Application of Speciated Isotopes Dilution Mass Spectrometry to the Assessment of Human Health and Toxic Exposure

Timothy Fahrenholz

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APPLICATION OF SPECIATED ISOTOPE DILUTION MASS SPECTROMETRY TO
THE ASSESSMENT OF HUMAN HEALTH AND TOXIC EXPOSURE

A Dissertation
Submitted to the Bayer School of Natural and Environmental Sciences

Duquesne University

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

By
Timothy M. Fahrenholz

December 2011
APPLICATION OF SPECIATED ISOTOPE DILUTION MASS SPECTROMETRY TO THE ASSESSMENT OF HUMAN HEALTH AND TOXIC EXPOSURE

By

Timothy M. Fahrenholz

Approved November 3, 2011

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ABSTRACT

APPLICATION OF SPECIATED ISOTOPE DILUTION MASS SPECTROMETRY TO
THE ASSESSMENT OF HUMAN HEALTH AND TOXIC EXPOSURE

By

Timothy M. Fahrenholz

December 2011

Dissertation supervised by Professor H. M. “Skip” Kingston

Previous work by our research group demonstrated that quantitative chemical
analysis of analytes, such as mercury and chromium species, in environmental matrices
could be successfully carried out without using calibration curves and with correction for
species interconversion by using EPA Method 6800A. This method encompasses isotope
dilution mass spectrometry (IDMS) and speciated isotope dilution mass spectrometry
(SIDMS), both of which are described in detail in chapter 1. Research described in this
dissertation expands upon our earlier work by applying the method to the speciation of
mercury in biological matrices, the speciation of glutathione in red blood cells and whole
blood, and the analysis of enzyme activity in mammalian tissue.
For example, in an experiment involving mercury speciation in a hair sample (chapter 2), EPA Method 6800A corrected for the conversion of over 70% of the C$_2$H$_3$Hg$^+$ to Hg$^{2+}$, as well as other conversions that occurred, so that the correct concentrations of all mercury species present could be obtained.

EPA Method 6800A was also used for the analysis of glutathione in red blood cells in two autism studies (chapter 3). In one of the studies, analysis of 16 matched pairs of control and autistic samples demonstrated that red blood cell total glutathione concentrations were higher in samples from autistic patients than ones from controls ($p=0.012$), suggesting that the autistic patients studied may be making more glutathione in response to a larger toxic burden of heavy metals such as lead, antimony, aluminum, and cadmium, compared to controls.

Another application of EPA Method 6800A involved the analysis of alpha–Galactosidase A (GLA) activity in liver, kidney, and brain tissues harvested from mice as part of the development of a gene therapy animal model for Fabry’s disease (chapter 4). In this study, EPA Method 6800A successfully distinguished between samples from gene deficient and wild type mice, as well as mice exposed to two different dosages for gene therapy treatment.

The successful application of EPA Method 6800A in biological matrices described in this dissertation should serve to foster further usage of it in fields such as medicine, toxicology, and agriculture.
DEDICATION

I want to dedicate this dissertation to two people, in particular. First, I dedicate this dissertation to Steve Becker, a brother in Christ and friend from Baltimore who spent many years working on behalf of Mother Angelica and the Eternal Word Television Network (EWTN). During the last seven years of his life, he endured tremendous suffering, as he spent most of that time either in a hospital or a nursing home. Despite the pain he went through, he displayed a level of unwavering faith that served as an inspiration to everyone who had the privilege of knowing him. He was already a tremendous witness for Christ during his years working on behalf of EWTN; he was an even greater witness during his years of suffering. He went to his eternal reward in 2006.

I also dedicate this dissertation to Dr. Arthur Spanier, who was my supervisor, colleague, and friend from my days working for the U.S. Department of Agriculture. Like Steve, Art also displayed courage and persistence during his periods of suffering. Despite undergoing treatment for lung cancer, congestive heart failure, and symptoms of multiple sclerosis, he demonstrated tremendous resiliency by producing quality work as leader of our research team. The high quality of his work was, unfortunately, not appreciated by everyone at the time at which it was produced. His efforts were, however, justly recognized by the Royal Society of Chemistry, which accepted for publication much of the work completed by our research team in a book titled Food and Flavor Chemistry: Explorations Into the 21st Century. Art was also highly instrumental in enabling me to gain acceptance into the graduate program at Duquesne by writing a wonderful letter of recommendation on my behalf. He went to his eternal reward in 2005.
I first want to thank the Lord for blessing me with the ability to do the work described in this dissertation. All of the Glory belongs to Him.

I also want to thank my parents, Thomas and Donna Fahrenholz, for all of the support and encouragement that they have given me over the years. I owe them a debt of gratitude that can never be repaid. My two brothers, Thomas and David, and my sister, Kristen, have also supported and encouraged me, and I thank them as well.

I want to thank my research advisor, Dr. H.M. “Skip” Kingston, for his service to me during my graduate years at Duquesne. He has helped me in ways too numerous to mention. I also thank Dr. Lynne Divis, Dr. Rita Mihailescu, and Dr. Stephanie Wetzel for taking the time out of their busy schedules to serve on my dissertation committee. Dr. Wetzel also took time out of her schedule to train me on the mass spectrometry instrumentation that the Department of Chemistry at Duquesne has. I thank her for that as well.

I also want to acknowledge the members of the Kingston research group and collaborators, past and present, who have helped me over the years, such as Dr. G.M. Mizanur Rahman, Dr. John Kern, Greg Zinn, Becky Wagner, Panxi Zhao, Yosip Vargas, Hemasudha Chatragadda, Becky Peckar, Josh Seither, and Bryan Seybert.

I wish to acknowledge the members of the instrumentation staff at the Bayer School of Natural and Environmental Sciences, Dave Hardesty, Dan Bodnar, and Lance Crosby. The help that they have given over the years has been tremendous.
For the funding of our research, I want to acknowledge the Richard King Mellon Foundation and the Heinz Foundation for their support of our autism research. I also want to especially acknowledge Applied Isotope Technologies, Inc. (AIT), and Matt Pamuku, the CEO of that company, in particular, for supporting much of the research described in this dissertation.

Finally, I want to thank Jeff Thomas, Doug Orth, and other staff members from Agilent Technologies for the helpful training and suggestions that they gave pertaining to the Agilent instrumentation used to produce the data described in this dissertation.
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Introduction: Human Environmental Health and The Importance of Mercury and Glutathione Speciation: the toxicant and immune system

Environmental health is a relatively new field, and yet it has been known anecdotally for decades and is a result of our industrial revolution that has forever altered our environment. There is great concern about how toxicants which are being introduced into the environment are impacting lifespans and the quality of people’s lives. Of the over 80,000 chemicals registered with the U.S. Environmental Protection Agency, only about 200 have undergone extensive testing for neurotoxicity (1). It has been suggested by the National Academy of Sciences that 3% of developmental disorders may be attributed to exposure to environmental toxicants and that another 25% could result from a combination of environmental and genetic factors (2). As the environment evolves, it is necessary to respond to these new toxicological insults; however, new metrologies are only now being developed to keep pace with this evolution of environment and health related repercussions.

This dissertation is a study of both the toxicants and the immune system components. It is both the development of metrology and its application to children’s neurological health. We will begin in this introductory chapter by discussing a specific area of environmental concern (mercury) and progress to the immune system and the human response. In subsequent chapters, research pertaining to the metrology enabling the assessment of both will also be discussed.
I. The Toxicant and Species of Mercury

Mercury is a ubiquitous element in the environment which has been known for centuries. It was called “quicksilver” by Aristotle (3), and historical evidence suggests that it was used for gold extraction as far back as about 290 A.D. (4). The uptake, distribution, and excretion pathway of mercury in the body depends on its species (3). Elemental mercury (Hg⁰), which can enter the atmosphere from the activity of volcanoes and from coal fired power plants (5), can come into the body through inhalation and penetrate the blood-brain barrier, with the main non-occupational exposure of it coming from dental amalgams (5). Inorganic mercury (Hg²⁺) primarily targets the kidney, from which it can be eliminated through the urine (5). Sources of Hg²⁺ include cosmetic creams and batteries (5). Methylmercury (CH₃Hg⁺) is a neurotoxin (6), and the main source of human exposure to it comes from the consumption of fish (7). Tables 1 - 3 are taken from the FDA website and show the mercury levels in various types of fish (8). It should be noted that the legal limit for mercury in commercial fish in the United States is 1 ppm (9). For pregnant mothers, nursing mothers, women of childbearing age, and small children, the FDA recommends avoiding fish that is high in mercury, such as swordfish, and limiting intake of low mercury fish, such as salmon, to 12 ounces per week (10).
### Table 1. Fish and Shellfish With Highest Levels of Mercury

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>MERCURY CONCENTRATION (PPM)</th>
<th>NO. OF SAMPLES</th>
<th>SOURCE OF DATA</th>
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<tbody>
<tr>
<td></td>
<td>MEAN</td>
<td>MEDIAN</td>
<td>STDEV</td>
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<tr>
<td>Mackerel King</td>
<td>0.730</td>
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<td>N/A</td>
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<td>Shark</td>
<td>0.968</td>
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<td>Swordfish</td>
<td>0.976</td>
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<tr>
<td>Tilefish (Gulf of Mexico)</td>
<td>1.450</td>
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<td>N/A</td>
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### Table 2. Fish and Shellfish With Lower Levels of Mercury

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<td>STDEV</td>
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<td>N/A</td>
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<td>N/A</td>
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<td>0.084</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Cod</td>
<td>0.005</td>
<td>0.087</td>
<td>0.080</td>
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<td>Crab 1</td>
<td>0.069</td>
<td>0.030</td>
<td>0.112</td>
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<td>Crawfish</td>
<td>0.033</td>
<td>0.035</td>
<td>0.012</td>
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<td>Croach Atlantic</td>
<td>0.072</td>
<td>0.073</td>
<td>0.036</td>
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<tr>
<td>Flatfish 2*</td>
<td>0.045</td>
<td>0.035</td>
<td>0.049</td>
</tr>
<tr>
<td>Haddock (Atlantic)</td>
<td>0.031</td>
<td>0.041</td>
<td>0.021</td>
</tr>
<tr>
<td>Hake</td>
<td>0.014</td>
<td>ND</td>
<td>0.021</td>
</tr>
<tr>
<td>Herring</td>
<td>0.044</td>
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<td>N/A</td>
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<tr>
<td>Jacksmelt</td>
<td>0.108</td>
<td>0.060</td>
<td>0.115</td>
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<td>Lobster (Spiny)</td>
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<td>Mackerel Atlantic</td>
<td>0.050</td>
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<td>Mackerel Chub (Pacific)</td>
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<td>N/A</td>
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<td>ND</td>
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<td>ND</td>
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<td>ND</td>
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<td>Salmon (Fresh/Frozen) *</td>
<td>0.014</td>
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<td>0.013</td>
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<td>Scallop</td>
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<td>N/A</td>
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<tr>
<td>Shad American</td>
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<td>N/A</td>
</tr>
<tr>
<td>Shrimp *</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Squid</td>
<td>0.070</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Tilapia *</td>
<td>0.010</td>
<td>ND</td>
<td>0.023</td>
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<tr>
<td>SPECIES</td>
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<td>NO. OF SAMPLES</td>
<td>SOURCE OF DATA</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-------------------------------</td>
<td>----------------</td>
<td>---------------------</td>
</tr>
<tr>
<td></td>
<td>MEAN</td>
<td>MEDIAN</td>
<td>STDEV</td>
</tr>
<tr>
<td>TROUT (FRESHWATER)</td>
<td>0.072</td>
<td>0.025</td>
<td>0.143</td>
</tr>
<tr>
<td>TUNA (CANNED, LIGHT)</td>
<td>0.118</td>
<td>0.075</td>
<td>0.119</td>
</tr>
<tr>
<td>WHITEFISH</td>
<td>0.069</td>
<td>0.054</td>
<td>0.067</td>
</tr>
<tr>
<td>WHITING</td>
<td>ND</td>
<td>ND</td>
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<table>
<thead>
<tr>
<th>SPECIES</th>
<th>MERCURY CONCENTRATION (PPM)</th>
<th>NO. OF SAMPLES</th>
<th>SOURCE OF DATA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEAN</td>
<td>MEDIAN</td>
<td>STDEV</td>
</tr>
<tr>
<td>BASS (SALTWATER, BLACK, STRIPED)</td>
<td>0.219</td>
<td>0.130</td>
<td>0.227</td>
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<tr>
<td>BASS CHILEAN</td>
<td>0.386</td>
<td>0.303</td>
<td>0.564</td>
</tr>
<tr>
<td>BLUEFISH</td>
<td>0.337</td>
<td>0.303</td>
<td>0.127</td>
</tr>
<tr>
<td>BUFFALOFISH</td>
<td>0.19</td>
<td>0.14</td>
<td>#</td>
</tr>
<tr>
<td>CARP</td>
<td>0.14</td>
<td>0.14</td>
<td>#</td>
</tr>
<tr>
<td>CROAKER WHITE (Pacific)</td>
<td>0.287</td>
<td>0.280</td>
<td>0.069</td>
</tr>
<tr>
<td>GROUPER (ALL SPECIES)</td>
<td>0.465</td>
<td>0.410</td>
<td>0.293</td>
</tr>
<tr>
<td>HALIBUT</td>
<td>0.252</td>
<td>0.200</td>
<td>0.233</td>
</tr>
<tr>
<td>LOBSTER (NORTHERN/AMERICAN)</td>
<td>0.310</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>LOBSTER (Species Unknown)</td>
<td>0.169</td>
<td>0.182</td>
<td>0.089</td>
</tr>
<tr>
<td>MACKEREL SPANISH (Gulf of Mexico)</td>
<td>0.454</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>MACKEREL SPANISH (S. Atlantic)</td>
<td>0.182</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>MARLIN *</td>
<td>0.485</td>
<td>0.390</td>
<td>0.237</td>
</tr>
<tr>
<td>MONKFISH</td>
<td>0.180</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>ORANGE ROUGHy</td>
<td>0.554</td>
<td>0.563</td>
<td>0.148</td>
</tr>
<tr>
<td>PERCH (Freshwater)</td>
<td>0.14</td>
<td>0.15</td>
<td>#</td>
</tr>
<tr>
<td>SABLEFISH</td>
<td>0.220</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>SCORPIONFISH</td>
<td>0.286</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>SHEEPSHEAD</td>
<td>0.128</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>SKATE</td>
<td>0.137</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>SNAPPER</td>
<td>0.189</td>
<td>0.114</td>
<td>0.274</td>
</tr>
<tr>
<td>TILEFISH (Atlantic)</td>
<td>0.144</td>
<td>0.099</td>
<td>0.122</td>
</tr>
</tbody>
</table>
Another form of mercury, ethylmercury ($\text{C}_2\text{H}_5\text{Hg}^+$), is a metabolic product of Thimerosal, a preservative that was commonly used in childhood vaccines and is still used in the influenza vaccine (11). The structure of Thimerosal is shown in Figure 1:

![Thimerosal structure](image)

**Table 3. Mercury Levels of Other Fish and Shellfish**

<table>
<thead>
<tr>
<th>Fish Type</th>
<th>Mercury Level (ppm)</th>
<th>ND</th>
<th>FDA Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>TUNA (CANNED, ALBACORE)</td>
<td>0.353</td>
<td>0.339</td>
<td>0.126</td>
</tr>
<tr>
<td>TUNA (FRESH/FROZEN, ALBACORE)</td>
<td>0.303</td>
<td>0.322</td>
<td>0.269</td>
</tr>
<tr>
<td>TUNA (FRESH/FROZEN, BIGEYE)</td>
<td>0.357</td>
<td>0.355</td>
<td>0.152</td>
</tr>
<tr>
<td>TUNA (FRESH/FROZEN, SKIPJACK)</td>
<td>0.639</td>
<td>0.560</td>
<td>0.184</td>
</tr>
<tr>
<td>TUNA (FRESH/FROZEN, YELLOWFIN)</td>
<td>0.205</td>
<td>N/A</td>
<td>0.073</td>
</tr>
<tr>
<td>TUNA (FRESH/FROZEN, Species Unknown)</td>
<td>0.325</td>
<td>0.270</td>
<td>0.220</td>
</tr>
<tr>
<td>TUNA (FRESH/FROZEN, Species Unknown)</td>
<td>0.414</td>
<td>0.339</td>
<td>0.316</td>
</tr>
<tr>
<td>WICKFISH (SEA TROUT)</td>
<td>0.250</td>
<td>0.168</td>
<td>0.225</td>
</tr>
</tbody>
</table>


Mercury was measured as Total Mercury except for species (*) when only Methylmercury was analyzed.

