapid Communications in Mass Spectrometry 1999, 13, 1231-1236.


110. *Strengthening forensic science in the United States: A path forward*. National research council: Committee on identifying the needs of the forensic sciences community; committee on applied and theoretical statistics, **2009**.


167. Petzke, K.; Lemke, S., Hair protein and amino acid $^{13}$C and $^{15}$N abundances take more than 14 weeks to clearly prove influences of animal protein intake in young women with a habitual daily protein consumption of more than 1 g per kg body weight. *Rapid Communications in Mass Spectrometry* **2009**, 23, 2411-2420.