ND - mercury concentration below detection level (Level of Detection (LOD)=0.01 ppb)
N/A - data not available

*The following species have been removed from the tables:
- Bass (freshwater) - not commercial
- Pickerel - not commercial

\(^2\) Standard deviation data generated for new data 2004 or later only.

\(^1\) Includes: Blue, King, Snow
\(^2\) Includes: Flounder, Plaice, Sole
\(^3\) Includes: Sea bass/ Striped Bass/ Rockfish

**NOTE:** On February 8, 2005, technical changes were made to the data that was posted on January 19, 2005. The changes corrected data or more properly characterized the species of fish or shellfish sampled.
There has been much controversy over the years regarding the possible link between autism and the usage of Thimerosal in vaccines. Recent large scale studies seem to suggest the absence of such a link, although some have speculated that certain autistic children may have greater genetic susceptibility to mercury toxicity, which could in turn activate latent autism upon exposure (12).

So far in this introduction, several species of mercury have been described. One of the biomolecules involved in the body’s defense against mercury species and other toxicants is glutathione. This important biomolecule is described in the next section.

II. Species of Glutathione

Glutathione (GSH) is a tripeptide consisting of glutamate, cysteine, and glycine and is synthesized in every cell the body (13). The structure of GSH is shown in Figure 2:

![Figure 2 – GSH](image)

It is involved in several important functions, such as antioxidant activity and xenobiotic detoxification (14). The key functional role is played by the cysteine thiol group, which is involved in conjugation and reduction reactions (15). Cysteine (the rate limiting amino
acid for GSH synthesis), can be synthesized by the body through a series of steps from methionine, but according to Dr. S. Jill James, et al., this pathway appears to function poorly in autistic children, thereby effectively rendering cysteine an essential amino acid for them (16). The James research group has also analyzed GSH, glutathione disulfide (GSSG – disulfide dimer formed from two GSH molecules, shown in Figure 3), and total GSH (tGSH) in plasma samples from autistic children and healthy controls and found that autistic children have lower plasma tGSH levels as well as lower plasma GSH/GSSG ratios (often used as an indication of oxidative stress level) compared to the healthy controls (13).

Figure 3 – GSSG
Strategies that are used to boost GSH levels include the consumption of protein foods with cysteine (17) and the intake of supplements which function as cysteine precursors, such as N-acetylcysteine (NAC), the structure of which is shown in Figure 4 (18).

III. Mercury – GSH Conjugates

As mentioned earlier, glutathione plays an important role in the detoxification of xenobiotics. For example, Hg\textsuperscript{2+} can bind to two GSH molecules as indicated in Figure 5, at which point it is either eliminated in the urine in that form or further metabolized and then eliminated in the urine (21).

Figure 4 – N-acetylcysteine (NAC)

Figure 5 – Hg\textsuperscript{2+} conjugated with two GSH molecules
With respect to CH$_3$Hg$^+$, evidence suggests that an important step in its detoxification is the secretion of a CH$_3$Hg$^+$ - GSH conjugate, shown in Figure 6, from liver cells into bile (20).

![Figure 6 – CH$_3$Hg$^+$ conjugated with GSH](image)

In the body, the CH$_3$Hg$^+$ - GSH conjugate can be converted to a CH$_3$Hg$^+$ - cysteine conjugate, which structurally resembles the amino acid methionine (20), and evidence suggests that it is this resemblance which enables the CH$_3$Hg$^+$ - cysteine conjugate to penetrate the blood-brain barrier (21). As shown in Figure 7 below, in a pregnant woman, some of the CH$_3$Hg$^+$ taken in through the diet can get converted to Hg$^{2+}$ and eliminated in the feces, some can enter the brain, some can be eliminated through hair, and some make it past the placenta into the unborn child (20). It can also enter the kidney, liver, cardiac muscle, and skeletal muscle as well (21), and in blood, most of the CH$_3$Hg$^+$ is concentrated in the red blood cells, rather than the plasma (20).
A study by Adams et al. in which GSH in red blood cells and heavy metals in urine from autistic children were analyzed before and after chelation therapy suggested that exposure to toxic metals other than mercury, such as lead, aluminum, cadmium, and antimony results in higher than normal GSH levels due to the body’s increased GSH synthesis in response to exposure to those metals, while exposure to mercury results in lower than normal GSH levels, possibly due to the inhibition of GSH synthesis by the mercury (22). The study also suggested that, as the body burden of toxic metals is lowered, the concentration of GSH in red blood cells normalizes (22). Additionally, a separate study by James et al. showed that depleted levels of GSH in neuroblastoma and glioblastoma cells resulted from the exposure of those cells to Thimerosal (23), the preservative with the C_2H_5Hg^+ group mentioned the first part of this introduction which some have suggested might play a role in the onset of autism in a vulnerable genetic subset of the population.
IV. Metallothionein and GSH

Studies also show that there is a synergistic relationship between GSH and Metallothionein (MT), a cysteine rich protein which stores zinc and is also involved in heavy metal detoxification. Specifically, GSH inhibits the release of zinc from MT while GSSG activates it, and the greater the level of oxidative stress, the greater the amount of zinc that is released from MT in response, which may be due to the need for zinc as part of an antioxidant defense mechanism (24). This relationship is illustrated in Figure 8, which was modified from Kang, Y. (24) by Hemasudha Chatragadda, a graduate student in the Kingston Research group at Duquesne University, in collaboration with Dr. Martha Herbert from Harvard University.

![Metallothionein Redox Cycle](image)

Figure 8 – Relationship between MT and GSH, modified from Kang, et al. (24) by Hemasudha Chatragadda, a graduate student in the Kingston Research group at Duquesne University
V. Goals of the Research Projects Described in this Dissertation

According to the Center for Disease Control, on average, 1 in 110 children in the United States has an autism spectrum disorder (ASD) (25). The prevalence is even higher in other countries, such as South Korea, where about 1 in 38 children has an ASD (26). With the high prevalence of autism in several locations, a significant amount of research is being conducted to try to determine what is causing such a high prevalence, as well as how to detect autism early in life, as evidence suggests that early intervention can have a positive impact on these autistic children (27). The discovery and use of autism biomarkers may enable medical professionals to make an early diagnosis of autism so that they can implement early intervention strategies. One of the goals of the research described in this dissertation is to follow up on the work of Dr. S. Jill James and others with respect to the potential use of GSH as a biomarker for autism.

Another goal of the research described in this dissertation is to improve upon already existing technology for the speciation of mercury in biological matrices such as hair and blood. This can help with clinical evaluation of one’s level of mercury toxicity, as some forms of mercury are more toxic than others.

An additional goal of the research described in this dissertation is to improve upon existing technology for the evaluation of enzyme activity. The successful achievement of this goal can also have applications in the diagnosis and treatment of diseases.
The research undertaken to achieve all of these goals involved the implementation of advanced methodology using special sample preparation techniques along with mass spectrometry. This advanced methodology is described chapter 1.
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Chapter 1

Metrology: Overview of Isotope Dilution Mass Spectrometry (IDMS) and Speciated Isotope Dilution Mass Spectrometry (SIDMS)

Metrology is the study of measurement. The International Union of Pure and Applied Chemistry (IUPAC) recognizes only four fundamental measurement types that are definitive. Definitive by definition means that all the biases and errors can be accessed, measured and known and corrected for to achieve a reliable and bias free measurement possessing known accuracy and precision. Over the past 60 years the science and art of Isotope Dilution Mass Spectrometry (IDMS), has evolved and developed. IDMS, which involves spiking a given sample with a known concentration and amount of a stable isotopically enriched form of the analyte of interest, equilibrating the elemental or molecular forms so that both spike and natural are in exactly the same species form, then performing a mass spectrometry measurement on the equilibrated sample, and determining the concentration of the analyte based upon peak ratios, was originally developed during the 1950s for elemental analysis (1). Frequently in IDMS, the analyte species are converted to one form so that there is only one species for both the natural and the spike analyte. Over the years, the usage of enriched isotopes for quantitative mass spectrometry analysis has been applied to fields such as environmental chemistry (2), clinical chemistry, pharmacokinetics, and toxicology (3). An equation for IDMS is shown in Figure 1 solved for the concentration of the analyte (4):
\[ C_x = \left( \frac{C_s W_s}{W_x} \right) \left( \frac{A_s - R_m B_s}{R_m B_x - A_x} \right) \]

Figure 1 – IDMS equation

Where:

- \( A_x \) = abundance of isotope A in the natural abundant analyte in the sample
- \( B_x \) = abundance of isotope B in the natural abundant analyte in the sample
- \( A_s \) = abundance of isotope A in the enriched analyte in the spike
- \( B_s \) = abundance of isotope B in the enriched analyte in the spike
- \( C_x \) = concentration of the natural abundant analyte in the sample, which is what the equation is solving for
- \( C_s \) = concentration of enriched analyte in the spike in \( \mu \)mol/g
- \( W_x \) = weight of the sample
- \( W_s \) = weight of the spike
- \( R_m \) = measured ratio of isotope A to isotope B (obtained from the mass spectrometry measurements)

An example of an IDMS calculation with tGSH is shown next:
Application of IDMS to GSH

When GSH is analyzed by tandem electrospray mass spectrometry (ESI-MS-MS), the collision energy can be adjusted such that the cysteinylglycine product ion (resulting from the loss of pyroglutamate – 129 g/mol) is obtained and is the most abundant product ion (5), as shown below in Figure 2:

![Figure 2 – Fragmentation of GSH in the collision cell resulting in the formation of the cysteinylglycine product ion of interest (in the green box)](image)

Natural abundant GSH (307 g/mol):

- Most abundant isotope detected at 308 m/z in positive mode ESI-MS
- Main product ion (depending on collision energy) detected at 179 m/z in MS/MS mode (loss of pyroglutamate)

Enriched GSH - has two $^{13}$C’s and one $^{15}$N on the glycine (310 g/mol)

- Most abundant isotope detected at 311 m/z in positive mode ESI-MS
- Main product ion (depending on collision energy) detected at 182 m/z in MS/MS mode (loss of pyroglutamate)
**IDMS calculation of tGSH concentration:**

- \( A_x \) = abundance of 182 m/z product ion in the natural abundant analyte in the sample (0.00341)
- \( B_x \) = abundance of 179 m/z product ion in the natural abundant analyte in the sample (0.881)
- \( A_s \) = abundance of 182 m/z product ion in the enriched analyte in the spike (0.881)
- \( B_s \) = abundance of 179 product ion in the enriched analyte in the spike (0.00224)
- \( C_x \) = concentration of GSH in the sample (unknown)
- \( C_s \) = concentration of enriched GSH in the spike in µmol/g (2.09)
- \( W_x \) = weight of the sample (0.247 g)
- \( W_s \) = weight of the spike (0.251 g)
- \( R_m \) = measured ratio of 182 m/z product ion to 179 product ion (obtained from the mass spectrometry measurements) (1.21)
- \( C_x = [((2.09 \mu\text{mol/g})(0.251g))/(0.247g)] x [(((0.881 \text{–} (1.21 \times 0.00224)))/(1.21 \times 0.881) \text{–} 0.00341)) = 1.76 \mu\text{mol/g}]
- Molecular weight of GSH = 307 µg/µmol
- \( C_x \) in µg/g (or ppm) = (1.76 µmol/g)(307 µg/µmol) = 540 ppm

Speciated Isotope Dilution Mass Spectrometry (SIDMS) entails spiking a sample with known amounts of stable isotopically enriched analyte species, achieving
equilibration of spike and analyte species, performing mass spectrometry on the
equilibrated sample, and determining concentrations based upon ratios and the extent of
interconversion between or among species (4). This method, which was patented by H.M.
Skip Kingston, represented an advancement beyond traditional IDMS by correcting for
species interconversions that occurred during sample preparation and/or measurement
(6). These two methods (IDMS and SIDMS) constitute EPA Method 6800A (4).

Examples of SIDMS equations will be presented later in this section.

EPA Method 6800 A does not use traditional calibration curves and has several
advantages over the more traditional calibration curve method of analysis. Among them
include the absence of several sources of error common to the calibration curve method,
as illustrated in Figure 3 below:

Figure 3 Comparing error sources from calibration curve analysis vs EPA Method 6800A

As can be seen in Figure 3, the number of error sources from the usage of EPA 6800A is
significantly less than that from the usage of calibration curves. Only mass bias, dead
time correction, isobaric interferences, and possible signal suppression of the excess isotope over the limited isotope remain for the former method.

EPA Method 6800A also represents an improvement over the more common internal standard method, which involves spiking the sample with a known amount of an element or a molecular compound (depending on the application), performing mass spectrometry on the sufficiently prepared sample (in its mass spectrometry application), and calculating the concentration of the analyte based upon the intensity of the analyte signal relative to the intensity of the internal standard signal (similar to, but not exactly the same as, the method which utilizes the equation in figure 1). Both methods have the advantage over the calibration curve method of enabling the analyst to correct for fluctuations in signal intensity and for reductions in sample recovery; however, if one is attempting to measure two or more species of an element or a molecular compound, and there are interconversions between or among the species of interest, SIDMS can correct for these interconversions.

SIDMS is particularly suited for the measurement of two species in which one can convert to the other, such as the related species pair of GSH and GSSG. One of the more common analyses that has given researchers difficulty with respect to conversion of one species to another over the years has been that of GSH and GSSG, a particularly important analysis from which one can obtain the GSH/GSSG ratio. As mentioned in the introduction, this ratio is often used as an indicator of oxidative stress and has been shown to be lower in plasma samples from autistic children than in ones from healthy controls (7).
To elaborate on the challenges with GSH and GSSG analysis, a potential difficulty that researchers have had has been the conversion of the former to the latter during sample preparation (8). A reagent often used to prevent this conversion is N-Ethylmaleimide (abbreviated NEM) (9), which blocks the thiol group on the GSH as shown below in Figure 4:

![Figure 4 – GSH NEM reaction](image)

An additional characteristic of NEM is that it inhibits Glutathione reductase (GR), an enzyme which converts GSSG back to GSH (8). SIDMS can complement the usage of NEM by correcting for imperfections in its usage, such as the possible conversion of
GSH to GSSG before the NEM had a chance to react fully and the reduction in recovery of GSSG, which can result from the reaction of NEM with its amines (7).

In describing the SIDMS equations for GSH and GSSG analysis that are designed to correct for conversion of GSH to GSSG, it is best to start off with a simple analogy. If one has several slices of wheat bread and several slices of white bread, one can make three types of sandwiches: ones with two slices of wheat bread, ones each with a slice of wheat bread and a slice of white bread ("hybrid" sandwiches), and ones with two slices of white bread. In a similar manner, if natural abundant GSH (307 g/mol) and isotopically enriched GSH with two $^{13}\text{C}$'s and one $^{15}\text{N}$ (310 g/mol) are equilibrated and oxidation occurs, one can end up with three types of oxidized GSSG: natural abundant GSSG (612 g/mol) from two molecules of natural abundant GSH (analogous to wheat bread sandwiches), partially enriched GSSG (615 g/mol) with two $^{13}\text{C}$’s and one $^{15}\text{N}$ from the combination of one natural abundant GSH molecule and one isotopically enriched GSH molecule (analogous to the "hybrid" sandwiches), and GSSG with four $^{13}\text{C}$’s and two $^{15}\text{N}$’s (618 g/mol) from two molecules of enriched GSH (analogous to the white bread sandwiches). One can determine how much of the GSH converted to GSSG by spiking the sample with enriched GSSG in which the enrichment is different from the enriched GSSG that comes from oxidation (such as GSSG that has four $^{13}\text{C}$’s (616 g/mol).

Suppose one has 1 mole of wheat bread slices ($6.02 \times 10^{23}$ slices), referred to as “n” and the same number of white bread slices, referred to as “m,” that gets converted to sandwiches. Mathematical relationships pertaining to the number of each kind of sandwich formed, which were worked out by Dr. John Kern from the Mathematics department at Duquesne University (10), are described on the following pages.
\( \phi = \# \text{ of ways to select } k \text{ items from } w = w!/[(k! \cdot (w-k)!)] \)

e.g. if \( w=9 \) and \( k=2 \),

\[
\phi = (9 \times 8 \times 7 \times 6 \times 5 \times 4 \times 3 \times 2 \times 1)/(2!)(7 \times 6 \times 5 \times 4 \times 3 \times 2 \times 1) = (9 \times 8)/2 = 36
\]

If \( w \) is an extremely large number (such as \( 6.02 \times 10^{23} \)) and \( k = 2 \), for all practical purposes, \( \phi = w^2/2 \)

The total number of sandwich combinations = \(((n + m)!)/k! \cdot ((n + m)-k)!\)

\[
= [(6.02 \times 10^{23} + 6.02 \times 10^{23}) \cdot (6.02 \times 10^{23} + 6.02 \times 10^{23} - 1)]/2
\]

\[
= [(12.04 \times 10^{23})(12.04 \times 10^{23} - 1)]/2
\]

(neglect -1) = \((12.04 \times 10^{23})^2/2 = 7.25 \times 10^{47} \) sandwiches

\[
= (n + m)^2/2
\]

The probability (expressed as a decimal) of getting a wheat bread sandwich

\[
= [(n^2)/2]/[(n + m)^2/2]
\]

\[
= [(6.02 \times 10^{23})^2/2]/[(12.04 \times 10^{23})^2/2]
\]

\[
= (6.02 \times 10^{23})^2 / (12.04 \times 10^{23})^2 = 0.250
\]

The probability (expressed as a decimal) of getting a white bread sandwich

\[
= [(m^2)/2]/[(n + m)^2/2]
\]

\[
= [(6.02 \times 10^{23})^2/2]/[(12.04 \times 10^{23})^2/2]
\]

\[
= (6.02 \times 10^{23})^2 / (12.04 \times 10^{23})^2 = 0.250
\]

The probability (expressed as a decimal) of getting a sandwich with one slice of wheat bread and one slice of white bread = \(n \cdot m/[(n + m)^2/2]\)

\[
= 2mn/(n + m)^2
\]
\[
\frac{(2)(6.02 \times 10^{23})(6.02 \times 10^{23})}{(6.02 \times 10^{23} + 6.02 \times 10^{23})^2}
\]

= 0.500

The same 0.500 answer can be obtained by using units of moles instead

\[
\frac{(2)(1\text{mole})(1\text{mole})}{(1\text{mole}+1\text{mole})^2}
\]

= 0.500

These mathematical relationships can be applied to GSH and GSSG analysis. Let “n” = the µmol of natural abundant 307 g/mol GSH that became oxidized either to natural abundant 612 g/mol GSSG or “hybrid” 615 g/mol GSSG. Let “m” = the µmol of isotopically enriched 310 g/mol GSH that became oxidized to either “hybrid” 615 g/mol GSSG or fully enriched 618 g/mol GSSG. The ratio of the probability of getting “hybrid” 615 g/mol GSSG (sandwich with one slice of wheat bread and one slice of white bread) to the probability of getting fully enriched 618 g/mol GSSG (sandwich with two slices of white bread) equals the ratio of the peak areas from LC/MS/MS analysis of the peaks in the chromatogram representing those species of GSSG (after taking into account the abundances of those isotopes and subtracting out the contribution coming from natural abundant GSSG).

In positive mode ESI-MS, the most abundant isotope from natural abundant 612 g/mol GSSG is detected at 613 m/z, that from “hybrid” 615 g/mol GSSG with two $^{13}$C’s and one $^{15}$N is detected at 616 m/z, that from enriched 616 g/mol GSSG with four $^{13}$C’s is detected at 617 m/z, and that from enriched 618 g/mol GSSG with four $^{13}$C’s and two $^{15}$N’s (which came from two 310 g/mol GSH’s that each had two $^{13}$C’s and one $^{15}$N) is detected at 619 m/z. In positive mode electrospray tandem mass spectrometry (ESI-MS/MS) with the collision energy adjusted so as to make the most abundant product ion
that which results from the loss of two pyroglutamates from GSSG (loss of 258 g/mol),
the numbers above change to 355 m/z, 358 m/z, 359 m/z, and 361 m/z, respectively. The
mathematical relationship described on the next two pages can be used to determine GSH
and GSSG concentrations.

(358 m/z peak area)/(361 m/z peak area)
= (probability of getting “hybrid” 615 g/mol GSSG)/(that of getting 618 g/mol GSSG)
= [(2mn)/(n+m)^2]/[(m^2)/(m+n)^2]
= 2n/m

m = µmol of 310 g/mol GSH that became oxidized
= (2 x µmol of 618 g/mol GSSG present) + (µmol of 615 g/mol GSSG present)

The mass of the solution of the enriched 310 g/mol GSH that was added is known,
as is the mass and concentration of the enriched 616 g/mol GSSG solution. One can
determine the concentration (and, thus, µmol) of the 618 g/mol GSSG and 615 g/mol
GSSG present by performing IDMS on both with the 616 g/mol GSSG representing the
enriched analyte. One µmol of 618 g/mol GSSG comes from two µmols of 310 g/mol
GSH, and one µmol of 615 g/mol GSSG comes from one µmol each of 307 g/mol GSH
and 310 g/mol GSH. Using this relationship, one can determine “m” (the µmol of 310
g/mol GSH that became oxidized). Once “m” is known, one can determine “n” (the µmol
of natural abundant 307 g/mol GSH that converted to either 612 g/mol GSSG or 615
g/mol GSSG) using the following relationship

[(358 m/z peak area)/((361 m/z peak area)*2)]*m
Since the mass of the sample (e.g. blood sample, plasma sample, etc.) is known, one can determine the concentration of GSH that became oxidized to GSSG in units of µmol/g (and, subsequently, µg/g) and perform the necessary subtraction from the uncorrected GSSG concentration and the necessary addition to the uncorrected GSH concentration.

Another area in which SIDMS can be applied is mercury speciation. As mentioned in the introduction, different species of mercury have different levels of toxicity and different target organs, hence the need for the analysis of the individual mercury species, rather than just total mercury, in order to determine one’s level of toxic exposure. Dr. G.M. Mizanur Rahman from Duquesne University described in his doctoral dissertation from 2004 the SIDMS equations that can be used for Hg$^{2+}$ and CH$_3$Hg$^+$ speciation, which are shown below and on the next pages (11):

For Hg$^+$

$$R_{199/202}^{Hg^{2+}} = \left[ (199) A_x C_{x}^{Hg^{2+}} W_x + 199 A_s^{Hg^{2+}} C_{s}^{Hg^{2+}} W_s^{Hg^{2+}} \right] (1 - \alpha) + \left[ (199) A_x C_{x}^{CH,Hg^{+}} W_x + 199 A_s^{CH,Hg^{+}} C_{s}^{CH,Hg^{+}} W_s^{CH,Hg^{+}} \right] \beta$$

$$R_{201/202}^{Hg^{2+}} = \left[ (201) A_x C_{x}^{Hg^{2+}} W_x + 201 A_s^{Hg^{2+}} C_{s}^{Hg^{2+}} W_s^{Hg^{2+}} \right] (1 - \alpha) + \left[ (201) A_x C_{x}^{CH,Hg^{+}} W_x + 201 A_s^{CH,Hg^{+}} C_{s}^{CH,Hg^{+}} W_s^{CH,Hg^{+}} \right] \beta$$

- $n$ = µmol of 307 g/mol GSH that became oxidized
For $\text{CH}_3\text{Hg}^+$

$$R_{199/202}^{\text{CH}_3\text{Hg}^+} = \left[ (109 A_{s} C_{x}^{\text{CH}_3\text{Hg}^+} W_{x} + 199 A_{s}^{\text{Hg}^2+} C_{s}^{\text{Hg}^2+} W_{s}^{\text{Hg}^2+}) \alpha + (199 A_{s}^{\text{CH}_3\text{Hg}^+} C_{s}^{\text{CH}_3\text{Hg}^+} + 199 A_{s}^{\text{CH}_3\text{Hg}^+} C_{s}^{\text{CH}_3\text{Hg}^+} W_{s}^{\text{CH}_3\text{Hg}^+}) (1 - \beta) \right] /$$

$$\left[ (202 A_{s} C_{x}^{\text{Hg}^2+} W_{x} + 202 A_{s}^{\text{Hg}^2+} C_{s}^{\text{Hg}^2+} W_{s}^{\text{Hg}^2+}) \alpha + (202 A_{s}^{\text{CH}_3\text{Hg}^+} C_{s}^{\text{CH}_3\text{Hg}^+} + 202 A_{s}^{\text{CH}_3\text{Hg}^+} C_{s}^{\text{CH}_3\text{Hg}^+} W_{s}^{\text{CH}_3\text{Hg}^+}) (1 - \beta) \right]$$

$$R_{201/202}^{\text{CH}_3\text{Hg}^+} = \left[ (201 A_{s} C_{x}^{\text{Hg}^2+} W_{x} + 201 A_{s}^{\text{Hg}^2+} C_{s}^{\text{Hg}^2+} W_{s}^{\text{Hg}^2+}) \alpha + (201 A_{s}^{\text{CH}_3\text{Hg}^+} C_{s}^{\text{CH}_3\text{Hg}^+} + 201 A_{s}^{\text{CH}_3\text{Hg}^+} C_{s}^{\text{CH}_3\text{Hg}^+} W_{s}^{\text{CH}_3\text{Hg}^+}) (1 - \beta) \right] /$$

$$\left[ (202 A_{s} C_{x}^{\text{Hg}^2+} W_{x} + 202 A_{s}^{\text{Hg}^2+} C_{s}^{\text{Hg}^2+} W_{s}^{\text{Hg}^2+}) \alpha + (202 A_{s}^{\text{CH}_3\text{Hg}^+} C_{s}^{\text{CH}_3\text{Hg}^+} + 202 A_{s}^{\text{CH}_3\text{Hg}^+} C_{s}^{\text{CH}_3\text{Hg}^+} W_{s}^{\text{CH}_3\text{Hg}^+}) (1 - \beta) \right]$$

Where,

$R_{199/202}^{\text{Hg}^2+}$ is the isotope ratio of $^{199}\text{Hg}$ to $^{202}\text{Hg}$ of $\text{Hg}^2+$ in the spiked sample (unknown)

$R_{201/202}^{\text{Hg}^2+}$ is the isotope ratio of $^{201}\text{Hg}$ to $^{202}\text{Hg}$ of $\text{Hg}^2+$ in the spiked sample (unknown)

$R_{199/202}^{\text{CH}_3\text{Hg}^+}$ is the isotope ratio of $^{199}\text{Hg}$ to $^{202}\text{Hg}$ of $\text{CH}_3\text{Hg}^+$ in the spiked sample (unknown)

$R_{201/202}^{\text{CH}_3\text{Hg}^+}$ is the isotope ratio of $^{201}\text{Hg}$ to $^{202}\text{Hg}$ of $\text{CH}_3\text{Hg}^+$ in the spiked sample (unknown)

$A_{s}$ is the relative isotope abundance of $^{199}\text{Hg}$ in the sample

$A_{s}$ is the relative isotope abundance of $^{202}\text{Hg}$ in the sample

$A_{s}$ is the relative isotope abundance of $^{201}\text{Hg}$ in the sample

$A_{s}^{\text{Hg}^2+}$ is the relative isotope abundance of $^{199}\text{Hg}$ in the $^{199}\text{Hg}^2+$ spike

$A_{s}^{\text{Hg}^2+}$ is the relative isotope abundance of $^{202}\text{Hg}$ in the $^{199}\text{Hg}^2+$ spike

$A_{s}^{\text{Hg}^2+}$ is the relative isotope abundance of $^{201}\text{Hg}$ in the $^{199}\text{Hg}^2+$ spike
\[ ^{199}A_s \text{CH}_3\text{Hg}^+ \] is the relative isotope abundance of \(^{199}\text{Hg}\) in the \(\text{CH}_3\text{^{201}Hg}^+\) spike

\[ ^{202}A_s \text{CH}_3\text{Hg}^+ \] is the relative isotope abundance of \(^{202}\text{Hg}\) in the \(\text{CH}_3\text{^{201}Hg}^+\) spike

\[ ^{201}A_s \text{CH}_3\text{Hg}^+ \] is the relative isotope abundance of \(^{201}\text{Hg}\) in the \(\text{CH}_3\text{^{201}Hg}^+\) spike

\[ C_x^{\text{Hg}^2+} \] is the concentration of \(\text{Hg}^2+\) in the sample (µmol/g, unknown)

\[ C_x^{\text{CH}_3\text{Hg}^+} \] is the concentration of \(\text{CH}_3\text{Hg}^+\) in the sample (µmol/g, unknown)

\[ W_s \] is the weight of the sample (g)

\[ C_s^{\text{Hg}^2+} \] is the concentration of \(\text{Hg}^2+\) in the \(^{199}\text{Hg}^2+\) spike (µmol/g)

\[ W_s^{\text{Hg}^2+} \] is the weight of the \(^{199}\text{Hg}^2+\) spike (g)

\[ C_s^{\text{CH}_3\text{Hg}^+} \] is the concentration of \(\text{CH}_3\text{Hg}^+\) in the \(\text{CH}_3\text{^{201}Hg}^+\) spike (µmol/g)

\[ W_s^{\text{CH}_3\text{Hg}^+} \] is the weight of the \(\text{CH}_3\text{^{201}Hg}^+\) spike (g)

\[ \alpha \] is the proportion of \(\text{Hg}^2+\) transformed to \(\text{CH}_3\text{Hg}^+\) (unknown)

\[ \beta \] is the proportion of \(\text{CH}_3\text{Hg}^+\) transformed to \(\text{Hg}^2+\) (unknown)

In more recent years, our laboratory has used \(\text{CH}_3\text{^{200}Hg}^+\) in place of \(\text{CH}_3\text{^{201}Hg}^+\), in part because the \(^{201}\text{Hg}\) isotope is now used for enriched \(\text{C}_2\text{H}_5\text{Hg}^+\) (\(\text{C}_2\text{H}_5\text{^{201}Hg}^+\)) so as to enable one to perform SIDMS with three Hg species (\(\text{Hg}^2+\), \(\text{CH}_3\text{Hg}^+\), and \(\text{C}_2\text{H}_5\text{Hg}^+\)) using SIDMS equations for three-species analysis that were also described in Dr. Rahman’s dissertation (11). It was considered important to add \(\text{C}_2\text{H}_5\text{Hg}^+\) to the number of mercury species analyzed is because, as mentioned in the introduction, it is a metabolic product of Thimerosal, a preservative still used in some vaccines, such as the influenza vaccine (12).

In summation, the speciation of glutathione and mercury is of critical toxicological importance and can be achieved without calibration curves by using EPA
Method 6800A. The following chapters will describe these applications and developments for these specific analyses.
References:


4. EPA Method 6800 website link:


10. Personal communication with Dr. John Kern, professor of Mathematics at Duquesne University, Pittsburgh, PA.


12. A.W. McMahon, J.K. Iskander, P. Haber, M.M. Braun, R. Ball, Inactivated influenza vaccine (IIV) in children <2 years of age: Examination of selected adverse events reported to the Vaccine Adverse Event Reporting System (VAERS) after thimerosal-free or thimerosal-containing vaccine, Vaccine, 2008, 26, 427 – 429.
Chapter 2 – Development and Refinements of Application of IDMS and SIDMS to Mercury Speciation in Specific Matrices

2 A. Mercury Speciation in Hair

As mentioned in the introduction, one of the fates of CH\textsubscript{3}Hg\textsuperscript{+} in the body is elimination through the hair (1). A normal concentration of hair mercury is between 0.4 and 6 ppm (2), with most (over 90%) of the mercury consisting of CH\textsubscript{3}Hg\textsuperscript{+} (3). According to the EPA, a toxic threshold for hair mercury is 10 ppm (4). Evidence indicates hair mercury concentration increases with increasing fish consumption (5).

During the sample preparation and analysis of samples with both CH\textsubscript{3}Hg\textsuperscript{+} and Hg\textsuperscript{2+}, methylation of inorganic mercury and demethylation of CH\textsubscript{3}Hg\textsuperscript{+} occur. IDMS is capable of analyzing total mercury but it is not capable of correcting for these interconversions of the two related species of mercury. For these corrections, SIDMS, in its most applicable form as described in EPA method 6800A, must be applied. In the application of 6800A, there are optimizations that can be applied, and these are part of the scientific contribution demonstrated in this chapter.

In addition to CH\textsubscript{3}Hg\textsuperscript{+}and Hg\textsuperscript{2+}, C\textsubscript{2}H\textsubscript{5}Hg\textsuperscript{+}, which is a metabolic product of Thimerosal, has also been detected in human hair (6,7), as well as blood (7) from persons exposed to Thimerosal. Studies from mice and rats suggest that C\textsubscript{2}H\textsubscript{5}Hg\textsuperscript{+}from Thimerosal can be converted to Hg\textsuperscript{2+} in mammals (8,9), and a study of infants exposed to Thimerosal suggests that mercury from the Thimerosal exposure can be eliminated through the stools (10). More research needs to be undertaken to determine the fates of C\textsubscript{2}H\textsubscript{5}Hg\textsuperscript{+} in the body.
To illustrate the importance of accounting for possible species interconversion, as well as the importance of taking into consideration percent recovery of the analytes of interest, a set of experiments was completed in our laboratory in which several literature hair sample preparation protocols were assessed by traditional external calibration. This set of experiments is described in the next section.

2A i. Analysis of Mercury Species in hair using published sample preparation protocols with traditional external calibration

Materials and Methods

Reagents/standards:

The hair standard used was a certified reference material (IAEA – 085) from the International Atomic Energy Agency (Vienna, Austria) that was certified for total mercury and methylmercury. The water used for solutions was double deionized (DDI) water from a Barnstead NANOpure Ultrapure Water System (Dubuque, IA). Reagent grade nitric acid, hydrochloric acid, hydrogen peroxide, L-cysteine, and ethanol were obtained from Fisher Scientific (Pittsburgh, PA). Both nitric acid and hydrochloric acid were purified using sub-boiling distillation in a quartz still (Milestone, Monroe, CT).

Instrumentation/analytical methods:

For analysis of the prepared samples, liquid chromatography-inductively coupled plasma-MS (LC-ICP-MS) analysis was performed using isocratic elution with a Waters (Milford, MA) Novapak C18 reversed phase column (150 x 3.9 mm, 4 µm) and a 709 IC
polymeric inert pump with an 838 advanced sample processor for automatic sample
delivery from Metrohm Peak (Houston, TX) coupled to an Agilent 4500 ICP-MS
(Agilent Technologies, Palo Alto, CA). The mobile phase consisted of 0.4% cysteine, pH 5. The flow rate was 1.0 ml/min, and the injection volume was 100 µl. The
chromatography and automation equipment were situated in a high efficiency particulate air (HEPA) filter class 100 clean hood chamber next to the ICP-MS in a class 1000 clean room laboratory. For data evaluation using external calibration, quantification was based on chromatographic peak area by monitoring the signal for the $^{202}\text{Hg}$ isotope. For each sample preparation method, four sample preparation replicates and four analysis replicates were completed (n=16).

Sample preparation methods used from the literature (note – four sub samples of the mercury hair samples were prepared in each case, procedural blanks were prepared with the samples for quality assurance purposes):

Method 1 (from reference 11)

For acid leaching with 2 M HCl, four 50 mg sub-samples of the hair reference material were each placed into a 15-mL disposable glass centrifuge tube with a snap-on cap, to which two drops of ethanol and 5 ml of 2 M HCl were added. A small amount of mercury-free cotton was added to prevent the hair from floating. Each centrifuge tube was tightly capped and heated at 100°C in a water bath for 5 minutes. After cooling, the sample solutions were then stirred well using a vortex mixer and centrifuged for 5
minutes at 3200 rpm to separate the hair. Each cooled extract was transferred to a tarred 50-mL polypropylene centrifuge tube and diluted to 20 mL by adding DDI water.

**Method 2 (from reference 12)**

For acid leaching with 2 M HCl, four 100 mg portions of hair sample were placed into 15 mL disposable glass centrifuge tubes with a snap on caps, to which 2 ml of 2M HCl were added. The centrifuge tubes were tightly capped and heated at 100°C in a water bath for 15 minutes. The sample solutions were then centrifuged for 5 minutes at 3200 rpm to separate the hair. The cooled extracts were transferred into tarred 50 mL polypropylene graduated centrifuge tubes and diluted to 20 mL by adding DDI water.

**Method 3 (from reference 13)**

For acid leaching with 4% (v/v) HCl, a 3 ml portion of 4% (v/v) HCl was shaken on a mechanical shaker with 200 mg of hair for 2 minutes. The hair was then separated by centrifugation at 2700 rpm for 20 minutes. The supernatant containing the methylmercury was transferred into a tarred 50 mL polypropylene graduated centrifuge tube. The process was repeated with 2 ml of 4% (v/v) hydrochloric acid, and the combined supernatants were diluted to 20 mL with DDI water. Four sample replicates were prepared.

**Method 4 (from reference 14)**

For acid leaching with 2 M HCl, approximately 100 mg of hair sample was put into a 50 mL polypropylene graduated centrifuge tube, and 5 mL of 2 M HCl were added.
The tubes were closed and shaken on a mechanical shaker for 4 hours. The tubes were then centrifuged at 2500 rpm for 20 minutes to separate the hair. The supernatant containing the methylmercury was transferred into a tarred 50 mL polypropylene centrifuge tube and diluted with DDI water to 20 mL. Four sample preparation replicates were prepared.

**Method 5 (from reference 15)**

For cold acid digestion with 2 M HCl, to prepare four sample preparation replicates, approximately 200 mg of hair sample were weighed into four 50 ml polypropylene graduated centrifuge tubes, and 5 mL of 2 M HCl were added to each tube. The tubes were closed and kept on the bench top for 24 hours. These tubes were then centrifuged at 2500 rpm for 20 minutes to separate the hair. The supernatants containing the methylmercury were transferred into tarred 50 mL polypropylene graduated centrifuge tubes and diluted with DDI water to 20 mL.

**Method 6 (from reference 16)**

For acid leaching with 5 M HNO₃, approximately 50 mg of hair sample was placed into a 15 mL disposable glass centrifuge tube with a snap on cap, to which 350 µL of 5 M HNO₃ were added. The centrifuge tube was loosely capped and heated at 100°C in a water bath for 120 minutes. The sample solution was then stirred well using a vortex mixer and then centrifuged for 5 minutes at 3200 rpm. The cooled extract was transferred into a tarred 50 ml polypropylene centrifuge tube and diluted to 20 mL by adding DDI water. Four sample preparation replicates were completed.
Method 7 (from reference 17)

For cold acid digestion with concentrated HNO$_3$ and 30% H$_2$O$_2$, approximately 100 mg portions of the sample were weighed into four 15 ml centrifuge tubes; 2 mL of concentrated HNO$_3$ and 1 mL of 30% H$_2$O$_2$ were added to each tube. The mixtures were then vortexed and kept on the bench top overnight. The next day, the mixtures were vortexed for 1 minute and then centrifuged for 5 minutes at 3500 rpm. The extracts were transferred into 50 ml polypropylene sample vials and diluted with DDI water to 20 ml.

Results and Discussion

The certified value for total mercury in hair standard IAEA-085 is 23.2 ± 0.8 ppm, and that for methylmercury is 22.9 ± 1.0 ppm. Table 1 shows the results obtained using the seven sample preparation methods described above from the literature and traditional external calibration with LC-ICP-MS. Concentrations are given in ppm, and results are reported at the 95 % C.I. The percent recoveries are given in the parentheses.

<table>
<thead>
<tr>
<th>Method</th>
<th>[Hg$^{2+}$] in ppm (as Hg)</th>
<th>[CH$_3$Hg$^+$] in ppm (as Hg), percent recoveries in parentheses</th>
<th>Sum of species (ppm), percent recoveries in parentheses</th>
</tr>
</thead>
<tbody>
<tr>
<td>certified value</td>
<td>22.9 ± 1.0</td>
<td>23.2 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.63 ± 0.18</td>
<td>20.10 ± 0.26 (88 ± 1)</td>
<td>20.73 ± 0.32 (89 ± 1)</td>
</tr>
<tr>
<td>2</td>
<td>0.23 ± 0.02</td>
<td>19.88 ± 1.36 (87 ± 6)</td>
<td>20.11 ± 1.36 (87 ± 6)</td>
</tr>
<tr>
<td>3</td>
<td>not detected</td>
<td>20.36 ± 1.05 (89 ± 5)</td>
<td>20.36 ± 1.05 (88 ± 5)</td>
</tr>
<tr>
<td>4</td>
<td>0.27 ± 0.29</td>
<td>22.36 ± 0.86 (98 ± 4)</td>
<td>22.63 ± 0.91 (98 ± 4)</td>
</tr>
<tr>
<td>5</td>
<td>not detected</td>
<td>18.38 ± 0.93 (80 ± 4)</td>
<td>18.38 ± 0.93 (79 ± 4)</td>
</tr>
<tr>
<td>6</td>
<td>8.89 ± 0.69</td>
<td>12.83 ± 0.68 (56 ± 3)</td>
<td>21.72 ± 0.97 (94 ± 4)</td>
</tr>
<tr>
<td>7</td>
<td>23.57 ± 0.78</td>
<td>1.62 ± 0.15 (7 ± 1)</td>
<td>25.19 ± 0.79 (109 ± 3)</td>
</tr>
</tbody>
</table>

Table 1 – results from mercury species analysis using literature methods
As can be seen in Table 1, there appears to have been a significant amount of conversion of CH$_3$Hg$^+$ to Hg$^{2+}$ in methods 6 and 7, much more so than in the other five methods. It is interesting to note that the latter two methods involved the usage of HNO$_3$ along with either an elevated temperature (100°C for 2 hours) or a relatively long reaction time (overnight, ~ 16 hours), whereas the other five methods used HCl instead of HNO$_3$. It was also observed that, with the exception of method 4, the percent recovery of CH$_3$Hg$^+$ was less than 100%. With respect to methods 1, 2, 3, and 5, this result was most likely due to incomplete extraction of the CH$_3$Hg$^+$; for methods 6 and 7, this result was likely due, as mentioned above, to the conversion of CH$_3$Hg$^+$ to Hg$^{2+}$.

The results in table 1 clearly indicate the importance of accounting for both species interconversion and sample recovery, as failure to do so would decrease the level of confidence that one has in the final reported results. SIDMS is designed to address both of these issues. In addition, for field applications, a sample preparation method involving the equilibration of natural abundant and enriched analytes on a solid phase extraction (SPE) column for the purpose of using SIDMS technology would be useful as well so that, if unstable analytes partially decompose or interconvert with other analytes before they reach the laboratory, EPA Method 6800A will correct for the transformations. The next section describes experiments used to demonstrate these applications as they pertain specifically to hair mercury speciation.

2 A ii – Analysis of Mercury Species in hair using SIDMS with iSPIKE Methodology

In his 2006 PhD dissertation, David Lineman described the procedure of loading an SPE column with an isotopically enriched analyte and then loading that same column
at some later time with the natural abundant analyte such that equilibration between the former and latter analytes occurred on the column. At that time, only the GC-MS application of this concept was implemented and described. It had been envisioned to follow up on this concept with LC-MS or other types of mass spectrometry applications to investigate the general application of this novel method of IDMS equilibration and spiking. Following elution, mass spectrometry analysis of the eluent would enable one to implement a new type of novel, complete IDMS analysis (18). This procedure is known as the iSpike ® method (registered trademarked of Applied Isotope Technologies, AIT, the “i” refers to “isotope”), and is especially applicable to field sample preparation. It also has been envisioned for clinical and homeland defense applications and patent are pending on these applications. To follow up on this work, an experiment was completed involving the speciation of mercury in hair. In this experiment, an attempt to achieve equilibration between the natural abundant and enriched mercury species on a specialized SPE column was made, and the results using that sample preparation method were compared to those from a method in which equilibration occurred in a microwave vessel. This experiment is described below.

**Materials and Methods**

**Reagents/standards:**

The hair standard used was certified reference material (IAEA – 085) from the International Atomic Energy Agency (Vienna, Austria) that was certified for total mercury and CH$_3$Hg$^+$. The water used for solutions was double deionized (DDI) water from a Barnstead NANOpure Ultrapure Water System (Dubuque, IA). Reagent grade
nitric acid and L-cysteine were obtained from Fisher Scientific (Pittsburgh, PA). Nitric acid was purified using sub-boiling distillation in a quartz still (Milestone, Monroe, CT). Isotopically enriched $^{199}$Hg$^{2+}$ and CH$_3$$^{200}$Hg$^+$ and sulfhydryl cotton fiber SPE were obtained from Applied Isotope Technologies, Inc. (Pittsburgh, PA).

**Instrumentation/analytical methods:**

For microwave sample preparation, a Milestone (Monroe, CT) Ethos 1600 microwave system was used. This system was equipped with temperature feedback control and magnetic stirring, as well as high pressure (maximum pressure – 100 bar) closed vessel microwave vessels made of high purity TFM (a thermally resistant form of fluoropolymer) with a capacity of 100 ml.

For analysis of the prepared samples, direct analysis of the SPE eluents were made with an Agilent 4500 ICP-MS (Agilent Technologies, Palo Alto, CA). Quantification was performed by monitoring the $^{199}$Hg, $^{200}$Hg, and $^{202}$Hg isotopes for both the eluent with the Hg$^{2+}$ and that with the CH$_3$Hg$^+$, and the equations described in chapter 1 were used for SIDMS analysis. For each sample preparation method, three sample preparation replicates and four analysis replicates were completed (n=12). The isotope ratios were corrected for dead time, and a solution with natural abundant Hg$^{2+}$ and CH$_3$Hg$^+$ was also analyzed periodically between samples to obtain the mass bias correction factor.
Sample preparation Method A – equilibration of natural abundant and enriched analytes in the microwave vessel

Procedures described in EPA Method 3200 (19) were used for this experiment. To three microwave vessels were each added a stir bar, 100 mg of certified reference material hair (IAEA-085), known amounts of $^{199}$Hg$^{2+}$ and CH$_3$Hg$^+$, and 10 ml of 4 M HNO$_3$. To a fourth vessel were added everything that was in the other three except the hair standard in order to serve as a procedural blank. Microwave irradiation was applied to the samples with stirring (2 minute ramp to 100$^0$C, 10 minute hold at 100$^0$C). After cooling, each sample was filtered through a 0.22 µm glass filter. To each sample were then added 2 mL of 0.2 M of pH 3.0 acetate buffer, followed by enough 10 N NaOH to adjust the pH of the sample solution to ~ 3 – 4.

For SPE, four sulfhydryl cotton fiber SPE columns were conditioned with 10 mL of H$_2$O, then 10 mL of 6 N HCl, and lastly 15 mL of H$_2$O. The pH adjusted samples were applied to the columns, and 8 ml of a solution containing 1.0 M HCl and 1.0 M NaCl were run through each column, followed by 2 mL of H$_2$O. The eluents were collected and referred to as “eluent 1a, 1b,” etc. Another 8 mL of a solution consisting of 6M HCl, saturated NaCl, and 0.1% CuCl$_2$·2H$_2$O were applied to each column, followed by 2 mL of H$_2$O, and the eluents from the application of this solution was collected as well (referred to as “eluent 2a, 2b,” etc.).
Sample Preparation Method B – Equilibration of natural abundant and enriched analytes on the SPE column

The samples were prepared exactly the way they were in method A except that no $^{199}\text{Hg}^{2+}$ and $\text{CH}_3^{200}\text{Hg}^+$ were added to the microwave vessel; instead, they were added to the conditioned SPE columns. After implementing the microwave protocol and adjusting the pH of the samples to ~ 3 – 4 with the acetate buffer and the 10 N NaOH, the samples were applied to the conditioned and double spiked columns, and the eluents were collected just like in method A.

Results and Discussion

The $\text{CH}_3\text{Hg}^+$, which was less strongly adsorbed to the stationary phase than $\text{Hg}^{2+}$, was detected in eluent 1, and the $\text{Hg}^{2+}$, more strongly adsorbed to the stationary phase, was detected in eluent 2. The reason for this difference in adsorption strength can be seen in the schematic representation of the interaction between these analytes and the stationary phase shown in Figures 1 and 2 below:

Figure 1 – Interaction between $\text{CH}_3\text{Hg}^+$ and the stationary phase – weaker adsorption due to analyte bonding to only one Sulfur on the stationary phase

Figure 2 – Interaction between $\text{Hg}^{2+}$ and the stationary phase – stronger adsorption due to analyte bonding to two Sulfurs on the stationary phase
The results from the analysis of the mercury speciation in hair using methods A and B are shown in Table 2 (values reported at 95% C.I.):

<table>
<thead>
<tr>
<th></th>
<th>Hg$^{2+}$ concentration in ppm</th>
<th>CH$_3$Hg$^+$ concentration in ppm</th>
<th>Sum of Species in ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Certified value</td>
<td>22.9 ± 1.2</td>
<td>23.2 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>Method A –</td>
<td>1.617 ± 0.104</td>
<td>22.698 ± 0.990</td>
<td>23.315 ± 0.995</td>
</tr>
<tr>
<td>equilibration in the microwave vessel</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Method B –</td>
<td>1.400 ± 0.092</td>
<td>22.766 ± 1.301</td>
<td>24.166 ± 1.304</td>
</tr>
<tr>
<td>equilibration on the SPE column</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2 – Mercury Speciation in hair using equilibration in the microwave vessel and equilibration on the SPE column

The results from the mercury speciation in hair using methods A and B were consistent with each other as well as with the certified values. These results demonstrate the usefulness of SIDMS for mercury speciation as well as the fact that equilibration between natural abundant and isotopically enriched mercury species can occur on the SPE column. This equilibration enables one to obtain the correct concentration of the analytes, even if the recovery is less that 100%. This is because the “apparent” recovery remains the same (i.e. if the recovery of the natural abundant analyte is 80%, that of the enriched analyte will also be 80%, so the ratio of the former to the latter will remain constant). Later on in this chapter, an experiment mimicking a 25% reduction in recovery of the analytes of interest will be described to demonstrate these concepts further.

As mentioned earlier, a method capable of analyzing C$_2$H$_5$Hg$^+$ in addition to Hg$^{2+}$ and CH$_3$Hg$^+$ would be useful. In the next section is a description of an experiment in
which these three mercury species are interconverting, and a set of equations based on work done by Dr. Rahman, which are contained in his dissertation (20) and were referred to in the previous chapter, were used to quantitatively successfully determine the concentrations of the three mercury species. These equations are an extension of the two species equations shown earlier, and are available in the literature from the Kingston Research Group in several publications and presentations.

2 A iii – Analysis of three Mercury Species in Hair using SIDMS

As mentioned on p. 17, there is documented evidence of ethylmercury being detected in hair (6,7) and blood (7). To expand upon this work which is already in the literature, an experiment was carried out to demonstrate how SIDMS can correct for interconversions among mercury species, such as \( \text{C}_2\text{H}_5\text{Hg}^+ \), \( \text{Hg}^{2+} \) and \( \text{CH}_3\text{Hg}^+ \) during sample preparation and/or analysis. This experiment is described next.

Materials and Methods

Reagents/standards:

The hair standard used was certified reference material (IAEA – 085) from the International Atomic Energy Agency (Vienna, Austria) that was certified for total mercury and \( \text{CH}_3\text{Hg}^+ \). The water used for solutions was double deionized (DDI) water from a Barnstead NANOpure Ultrapure Water System (Dubuque, IA). Reagent grade nitric acid and L-cysteine were obtained from Fisher Scientific (Pittsburgh, PA). Nitric acid was purified using sub-boiling distillation in a quartz still (Milestone, Monroe, CT). A solution of known concentration of \( \text{C}_2\text{H}_5\text{Hg}^+ \) was supplied by Applied Isotope
Technologies (Pittsburgh, PA). Isotopically enriched $^{199}\text{Hg}^{2+}$, $^{200}\text{Hg}^{+}$, $^{201}\text{Hg}^{+}$ and $^{202}\text{Hg}^{+}$ were also supplied by Applied Isotope Technologies.

**Instrumentation/analytical methods:**

For microwave sample preparation, a Milestone (Monroe, CT) Ethos 1600 microwave system was used. This system was equipped with temperature feedback control and magnetic stirring, as well as high pressure (maximum pressure – 100 bar) closed vessel microwave vessels made of high purity TFM (a thermally resistant form of fluoropolymer) with a capacity of 100 mL.

For analysis of the prepared samples, LC-ICP-MS analysis was performed using isocratic elution with a Waters (Milford, MA) Novapak C$_{18}$ reversed phase column (150 x 3.9 mm, 4 µm) and a 709 IC polymeric inert pump with an 838 advanced sample processor for automatic sample delivery from Metrohm Peak (Houston, TX) coupled to an Agilent 4500 ICP-MS (Agilent Technologies, Palo Alto, CA). The mobile phase consisted of 0.2% cysteine. The flow rate was 1.0 mL/min, and the injection volume was 100 µl. The chromatography and automation equipment were situated in a high efficiency particulate air (HEPA) filter class 100 clean hood chamber next to the ICP-MS in a class 1000 clean room laboratory. Quantification was performed by monitoring the $^{199}\text{Hg}$, $^{200}\text{Hg}$, $^{201}\text{Hg}$ and $^{202}\text{Hg}$ isotopes for the Hg$^{2+}$, CH$_3$Hg$^+$, and C$_2$H$_5$Hg$^+$ peaks in the chromatograms, and isotope ratios for SIDMS analysis were based upon peak area ratios. Two sample preparation replicates and four analysis replicates were completed (n = 8). The isotope ratios were corrected for dead time, and a solution with natural abundant
Hg$^{2+}$, CH$_3$Hg$^+$, and C$_2$H$_5$Hg$^+$ was also analyzed periodically between samples to obtain the mass bias correction factors.

**Sample Preparation**

To a microwave vessel were added a stir bar and 50 mg of IAEA-085 hair standard, and this hair was spiked with a known amount of natural abundant ethylmercury to mimic a hair sample with three mercury species instead of two. To the vessel were then added known amounts of $^{199}$Hg$^{2+}$, CH$_3$$^{200}$Hg$^+$, and C$_2$H$_5$$^{201}$Hg$^+$, along with 10 mL of 4M HNO$_3$. Microwave irradiation was implemented following the protocol described in EPA Method 3200 (14) with a 2 minute ramp to 100$^0$C, followed by a 10 minute hold at 100$^0$C. After filtration through a 0.22 µm filter, the sample was analyzed by LC-ICP-MS as described above. Sample preparation was done in duplicate, with 4 analysis replicates for each sample preparation replicate (n=8). Procedural blanks that included everything except the certified hair standard and spiked natural abundant C$_2$H$_5$Hg$^+$ were prepared and analyzed in a likewise manner.

**Results and Discussion**

The results from the SIDMS analysis of the three mercury species in hair are shown in Table 3 (results reported in units of ppm at 95% C.I.):
<table>
<thead>
<tr>
<th></th>
<th>Hg^{2+}</th>
<th>CH$_3$Hg$^+$</th>
<th>C$_2$H$_5$Hg$^+$</th>
<th>% conv. of Hg$^{2+}$ to CH$_3$Hg$^+$</th>
<th>% conv. of CH$_3$Hg$^+$ to C$_2$H$_5$Hg$^+$</th>
<th>% conv. of C$_2$H$_5$Hg$^+$ to Hg$^{2+}$</th>
<th>% conv. of CH$_3$Hg$^+$ to Hg$^{2+}$</th>
<th>% conv. of C$_2$H$_5$Hg$^+$ to CH$_3$Hg$^+$</th>
<th>% conv. of CH$_3$Hg$^+$ to C$_2$H$_5$Hg$^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Certified Value</td>
<td>22.9 ± 1.4</td>
<td>*</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>SIDMS measured</td>
<td>1.30 ± 0.74</td>
<td>22.06 ± 0.44</td>
<td>21.10 ± 1.07</td>
<td>8.29 ± 0.87</td>
<td>5.36 ± 0.84</td>
<td>1.73 ± 0.33</td>
<td>6.78 ± 0.68</td>
<td>74.31 ± 4.03</td>
<td>1.21 ± 0.42</td>
</tr>
</tbody>
</table>

* Not present originally, spiked so as to obtain 22.7 ppm ± 1% RSD

Table 3 – Results - analysis of Hg$^{2+}$, CH$_3$Hg$^+$, and C$_2$H$_5$ $^{201}$Hg$^+$ in spiked hair standard
The expected values were obtained despite the interconversions among the mercury species, particularly the conversion of $\text{C}_2\text{H}_5\text{Hg}^+$ to $\text{Hg}^{2+}$ (over 70% conversion). These results demonstrate the usefulness of the SIDMS method for correcting for species interconversions so as to enable one to obtain the original analyte concentrations. In this context, the specific application was for mercury speciation in hair. The method can also be used for the determination of mercury species in other matrices as well, one of them being blood. This application of SIDMS to mercury speciation in blood is described in the next section.

**2B – Mercury Speciation in blood**

As in hair, normally, most of the mercury in blood is in the form of methylmercury. The analysis of mercury species in blood is somewhat more challenging than it is in hair, however, due to the lower concentration of mercury in blood, as literature values for the hair to blood mercury ratio have been found to be between 140 and 370 (21). According to the EPA, blood mercury levels are commonly below a level associated with possible health effects, 5.8 µg/ml (about 6 ppb) (22). Over the years, methods for analyzing mercury species in blood have included isotope dilution with alkaline digestion and sodium tetraethylborate derivatization combined with gas chromatography/ICP/MS (GC/ICP/MS) analysis (23) as well as ultrasound extraction combined with LC/ICP/MS analysis with quantification based on external calibration (9). SIDMS can expand upon existing methods like these by taking into account both sample
recovery and species interconversion. Experiments designed to demonstrate this concept are described in the next section.

2B i – Analysis of Mercury Species in Blood with Elevated Mercury Concentration

Materials and Methods

Reagents/Standards

The human blood sample used was obtained from the Stanford University blood bank and was screened for the absence of Hepatitis B and HIV before being sent to Duquesne University. The sample included EDTA as an anticoagulant and, after arriving at Duquesne, was stored at -20°C until usage. Reagent grade nitric acid and L-cysteine were obtained from Fisher Scientific (Pittsburgh, PA). Isotopically enriched $^{199}\text{Hg}^{2+}$ and $\text{CH}_3^{200}\text{Hg}^+$ were obtained from Applied Isotope Technologies, Inc. (Pittsburgh, PA).

Instrumentation/analytical methods:

For microwave sample preparation, a Milestone (Monroe, CT) Ethos 1600 microwave system was used. This system was equipped with temperature feedback control and magnetic stirring, as well as high pressure (maximum pressure – 100 bar) closed vessel microwave vessels made of high purity TFM (a thermally resistant form of fluoropolymer) with a capacity of 100 mL.

For total mercury analysis, a DMA-80 from Milestone (Monroe, CT) was used by following the guidelines stated in EPA Method 7473 (24). This instrument analyzes a sample, such as a blood sample, directly without need of sample preparation by thermally
decomposing it at 750°C such that all of the mercury species are converted to elemental mercury vapor. This mercury vapor then binds to and is subsequently released by a gold amalgamator, after which it is analyzed by atomic absorption mass spectrometry.

For analysis of the samples for mercury speciation, LC-ICP-MS analysis was performed using isocratic elution with a Waters (Milford, MA) Novapak C<sub>18</sub> reversed phase column (150 x 3.9 mm, 4 µm) and a 709 IC polymeric inert pump with an 838 advanced sample processor for automatic sample delivery from Metrohm Peak (Houston, TX) coupled to an Agilent 4500 ICP-MS (Agilent Technologies, Palo Alto, CA). The mobile phase consisted of 0.2% cysteine. The flow rate was 1.0 mL/min, and the injection volume was 100 µl. The chromatography and automation equipment were situated in a high efficiency particulate air (HEPA) filter class 100 clean hood chamber next to the ICP-MS in a class 1000 clean room laboratory. Quantification was performed by monitoring the <sup>199</sup>Hg, <sup>200</sup>Hg and <sup>202</sup>Hg isotopes for the Hg<sup>2+</sup> and CH<sub>3</sub>Hg<sup>+</sup> peaks in the chromatograms, and isotope ratios for SIDMS analysis were based upon peak area ratios. Sample preparation was done in triplicate, and four analysis replicates were completed for each sample preparation replicate (n=12). The isotope ratios were corrected for dead time, and a solution with natural abundant Hg<sup>2+</sup> and CH<sub>3</sub>Hg<sup>+</sup> was also analyzed periodically between samples to obtain the mass bias correction factors.

**Sample preparation**

The Stanford blood sample was tested for total mercury with the DMA-80 and found to have a concentration of 2.70 ± 0.76 ppm. This sample was then spiked with natural abundant Hg<sup>2+</sup> and CH<sub>3</sub>Hg<sup>+</sup> to represent a blood sample with a total mercury
concentration of ~ 400 ppb. Although this value is much higher than what a normal human blood sample would have (less than 5 µg/l, or ~ 5 ppb) (25), cases in which blood mercury concentrations in the triple or quadruple digits ppb have been documented. For example, Dr. Nilo Arnaiz from the UCLA Department of Medicine reported a case in which a 37 year old woman who consumed swordfish daily and ended up with symptoms of mercury poisoning as a result had a blood mercury concentration of 208 µg/l (approximately 200 ppb) (26). Another case was reported in which a 48 year old man deliberately consumed a solution of inorganic mercury salt and ended up in the hospital with mercury poisoning and a blood mercury concentration of 5,200 µg/l (25).

For mercury speciation, to three microwave vessels were each added 0.6000g of the blood with the elevated mercury, known amounts of $^{199}$Hg$^{2+}$ and CH$_3$ $^{200}$Hg$^+$, and 10 ml of 4M HNO$_3$. To a fourth vessel was added everything that was added to each of the other three except the blood so as to serve as a procedural blank. Microwave irradiation was applied to the samples according to the protocol described in EPA Method 3200 (19) (2 minute ramp to 100°C, 10 minute hold at 100°C). After cooling, the samples were filtered through a 0.22 µm filter, then stored at 4°C until analysis.

To mimic what would happen in case of a 25% reduction in recovery, aliquots were taken from the microwave prepared triplicate samples and diluted 25% with 4M HNO$_3$. These samples were analyzed using LC-ICP-MS using conditions similar the ones described earlier in this chapter for the hair studies.

**Results and discussion**

The results obtained from the SIDMS analyses of both the originally prepared
samples and the ones prepared so as to mimic a 25% reduction in recovery are shown in table 4 below (concentrations reported at the 95% C.I.):

<table>
<thead>
<tr>
<th></th>
<th>Hg$^{2+}$ conc. in ppb</th>
<th>CH$_3$Hg$^+$ conc. in ppb</th>
<th>Sum of Hg$^{2+}$ and CH$_3$Hg$^+$ in ppb</th>
<th>Hg$^{2+}$ to CH$_3$Hg$^+$</th>
<th>CH$_3$Hg$^+$ to Hg$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original Sample</td>
<td>220 ± 11</td>
<td>168 ± 9</td>
<td>388 ± 16</td>
<td>1.08% ± 3.01%</td>
<td>1.50% ± 1.55%</td>
</tr>
<tr>
<td>Sample mimicking 25% reduction in recovery</td>
<td>218 ± 9</td>
<td>161 ± 11</td>
<td>379 ± 17</td>
<td>2.02% ± 2.74%</td>
<td>0.14% ± 2.06%</td>
</tr>
</tbody>
</table>

Table 4 – Results from analysis of Hg$^+$ and CH$_3$Hg$^+$ in spiked Stanford blood sample

The elevated mercury sample was also analyzed for total mercury using EPA method 7473 as described earlier. The result obtained was 402 ± 39 ppb, which is in agreement with the results from sum of the mercury species in the SIDMS analyses shown above; therefore, mass balance was achieved.

Despite the fact that the second set of samples were prepared so as to mimic a 25% reduction in recovery when compared to the original samples, SIDMS analysis yielded the same results for Hg$^{2+}$ and CH$_3$Hg$^+$ concentrations from both sets of samples. This is because the calculations for the concentrations were based, not upon calibration curves, but upon isotopic ratios which remain constant regardless of the percentage recovery or the intensity of the signal coming from the mass spectrometer.

Since most human blood samples have mercury species concentrations below 10 ppb, for improved sensitivity, gas chromatography, rather than liquid chromatography, is often used with ICP-MS for this type of analysis (although, as mentioned earlier, an LC-
ICP-MS method for speciation of mercury in blood has been published – see reference number 9). A description of mercury speciation analysis in blood using LC-ICP-MS was described in this section; the next section describes the same kind of analysis using SPME-GC-ICP-MS.

2B ii – Analysis of Mercury Species in Blood by SPME-GC-ICP-MS using SIDMS

Reagents/Standards

The blood standard used was SRM 966 level 2 bovine blood from the National Institute of Standards and Technology (NIST, Gaithersburg, MD). This blood standard was certified for total mercury and had reference values (values not certified due to all of the potential biases not being accounted for) for inorganic mercury and methylmercury. The water used for solutions was double deionized (DDI) water from a Barnstead NANOpure Ultrapure Water System (Dubuque, IA). Reagent grade nitric acid was obtained from Fisher Scientific (Pittsburgh, PA). Isotopically enriched $^{199}\text{Hg}^{2+}$ and $\text{CH}_3^{200}\text{Hg}^+$ were obtained from Applied Isotope Technologies, Inc. (Pittsburgh, PA). Sodium tetrapropylborate was obtained from ABCR (Karlsruhe, Germany).

Instrumentation/analytical methods:

For microwave sample preparation, a Milestone (Monroe, CT) Ethos 1600 microwave system was used. This system was equipped with temperature feedback control and magnetic stirring, as well as high pressure (maximum pressure – 100 bar) closed vessel microwave vessels made of high purity TFM (a thermally resistant form of
fluoropolymer) with a capacity of 100 mL. Quartz vessels (20 ml) were also used for implementation of the vessel within a vessel system (to be described in further detail in the sample preparation section).

For mercury speciation analysis of the prepared samples, SPME-GC-ICP-MS was used. The SPME equipment consisted of an 85 µm Carboxen/PDMS fiber assembly (part number 57334-U, Supelco, subsidiary of Sigma-Aldrich, St. Louis, MO), a Micro-Extraction holder (part number 57330-U, Supelco, subsidiary of Sigma-Aldrich, St. Louis, MO), and 20 ml borosilicate glass headspace vials with septa caps (part number S326-0020, I-CHEM, Rockville, TN). The GC-ICP-MS system consisted of an Agilent 5890 GC connected through a transfer line to an Agilent 4500 ICPMS, and the column used was an Agilent DB-5, 30 m x 0.25 mm x 0.25 µm (Agilent p/n 122-5032). The injection port temperature was 150 °C, and the temperature gradient consisted of an initial 2 minute hold at 60 °C, followed by an increase from 60°C to 120°C (15°C/minute), then and increase from 120°C to 200°C (20°C/minute), and, lastly, a two minute hold at 200°C. Quantification was performed by monitoring the $^{199}$Hg, $^{200}$Hg, and $^{202}$Hg isotopes for the Hg$^{2+}$ and CH$_3$Hg$^+$ peaks in the chromatograms, and isotope ratios for SIDMS analysis were based upon peak area ratios. Three sample replicates and three analysis replicates were completed ($n=9$). A solution with natural abundant Hg$^{2+}$ and CH$_3$Hg$^+$ was also analyzed periodically between samples to obtain the mass bias correction factors.

Sample Preparation

To a quartz microwave vessel were added a Teflon stir bar, 0.300 g of SRM 966 level 2 Bovine blood (certified value for total Hg concentration = 29.8 ± 1.6 ppb, 0.0350 g
of 22.311 ppb $^{199}\text{Hg}^{2+}$, 0.0350g of 24.374 ppb $\text{CH}_3^{200}\text{Hg}^+$, and 7.2 g of 2 M HNO$_3$. For the analytical blank, 0.300g of H$_2$O was used in place of the blood. The small vessel was put into a larger Teflon vessel with 10 mL of 2 M HNO$_3$, and the vessel was subjected to microwave irradiation (2 minute ramp to 100°C, 10 minute hold at 100°C). After cooling, the contents of the vessel were transferred to a centrifuge tube and were centrifuged to separate particulate matter from the extract. 2 g of the extract were combined with 8 g of pH 5.2 ammonium acetate buffer (0.287M acetic acid, 0.710M ammonium acetate) and 100 mg of sodium tetrapropylborate in a 20 mL vial (for derivatization). This solution was stirred at room temperature for ~3 – 5 minutes, after which the SPME fiber (85 μm Carboxen/PDMS) was inserted into the headspace, and the solution was stirred at 65°C for 15 minutes. The SPME was then inserted into the GC-ICP-MS for analysis. Sample preparation was done in triplicate, as was the analysis (n = 9).

**Results and discussion**

The results from the mercury speciation analysis of SRM 966 level 2 are shown in Table 5 (results reported at 95% C.I.):

<table>
<thead>
<tr>
<th>Experimental Hg$^{2+}$ conc. (ppb)</th>
<th>Experimental CH$_3$Hg$^+$ conc. (ppb)</th>
<th>Conversion of CH$_3$Hg$^+$ to Hg$^{2+}$</th>
<th>Conversion of Hg$^{2+}$ to CH$_3$Hg$^+$</th>
<th>Experimental sum of Hg species (ppb)</th>
<th>Certified value for total Hg (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.7 ± 1.2</td>
<td>15.3 ± 1.5</td>
<td>4%</td>
<td>3%</td>
<td>31.9 ± 2.1</td>
<td>29.8 ± 1.6</td>
</tr>
</tbody>
</table>

Table 5 – Results from SIDMS analysis of Hg species in SRM 966 level 2 blood
The experimental value obtained for the sum of the mercury species (31.9 ± 2.1 ppb) was in agreement with the certified value for total mercury in SRM 966 level 2 blood (29.8 ±1.6 ppb).

Future work

Further work on the speciation of three mercury species (Hg$^{2+}$, CH$_3$Hg$^+$, and C$_2$H$_5$Hg$^+$) in blood will be carried out by our research group using SRM 955c level 3 Caprine blood from NIST as a standard, as this standard is certified for the three mercury species mentioned, as well as total mercury. Preliminary work has already been done with blood samples from Stanford University that were spiked with Hg$^{2+}$, CH$_3$Hg$^+$, and C$_2$H$_5$Hg$^+$ after they arrived at Duquesne University. The chromatogram from one of these samples in which the final concentration of each species in the blood was ~ 30 ppb is shown in Figure 3 (signal from $^{202}$Hg):
Preliminary evidence suggests that a significant percentage of the organic mercury species converted to Hg\(^{2+}\) during the measurement; however, the SIDMS technology can correct for these transformations. Additionally, to improve sensitivity, more advanced instrumentation will be used – specifically, an Agilent 7890A GC with an Agilent 7700 ICP-MS.

So far, this chapter has focused on the analyses of mercury species in human biological matrices to assess toxicological exposure. The methods described in this chapter can also be used for mercury speciation in environmental samples which can be potential sources of that exposure. One such matrix for which mercury speciation using EPA Method 6800 has already been successfully used is soil (27). Another environmental matrix for which mercury speciation would be important is crude oil, and the usage of SIDMS for such an analysis is described in the next section.

**2C – Mercury Speciation in Crude Oil**

The analysis of mercury species in crude oil is important not only for the purpose of assessing the impact that the oil producers have on the environment, but also for determining how best to handle the problems associated with mercury species being present in the equipment used to process the oil. For example, elemental mercury can form amalgams with certain metals in equipment made from those metals and damage that equipment as a result (28).
Our research group obtained two crude oil samples from a major oil company and analyzed them for total mercury and mercury species extractable by EPA Method 3200. The description of the procedures used for these analyses is described next.

2C i – Total Mercury and Mercury Speciation in Crude Oil

Reagents/standards:

The water used for solutions was double deionized (DDI) water from a Barnstead NANOpure Ultrapure Water System (Dubuque, IA). Reagent grade nitric acid, L-cysteine, hydrogen peroxide, pyridine, and methanol were obtained from Fisher Scientific (Pittsburgh, PA). Nitric acid was purified using sub-boiling distillation in a quartz still (Milestone, Monroe, CT). Isotopically enriched \(^{199}\text{Hg}^{2+}\), \(^{200}\text{Hg}^{+}\), and \(^{201}\text{Hg}^{2+}\) were supplied by Applied Isotope Technologies (Pittsburgh, PA). The two crude oil samples were obtained from a major oil company.

Instrumentation/analytical methods:

For microwave sample preparation, a Milestone (Monroe, CT) Ethos 1600 microwave system was used. This system was equipped with temperature feedback control and magnetic stirring, as well as high pressure (maximum pressure – 100 bar) closed vessel microwave vessels made of high purity TFM (a thermally resistant form of fluoropolymer) with a capacity of 100 mL.

For total mercury analysis of the prepared samples, direct analysis was made with an Agilent 4500 ICP-MS (Agilent Technologies, Palo Alto, CA). Quantification was performed by monitoring the \(^{199}\text{Hg}\) and \(^{202}\text{Hg}\) isotopes for IDMS analysis. Three sample
preparation replicates and four analysis replicates were made for each of the two crude oil samples.

For analysis of the EPA 3200 extractable mercury species, LC-ICP-MS analysis was performed using isocratic elution with a Metrohm Peak (Houston, TX) PS-DVB C_{18} reversed phase column (150 x 4.6 mm, 2 µm) and a model 818 IC polymeric inert pump with an 838 advanced sample processor for automatic sample delivery from Metrohm Peak coupled to an Agilent 4500 ICP-MS (Agilent Technologies, Palo Alto, CA). The mobile phase consisted of a pH 3 solution of 50 mmol/L pyridine, 0.5% (w/v) L-cysteine, and 5% (v/v) methanol. The flow rate was 1.0 mL/min, and the injection volume was 100 µl. The chromatography and automation equipment were situated in a high efficiency particulate air (HEPA) filter class 100 clean hood chamber next to the ICP-MS in a class 1000 clean room laboratory. Quantification was performed by monitoring the $^{199}\text{Hg}$, $^{200}\text{Hg}$, $^{201}\text{Hg}$ and $^{202}\text{Hg}$ isotopes for the Hg$^{2+}$, CH$_3$Hg$^+$, and C$_2$H$_5$Hg$^+$ peaks in the chromatograms, and isotope ratios for SIDMS analysis were based upon peak area ratios. Three sample preparation replicates and four analysis replicates were completed. The isotope ratios were corrected for dead time, and a solution with natural abundant Hg$^{2+}$, CH$_3$Hg$^+$, and C$_2$H$_5$Hg$^+$ was also analyzed periodically between samples to obtain the mass bias correction factors. Three sample preparation replicates and four analysis replicates were made for each of the two crude oil samples (n=12).

Sample Preparation for total Hg analysis

To a microwave vessel were added a stir bar, 0.5 g of crude oil, a known amount of $^{199}\text{Hg}^{2+}$, 9 mL of HNO$_3$, and 2 mL of 30% H$_2$O$_2$. The sample was subjected to
microwave irradiation using EPA 3052 (29) (ten minute ramp to 180°C, ten minute hold at 180°C). After cooling, the sample was filtered through a 0.22 mm glass fiber filter and then stored at 4°C until analysis. Sample preparation was done in triplicate, and two procedural blanks containing everything that was in the crude oil sample preparation replicates except the crude oil itself were also prepared.

**Sample Preparation for analysis of Hg species extractable by EPA Method 3200**

To a microwave vessel were added a Teflon stir bar, 0.5 g of crude oil, known amounts of $^{199}$Hg$^{2+}$, CH$_3$$^{200}$Hg$^+$, and C$_2$H$_5$$^{201}$Hg, and 10 mL of 4 M HNO$_3$. The sample was subjected to microwave irradiation using EPA Method 3200 (19) (two minute ramp to 100°C, ten minute hold at 100°C). After cooling, there was a mixture of aqueous solution and oily residue. The aqueous part of the sample was removed and filtered through a 0.22 mm glass fiber filter, diluted to 20 mL with DDI water, and then stored at 4°C until analysis. Sample preparation was done in triplicate, and two procedural blanks containing everything that was in the crude oil sample preparation replicates except the crude oil itself were also prepared.

**Sample preparation for nonextractable Hg Species**

The microwave vessels with the oily residues were spiked with known amounts of $^{199}$Hg$^{2+}$, and to each of them were also added 9 mL of concentrated HNO$_3$ and 1 mL of 30% H$_2$O$_2$. EPA Method 3052 was implemented (ten minute ramp to 180°C, ten minute hold at 180°C). After cooling and filtering the samples through the 0.22 mm glass fiber
filters, the samples were stored at 4°C until analysis. A procedural blank was also prepared with the samples.

**Results and Discussion**

The total mercury results, extractable mercury species results, and total mercury results for the oily residues are shown in tables 6, 7, and 8, respectively, on the next page (results reported at the 95% C.I.):
<table>
<thead>
<tr>
<th>Crude oil sample</th>
<th>Total Hg concentration in ppb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serial # 871833</td>
<td>414.1 ± 24.7</td>
</tr>
<tr>
<td>Serial # 870987</td>
<td>432.8 ± 25.4</td>
</tr>
</tbody>
</table>

Table 6 – Total Hg results in Crude Oil

<table>
<thead>
<tr>
<th>Sample</th>
<th>Deconvoluted concentration (ppb)</th>
<th>Interconversion %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hg^{2+}</td>
<td>CH\textsubscript{3}Hg\textsuperscript{+}</td>
</tr>
<tr>
<td>---------</td>
<td>--------</td>
<td>-----------------</td>
</tr>
<tr>
<td>Serial # 871833</td>
<td>17.3 ± 3.6</td>
<td>0.8 ± 0.5</td>
</tr>
<tr>
<td>Serial # 870987</td>
<td>23.6 ± 3.7</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

Table 7 – Extractable Hg Species

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total nonextractable Hg (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serial # 871833</td>
<td>342.5 ± 46.8</td>
</tr>
<tr>
<td>Serial # 870987</td>
<td>323.9 ± 30.7</td>
</tr>
</tbody>
</table>

Table 8 – Nonextractable Hg
In both crude oil samples, the extractable mercury species constituted about 7% of the total mercury in the sample. Further research is needed to determine the nonextractable mercury species. As with ethylmercury in hair, there was a significant amount of conversion of ethylmercury to inorganic mercury indicated in the speciation results. SIDMS successfully corrected for this conversion.

2D - Conclusion

The focus of this chapter has been the analysis of mercury species in several different matrices. Results showed that EPA Method 6800A could correct for interconversion among mercury species, as well as recoveries of less than 100%. A critical molecule which the body makes to combat the mercury species mentioned in this chapter, as well as other xenobiotics, is GSH. This molecule will be the focus of the next chapter.
**References:**


15. C. Chai; W. Feng; Q. Qian; M. Guan; X. Li; Y. Lu; X. Zhang; Nucl. Sci. Tech., 1994, 5(2), 65–70.


19. EPA Method 3200, website link:


22. EPA website: http://www.epa.gov/hg/exposure.htm


24. EPA 7473 website link:


26. UCLA Department of Medicine website:
http://www.med.ucla.edu/modules/wfsection/article.php?articleid=311


29. EPA Method 3052 website link:

GSH is a tripeptide occurring in most cells of the body, and is involved in several critical functions, such as antioxidation and heavy metal detoxification (1). Under conditions of oxidative stress, the active form of GSH, reduced GSH forms a disulfide dimer, thereby producing GSSG. It has been discussed in the literature that an important biomarker may be the GSH/GSSG ratio associated with one’s level of oxidative stress, with lower values for the GSH/GSSG ratio indicating greater oxidative stress levels (2). It has been observed that children with autism have lower plasma GSH/GSSG ratios, as well as lower plasma total GSH (tGSH) concentrations compared to healthy controls (3). Abnormal GSH metabolism has been implicated in other conditions as well, such as AIDS and diabetes (4).

Several analytical methods have been developed for the analysis of tGSH, GSH, and GSSG. For tGSH analysis, methods have included spectrophotometric assays (5) as well as hyphenated techniques, such as LC separation combined with either electrochemical, fluorescence (6), or mass spectrometry detection (7). Prior to tGSH analysis, sample preparation typically involves treating disulfide bonds with a reducing agent, such as dithiothreitol (DTT) (8). For analysis of GSH and GSSG, methods have included LC separation with either fluorescence or mass spectrometry detection (9). A derivatization agent, such as NEM, is often used during sample preparation to block the thiol group on GSH, thereby preventing its conversion to GSSG (10).

In quantitative analysis of tGSH, GSH, and GSSG, the traditional calibration curve method has been commonly used. Using internal standard (IS) with this method has
helped to correct for fluctuations in signal intensity. For mass spectrometry applications, one type of internal standard can be an isotopically enriched form of the analyte of interest. For example, Harwood, et al. used isotopically enriched GSH-NEM and isotopically enriched GSSG as internal standards for the quantitative calibration curve based analysis of GSH and GSSG in erythrocytes using LC/MS/MS (11). In their application, to plot the calibration curves, the x-axis represented the concentration of the analyte, while the y-axis represented the natural abundant analyte/internal standard peak area ratio.

Literature values for the GSH/GSSG ratio in healthy adult subjects range from about 10 to 1 (12), to 200 to 1 (13), to 1000 to 1 (11), as shown in Figure 1 below:

![Figure 1 – Literature values for GSH/GSSG ratio in red blood cells](image)

It should be noted that the method for calculating the GSSG concentration described in reference 12 was an indirect one which consisted of subtracting the reduced GSH
concentration from the tGSH concentration. According to Michaelsen, et al., indirect measurement of GSSG and inadequate prevention of artificial GSSG formation can lead to inaccurately high results for the GSSG concentration (13).

In our work, an advanced application of isotopically enriched analytes for quantitative analysis is, as mentioned in chapter 1, EPA Method 6800A (14). One of the procedures described in this method, IDMS, involves spiking the sample with a known amount of enriched analyte, performing mass spectrometry measurements on the prepared sample, and calculating the concentration of the analyte based on peak area ratios. No traditional calibration curves are used in this method. An application of IDMS for tGSH analysis in red blood cells will be described later in this chapter. A more advanced procedure described in EPA Method 6800A, speciated isotope dilution mass spectrometry (SIDMS), involves spiking the sample with known amounts of the enriched analytes of interest (and pairs of related analytes), analyzing the sample by mass spectrometry, and determining concentrations and extent of species interconversion by analyzing analyte MS peak ratios. This method can be used for GSH/GSSG analysis, as GSH to GSSG conversion can occur during sample preparation (although there are techniques for minimizing this conversion, which will be discussed later), and correction of the conversion is necessary to assess the analytes accurately. This application of SIDMS for optimizing GSH and GSSG analysis was a major part of my research and this PhD project, as was the application of IDMS for optimizing tGSH analysis.

In a collaborative project with the Kingston research group of Duquesne University and the Children’s Institute in Pittsburgh, IDMS was used for the analysis of tGSH, and SIDMS for the analysis GSH and GSSG, in red blood cells from autistic
children before and after exposure to a therapeutic cleanroom environment (this collaborative project will henceforth be referred to as the “cleanroom study”). A second collaborative project involving the same kinds of analyses for a set of 30 control and 30 autistic patients was also undertaken (this second collaborative project will henceforth be referred to as the “red blood cell study”). This application of the developed measurement methods in these studies is described next.

3A – Analysis of tGSH, GSH and GSSG in Red Blood Cells from Autistic Children Exposed to Cleanroom

Materials and Methods

Cleanroom Study and Red Blood Cell Study Samples

Both the cleanroom and red blood cell studies were funded with grants obtained by Professor Kingston in collaboration with Dr. Scott Faber (M.D.) of The Children’s Institute. Both studies were IRB approved, and the grants for them were registered with both institutions. In the cleanroom study, blood samples from ten autistic children were taken before and after a two week clean room exposure (12 hours per day). These samples were obtained in the child’s home from a registered phlebotomist, Ms. Becky Pekar. Following the sample handling procedure described by Michaelsen, et al. for minimizing GSH oxidation, the blood was drawn from the vein into tube containing anticoagulant (Na$_2$EDTA) and immediately put on ice and protected from light, (13). As these were regional studies, all patients were within a one-hour driving distance of Duquesne and The Children’s Institute; thus, samples could be transported to Duquesne
within 2 hours. All patients were within one hour driving distance of Duquesne and The Children’s Institute as these were regional studies. The samples were aliquoted into tared 1.7 mL polypropylene microcentrifuge tubes from VWR (Randor, PA, part number 87003-284) under inert atmosphere (Nitrogen) conditions. For the first 6 cleanroom samples, either a nitrogen glove box shared by others in the Chemistry Department at Duquesne or a nitrogen glove bag was used; for the rest of the samples, a dedicated nitrogen glove box specifically for the Kingston research group was used. The samples were then centrifuged using a Sorvall Discovery M150 SE centrifuge with an S120AT2 rotor (ThermoFisher, Asheville, NC) at 2,000 x g at 4°C for 15 minutes to separate the plasma from red blood cells. The plasma layer above the red blood cells was removed and transferred to microcentrifuge tubes, then the small buffy coat containing the white blood cells which remained above the red blood cells was removed and discarded. The red blood cell and plasma samples were weighed on an analytical balance, then, placed into the -80°C freezer for storage.

**Chemicals/Reagents**

Isotopically enriched GSH with two $^{13}$C’s and one $^{15}$N and isotopically enriched GSSG with four $^{13}$C’s were obtained from Cambridge Isotope Laboratories (Andover, MA). With both enriched standards, the enrichment was on the glycine. Natural abundant GSH was obtained from Sigma-Aldrich (St. Louis, MO, p/n G4251-10G). Natural abundant GSSG was obtained from Sigma (p/n G4501-1G). HPLC grade acetonitrile was obtained from Sigma-Aldrich (p/n 27,071-7). ammonium formate and 88% formic acid were obtained from Fisher Scientific (Pittsburgh, PA – p/n’s A666-500 and A118P-500,
respectively). DTT and NEM were both obtained from Sigma-Aldrich (p/n’s D5545-5G and E1271-5G, respectively).

**Equipment**

The column used for both the tGSH analysis and the GSH/GSSG analysis was a Waters Atlantis HILIC Silica, 3µm, 2.1 x 100 mm (Waters Corporation, Milford, MA, p/n 186002013). The system used for LC/MS/MS analysis was an Agilent 6460 Triple Quadrupole Mass Spectrometer with an Agilent 1200 HPLC system and Mass Hunter software (Agilent Technologies, Santa Clara, CA). For the analysis of the samples using the autosampler, polypropylene 96 well plates with 0.5 mL wells (Agilent p/n 5042-1386) and silicone coverings with pre-slit well caps (Thermo Scientific, p/n 276011) were used. For the pre-concentration of sample solutions by evaporating off the solvent and redissolving in a smaller volume, a SpeedVac (made by what was formerly Savant, now Thermo, model number SVC 100D) was used for evaporation purposes. For centrifugation of the samples after protein denaturation, the Sorvall Discovery M150 SE centrifuge with the S120AT2 rotor (Thermo Scientific) was used. Polypropylene microcentrifuge tubes (1.7 mL) were obtained from VWR (Randor, PA, part number 87003-294).

**Sample Preparation for the analysis of tGSH**

Frozen aliquots of the red blood cells were removed from the -80°C freezer and thawed. 30.0 mg of sample were added to 1.7 mL polypropylene microcentrifuge tubes and treated with 30.0 mg of 2.09 µmol/g isotopically enriched GSH. The concentration of
the enriched GSH in the solution that was added to the red blood cells was determined by a procedure known as reversed isotope dilution mass spectrometry (RIDMS), which will be described in more detail later in this chapter). Also added were 300 mg of a freshly made aqueous solution of ~ 10,000 ppm DTT. After 20 minutes, ~ 1 mL of acetonitrile was added to denature the proteins, and the samples were centrifuged at 4°C at 5,000 x g for 10 minutes. The supernatants were removed and used for analysis by LC/MS/MS. Sample preparation was done in triplicate, and analysis was done in duplicate for a total of six replicate analyses, (n=6). For method validation, an aqueous solution of GSH with a known concentration 2.30 µmol/g was analyzed as well.

Sample preparation for analysis of GSH and GSSG

The red blood cell sample in the 1.7 mL microcentrifuge tube (mass of sample in the tube was already known before removal from the freezer, usually ~400 mg) was treated with 800 µL of ~ 8,000 ppm NEM in a nitrogen glove box while thawing, and a 250 mg aliquot of this NEM treated sample was spiked with 40.0 mg of 2.95 µmol/g isotopically enriched GSH. The sample was also spiked with 35.0 mg of a solution of enriched GSSG that had been diluted 50 fold with H₂O just before spiking (original concentration of enriched GSSG stock solution – 1.95 µmol/g). The concentrations of enriched species were determined by RIDMS, described in more detail later in this chapter. Each sample was allowed to equilibrate for 30 min, after which it was treated with HPLC grade acetonitrile to denature the proteins, then centrifuged at 5,000 x g for 3 min at 4 °C. The supernatant was removed, the solvent from the resulting supernatant was evaporated off using a SpeedVac at medium heat (40 °C), and the resulting residue was
dissolved in 30 µL of DDI H₂O, after which ~ 100 µL of HPLC grade acetonitrile were added. This final solution was used for analysis. Sample preparation was done in triplicate, and analyses in duplicate (n=6). For method validation, an aqueous solution containing known concentrations of reduced GSH (2.00 µmol/g) and GSSG (0.0601 µmol/g) was made and analyzed as well.

Preparation of aqueous solutions of the enriched GSH and GSSG

Both enriched GSH and GSSG are very expensive. 50 mg of the former costs over $700, and 50 mg of the latter costs over $2,000. One way to use both in a cost efficient manner is to use only a few mg to make aqueous solutions of each of them, aliquot those solutions into 1.7 mL polypropylene tubes, and store those tubes with the solutions in the -20°C freezer. Studies have shown that aqueous solutions of GSH and GSSG to be used for analysis can be stored for months at -20°C (5, 15). Because the low mass of enriched material used can lead to greater uncertainty in the concentration calculation, one can instead analyze the concentrations of the solutions by taking aliquots of them out of the -20°C freezer and determining the concentrations of them using RIDMS, which is described in EPA Method 6800a (14). To use this method for the calibration and validation of the enriched GSH, for example, an aqueous solution of known concentration of natural abundant GSH (which is relatively inexpensive) was made, and a known amount of this solution was used to spike a known amount of the frozen and thawed solution of enriched GSH. The spiked solution was then treated with DTT, followed by Acetonitrile, just as in the tGSH sample preparation protocol. The solution was then analyzed by LC/MS/MS using the same method that was used for the tGSH analysis,
described below. Sample preparation was done in triplicate, and analysis in duplicate (n = 6). The RIDMS calculation is done exactly the same way as the IDMS calculation is with the exception that the natural abundant GSH is now considered the enriched form and the enriched GSH is now considered the natural abundant form. Enriched GSSG can be analyzed in a similar manner using inexpensive natural abundant GSSG, with the exception that no DTT is used, and can be analyzed using the same LC/MS/MS protocol as used for the analysis of the GSSG in the red blood cells, described in the next section.

**LC/MS/MS Analysis Conditions for the tGSH analysis**

As mentioned earlier, an Agilent (Santa Clara, CA) 6460 Triple Quadrupole Mass Spectrometer coupled to an Agilent 1200 Series LC system was used for the analysis. The column used was a Waters (Milford, MA) Atlantis HILIC Silica (3µm, 2.1 x 100 mm), with mobile phase A consisting of 20 mM ammonium formate, pH 3.8, and mobile phase B consisting of HPLC grade acetonitrile. The column temperature was 45°C, and the solvent flow rate was 0.35 mL/minute. The nebulizer gas (nitrogen) temperature was 300°C, and its flow rate was 8 L/minute. The sheath gas (nitrogen) temperature was 250°C, and its flow rate was 11 L/minute. The injection volume was 1 µl, and isocratic elution was used. Transitions monitored in positive mode included 308 m/z → 179 m/z (for natural abundant GSH) and 311 m/z → 182 m/z (for isotopically enriched GSH). The product ion of interest was one resulting from the loss of one pyroglutamate, 129 amu). The collision energy was 12V, the capillary voltage was 3500V, the fragmentor voltage was 135V, and the dwell time was 50 ms.
LC/MS/MS Analysis Conditions for the GSH/GSSG analysis

An Agilent (Santa Clara, CA) 6460 Triple Quadrupole Mass Spectrometer coupled to an Agilent 1200 Series LC system was used for the analysis. The column used was a Waters (Milford, MA) Atlantis HILIC Silica (3µm, 2.1 x 100 mm), with mobile phase A consisting of 20 mM ammonium formate, pH 3.8, and mobile phase B consisting of HPLC grade acetonitrile. The column temperature was 45°C, and the solvent flow rate was 0.35 mL/minute. The nebulizer gas (nitrogen) temperature was 300°C, and its flow rate was 8 L/minute. The sheath gas (nitrogen) temperature was 250°C, and its flow rate was 11 L/minute. The injection volume was 5 µl, and the gradient used is described in table 1 below:

<table>
<thead>
<tr>
<th>Time</th>
<th>% 20 mM Ammonium formate buffer</th>
<th>% Acetonitrile</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 minutes</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>10 minutes</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>16 minutes</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>20 minutes</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>25 minutes</td>
<td>25</td>
<td>75</td>
</tr>
</tbody>
</table>

Table 1 – Gradient system for HILIC method

Analysis was done in positive mode, and the collision energy used for tandem MS purposes for the GSH-NEM complex (which resulted in the primary product ion being one with the loss of pyroglutamate) was 12V, and that for GSSG (resulting in the loss of two pyroglutamates) was 25V. The transitions monitored included 433 m/z → 304 m/z (for natural abundant GSH-NEM), 436 m/z → 307 m/z (for isotopically enriched GSH-NEM), 613 m/z → 355 m/z (for natural abundant GSSG), 616 m/z → 358 m/z (for
possible “hybrid” GSSG), 617 m/z → 359 m/z (for isotopically enriched GSSG with four $^{13}$C’s), and 618 m/z → 361 m/z (for possible enriched GSSG completely from enriched GSH). The retention time for the GSH-NEM complex was ~ 3 minutes, and that for the GSSG was ~ 8 minutes. The capillary voltage was 3500V, the fragmentor voltage was 135V, and the dwell time was 50 ms.

Results and Discussion (Note: All results reported at the 95% C.I.)

Method Validation

For the validation of the method for the analysis of tGSH concentration, the experimental value obtained, 2.09 ± 0.30 µmol/g, was in agreement with the known value of 2.30 µmol/g. For validation of the method for analysis of reduced GSH and GSSG, the experimental values obtained, 2.27 ± 0.32 µmol/g and 0.0623 ± 0.0028 µmol/g, respectively, were in agreement with the corresponding known values of 2.00 µmol/g and 0.0601 µmol/g.

Results from cleanroom study

The tGSH and GSH/GSSG results from the cleanroom study are shown in Figures 2 and 3, respectively.
In the clean room study, in the tGSH analysis, no overall change was noticed in the before and after tGSH values for the children. Regarding the GSH/GSSG ratio analyses, children 6 and 8 are young identical twins girls. The former twin (child 6) had
an increase in the GSH/GSSG ratio. This twin (child 6) showed improvement with respect to behavioral characteristics as well (data for behavioral characteristics not shown), while the latter (child 8) did not. It was hypothesized that the improvement in behavior in child 6 and the absence of such an improvement in child 8 was related to the improvement or lack thereof in the GSH/GSSG ratio. An epigenetic relationship was also hypothesized, but a conclusive statement regarding such a relationship for the explanation of the results from children 6 and 8 is beyond the scope of this discussion. Such a relationship between development of autism and possible epigenetic factors has been documented in the literature and in the examples documented in “Ghost in Your Genes” a documentary relating epigenetics and environmental factors presented by NOVA (16). In the NOVA documentary, a case was described involving identical twin sisters of whom one twin had autism while the other did not. Environmental factors and epigenetics were considered possible contributing factors by researchers in the documentary, and these same factors are related to the study results discussed here.

Although there was a relatively small sample size (n=10) used in the cleanroom study that limited the statistical power and made it difficult to make strong, conclusive statements about the results obtained, our research group is, as of this writing, conducting a much larger study involving 30 autism patients and 30 healthy controls matched for age, gender, and socioeconomic status. This study is described next.
3B – Analysis of tGSH in Red Blood Cells from 16 Pairs of Age, Gender, and Socioeconomic status matched autistic and control samples

In an ongoing study which will eventually include 30 young autism patients and 30 matched healthy controls, blood samples from 16 autism patients and 16 age, gender, and socioeconomically matched healthy controls were delivered to Duquesne University and processed so as to obtain the red blood samples just like the cleanroom samples were. As of this writing, from the perspective of the author, this is still a blind study, as information pertaining to which children are the controls and which are autistic is being withheld until the end of the study. These 32 samples were prepared for tGSH in a manner similar to that used for the analysis of the tGSH in the cleanroom samples. To a 1.7 mL polypropylene microcentrifuge tube were added 20.0 mg of thawed and vigorously shaken (for ~ 3 – 5 seconds) 2.31 µmol/g isotopically enriched GSH, 18.0 mg of thawed red blood cell sample, and 160 mg of freshly made ~10,000 ppm DTT. After allowing the sample to equilibrate for 30 minutes, 0.6000 g of HPLC grade Acetonitrile were added to denature the proteins. The sample was centrifuged at 4°C at 5,000 x g for 10 minutes, and the supernatant was removed and used for analysis. Sample preparation was done in triplicate, and analysis of each replicate in duplicate (n=6). The LC/MS/MS method used for tGSH analysis was also similar to that used for the cleanroom study except that the concentration of pH 3.8 ammonium formate was 10 mM instead of 20 mM. It had been noticed by our research group that, as the concentration of ammonium formate in the solution used as solvent A for the LC/MS/MS method increases, peak in the chromatogram becomes more narrow and the signal intensity decreases; conversely,
as the concentration of ammonium formate decreases, the peak becomes broader and more intense (data not shown). The changing of the ammonium formate concentration was intended to optimize the peak width/signal intensity relationship, taking into account other parameters, such as the number of SRM transitions. The results of the analysis of these 16 pairs of samples are shown in Figure 4 on the next page:
Figure 4 – Results from the tGSH analysis of red blood cells from 16 matched pairs of control and autistic samples from the red blood cell study.
The results in Figure 4 were sent to our statistician, Dr. John Kern from Duquesne University. He reported that, overall, collectively, the autistic patients had higher red blood cell tGSH than the healthy controls (although there was some overlap), and the difference was statistically significant ($p=0.012$). As mentioned in the introduction, a study by Adams et al. in which GSH in red blood cells and heavy metals in urine from autistic children were analyzed before and after chelation therapy suggested that exposure to toxic metals other than mercury, such as lead, aluminum, cadmium, and antimony results in higher than normal GSH levels due to the body’s increased GSH synthesis in response to exposure to those metals, while exposure to mercury results in lower than normal GSH levels, possibly due to the inhibition of GSH synthesis by the mercury (17). The study also suggested that, as the body burden of toxic metals is lowered, the concentration of GSH in red blood cells normalizes (17). The results obtained thus far in our red blood cell study suggest the possibility, but do not yet prove, that the autistic patients in our study have, on average, a higher body burden of toxic metals other than mercury which resulted in their bodies making more GSH in response to exposure to those toxic metals. Further research is needed to confirm this hypothesis. Figure 5 is a statistical extension and interpretation of the logistic regression analysis of tGSH in red blood cells vs. autistic diagnosis probability for 32 children. The calculations used for Figure 5 were performed by the statistician on our research team, Dr. Kern.
Figure 5 – Statistical extension and interpretation of the logistic regression analysis of tGSH in red blood cells vs. autistic diagnosis probability for 32 children (n= 16 matched pairs).

The graph is based on the overall probability of a 1, 2 or 3\% chance of having an autistic child in a population such as Western Pennsylvania. To explain further, if the overall prevalence of autism in western Pennsylvania is, for example, 2\% (blue line), the probability of a child with a red blood cell tGSH concentration of 1.8 \( \mu \text{mol/g} \) having autism is extremely low (no more than a random sample from the general population), but increases as the tGSH concentration increases such that, at a concentration of 2.6 \( \mu \text{mol/g} \), for example, the diagnostic probability exceeds 15%.

Our research group is following up on this work by analyzing the remaining 14 pairs of control and autistic samples for red blood cell tGSH, as well as all 60 red blood cell samples (30 control and 30 autistic) for GSH/GSSG analysis. While the GSH/GSSG ratios for these same patients were not available at the time of this chapter presentation,
this data is being accumulated and will, very likely, be presented as a final addendum to this chapter.

In both the cleanroom study and the red blood cell study, although steps were taken to minimize the conversion of GSH to GSSG in the blood samples prior to their arrival at Duquesne, such as drawing the samples into blood draw tubes via vacuum suction without exposing them to atmospheric oxygen, keeping the samples in ice, and protecting them from light during transport, additional work is currently being carried out by our research group to improve this methodology so that whole blood samples obtained from nonlocal settings can also be analyzed for both tGSH and GSH/GSSG. In addition, research into enabling long distance transport of these blood tubes with correction by field and remote automatic spiking is being carried out at the present time. The progress made so far in this area by our research group is described next.

3C – Enabling sampling of tGSH and GSH/GSSG in blood sample from remote locations

A recent paper by Rossi, et al, describes using a blood draw tube containing Na₂EDTA (anticoagulant) and NEM for the purpose of preventing the conversion of GSH to GSSG after the blood is drawn into the tube and transported to the nonlocal laboratory for analysis (18). Although their experiments included only the analysis of reduced GSH (specifically, GSH conjugated with NEM) and did not include GSSG analysis, they did demonstrate the potential for obtaining samples from nonlocal settings for reduced GSH analysis. Our research group at Duquesne University, in collaboration with Applied Isotope Technologies (AIT – Pittsburgh, PA), and the Open Medicine Institute (Mountain
Materials and Methods

The same reagents used in the cleanroom and red blood cell studies for sampling and quantifying of tGSH and GSH/GSSG were used in this study. A solution containing ~ 4,000 ppm NEM, 0.919 µmol/g enriched GSH, and 0.00317 µmol/g enriched GSSG was made and put into a 7 ml capacity blue capped blood draw tube containing 10.5 mg of Na$_2$EDTA as an anticoagulant (tube provided by Kendall, which is now part of Covidien, Mansfield, MA). This prepared blood draw tube, along with a second and third tube containing just the Na$_2$EDTA, were weighed and then shipped frozen to the hospital clinical laboratory run by Dr. Andreas Kogelnik MD, PhD, Medical Director of the Open Medicine Institute in Mountain View California. The staff at the Open Medicine Institute followed the procedure given to them, which is shown below in a protocol that was provided as a standard operating procedure (SOP):

Standard Operating Procedure for Blood Draw Tubes for tGSH and GSH/GSSG

(begin official SOP document)
Blood Draw Tubes for total Glutathione, reduced Glutathione and Glutathione Disulfide Test

AIT-GSH-GSSG-α

PURPOSE  To measure the concentrations of total glutathione (tGSH), reduced glutathione (rGSH) and glutathione disulfide (GSSG) in whole blood.

PRINCIPLE  The values for tGSH and the rGSH/GSSG ratio are often used as indicators of the extent of one’s oxidative stress level and have applications to autism, chronic fatigue syndrome, cancer, and other conditions.
APPLICATION  Blood samples are drawn into specialized tubes by the phlebotomist, then, following the procedure below are sent to AIT for analysis.

The procedure involves the usage of N-Ethylmaleimide (NEM) already present in the blood draw tube designated for the analysis of the rGSH/GSSG ratio. The purpose of the NEM is to block the thiol group on rGSH and, thereby, prevent the conversion of it to GSSG. Isotopically enriched rGSH and GSSG, as well as Disodium EDTA, are also present in the tube designated for rGSH/GSSG analysis. Disodium EDTA is present in the tube for tGSH analysis.

LINKED DOCUMENTS  The MSDS’s for NEM, GSH, GSSG, and Disodium EDTA are on file and available upon request.

RELATED DOCUMENTS  The procedure for preparing the blood draw tube for rGSH/GSSG analysis is similar to one described by Rossi, et al. (Analytical Biochemistry, 2011, 415, 81-83).
SPECIMEN

Required specimen is the blood samples that need to be drawn to the correct tubes at the specified among, by following the SOP detailed in this document.

SUPPLIED BY AIT:

MATERIALS

1. One blood draw tube labeled “throw away,” with code “aaag”

EQUIPMENT

2. One blood draw tube labeled “tGSH” with code “aaah”

3. One blood draw tube labeled “rGSH/GSSG” with code “aaaj”

4. Three pieces of bubble wrap to be used for re-packing the tubes

5. Ziploc bags with special packing material, to be used for re-packing tubes

6. Federal Express form to be used to ship the tubes back to AIT

7. Cold packs

SUPPLIED BY THE CUSTOMER: Facility, blood draw equipment (syringe, needle, etc.), -20 °C freezer, phlebotomist
QUALITY CONTROL/ QUALITY ASSURANCE

This SOP is a part of final test development project conducted by AIT which supplies all the material and implement quality assurance protocols associated with this project.

DEFINITIONS

- rGSH → reduced glutathione,
- GSSG → glutathione disulfide,
- tGSH → total glutathione
- NEM → N-Ethylmaleimide, MSDS → Material Safety Data Sheet

PROCEDURE

1. Upon taking delivery, inspect the package for visible, substantial damage to the box.
   Call AIT (408-472-2333), if damage is observed.
2. Open the box without damaging it so that it can be re-used to return the samples. Examine the contents – especially the three blood draw tubes (one with a frozen solution inside) – for damage and/or leaks that can be observed by eye. Please see the packing list at the end of this document. Call AIT, if tubes are damaged or leak occurred during transit. Save all the contents of the box, including the packing material.

3. Put the entire box in a –20°C freezer until ready for use. Alternatively, remove the contents – blood draw tubes – in container or a tube rack, and put the container in a –20°C freezer until ready for use. Make sure the tubes are placed in the container stored upright in the freezer.

4. Approximately 20 minutes before the blood is scheduled to be drawn, take the three tubes out of the freezer.

   **Note:** Keep the ice-packs in the freezer, so they will be ready to use when samples are re-packed for shipment back to AIT.

5. Place the rGSH/GSSG tube (with the 3 mL of solution) into a Ziploc bag, then place the tube in the bag upright into a beaker with ambient temperature water to thaw the solution inside the tube. After the solution is thawed, gently invert the tube 3 to 4 times to facilitate mixing.
6. Draw approximately 3 mL of blood from the antecubital vein into the “throw-away” tube and discard the tube. The throw-away tube should be at least half full. This approximates to about 3 mL volume.

**Note:** If blood is to be drawn from the patient in sequence with another draw, the throw-away will not be needed as long as the draw for GSH test assay is done after another draw using the same needle under use.

7. Using the same blood draw needle in the antecubital vein, draw 6 mL of blood into the tGSH tube, then draw approximately 3 mL of blood into the rGSH/GSSG tube containing 3 mL of solution already in it.

8. Mix the contents of the tubes by gently inverting them several times, over the course of 5 minutes.

9. Put the tubes back into the -20°C freezer for storage for a minimum of 2 hours.

10. Ship the samples back to AIT, overnight, with ice packs, in the same insulated box that the tubes came in. Specifically, wrap each of the tubes in the supplied bubble wrap, put the wrapped tubes along with absorbent paper towels into the small Ziploc bag, seal the small Ziploc bag, put the small Ziploc bag along with two frozen cold packs into the large Ziploc bag, seal the large Ziploc bag, put the large Ziploc bag into the styrofoam box with the shock absorbent material so that the bag is in the center of the box, put the
Styrofoam cap on top of the Styrofoam box, put the Styrofoam box back into the cardboard box, and tape up the cardboard box with packing tape. Label the outside of the cardboard box with the following return address: *Applied Isotope Technologies, 2403 Sidney Street, Ste-280, Pittsburgh, PA 15203*. Also, label the outside of the box “Exempt Human Specimens.”

**CALCULATIONS**

Mass spectrometric sample analyses will be carried out by the AIT staff.

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**RESULTS/INTERPRETATION**

The data will be obtained and interpreted by the staff at AIT. Final, tabulated results will be reviewed and approved by Prof. Skip Kingston.

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**REFERENCES**

The GSH test was developed by applying Speciated Isotope Dilution Mass Spectrometry (SIDMS), a technology codified in RCRA EPA Method 6800. Additional information may be obtained by contacting AIT:

Matt Pamuku

Ph: 408-472-2333

E: matt@sidms.com
DOCUMENT CONTROL

Author: Timothy Fahrenholz  Reviewed by: Matt Pamuku  Approved by: Skip Kingston, Ph.D.

Signed: Signed copy on file  Signed: Signed copy on file  Signed: Signed copy on file

Date: August 30, 2011  Date: August 30, 2011  Date: August 30, 2011

Revision: V-1.02.01

Part No: TBD

ADDITIONAL INFORMATION

AIT reserves the right to correct all technical specifications, descriptions, protocols, material, deliveries, prices and correct typos, without notice.

This test is still under beta-level development. The recipient agrees to cooperate with AIT to accomplish its testing goals. AIT offers no warranties, implied or expressed, and fit-for-purpose statements associated with this test. The recipient agrees to cooperate with all terms and conditions.

PACKING LIST

<p>| Blood draw tube | Acceptable condition? (for the tubes, no |</p>
<table>
<thead>
<tr>
<th>Cracks, leaks, etc</th>
<th>Yes/No</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Tube labeled “Throw Away”</td>
<td>Yes/No</td>
</tr>
<tr>
<td>2. Tube labeled “tGSH”</td>
<td>Yes/No</td>
</tr>
<tr>
<td>3. Tube labeled “rGSH/GSSG”</td>
<td>Yes/No</td>
</tr>
<tr>
<td>4. Bubble Wrap</td>
<td>Yes/No</td>
</tr>
<tr>
<td>5. Ziploc Bags</td>
<td>Yes/No</td>
</tr>
<tr>
<td>6. FedEx Instructions</td>
<td>Yes/No</td>
</tr>
<tr>
<td>7. FedEx Shipping Form</td>
<td>Yes/No</td>
</tr>
<tr>
<td>8. Cold Packs</td>
<td>Yes/No</td>
</tr>
</tbody>
</table>

(End of official SOP document)

After receiving the three blood draw tubes with the blood in them from the Open Medicine Institute, the tubes were inspected, and no noticeable damage was observed. The tubes were stored at -20°C until sample preparation/analysis.

**Sample Preparation and Analysis of tGSH in whole blood sample from Open Medicine Institute**

The sample preparation protocol for the analysis of tGSH in the whole blood sample was similar to that used for the analysis of tGSH in red blood cells described earlier. The blood sample was removed from the -20°C freezer and thawed. To each of
three 1.7 mL polypropylene tube were added ~ 60.0 mg of whole blood, ~ 15.0 mg of 2.18 µmol/g enriched GSH, and 150 mg of 10,000 ppm DTT for triplicate sample preparation. After 30 minutes, 600 mg of acetonitrile were added to each tube to denature the proteins, and the sample preparation replicates were centrifuged at 5,000 x g at 4°C for 10 minutes, after which the supernatants were removed and used for LC/MS/MS analysis. Sample preparation was done in triplicate, and analysis in duplicate (n=6).

For LC/MS/MS analysis, the conditions used were similar to those used for the tGSH analysis in the red blood cell study described earlier in this chapter.

Sample Preparation and Analysis of GSH and GSSG in whole blood sample from Open Medicine Institute

In a personal communication with Dr. Andrew Alpert (one of the world experts in hydrophilic interaction chromatography, or HILIC) from Poly LC, Inc., at the 2011 Pittcon in Atlanta, to improve the sensitivity for the analysis of GSSG, he suggested that, in the sample preparation protocol, after evaporating off the solvent with the SpeedVac, instead of initially redissolving the residue in water, one should redissolve it in the ammonium formate buffer solution used as solvent A in the LC/MS/MS method to help with the ionization (19). In another personal communication with Brian Rappold from Labcorp at the 2011 American Society for Mass Spectrometry (ASMS) Conference in Denver, Colorado, he suggested the usage of a mixture of ethyl acetate and acetonitrile (e.g. 10% ethyl acetate), rather than 100% acetonitrile, as solvent B in the LC/MS/MS protocol, which would help in the evaporation of the solvent mixture in the electrospray droplet and, thus, help improve sensitivity (20). In a PowerPoint® presentation that he
gave at the conference, he described using this method to achieve up to a five-fold improvement in sensitivity. During a September 2011 mass spectrometry training course at Duquesne University given by Dr. Richard King from Pharmacadence Analytical Services, LLC, he suggested that, for improving the sensitivity in a given method, one way would be decrease the dwell time in the triple quadrupole mass spectrometer (e.g. from 50 ms to 5 ms) and, thus, obtain more points per peak (21). These three suggestions were implemented in the protocol for analyzing GSH and GSSG in the whole blood sample sent back to Duquesne by the Open Medicine Institute.

More specifically, for sample preparation, the GSH/GSSG tube from Dr. Kogelnik’s hospital clinical laboratory was thawed, and 200 µL aliquots of the spiked blood were added to five 1.7 mL polypropylene microcentrifuge tubes. To each tube were added 800 µl of acetonitrile to denature the proteins, and these five tubes were centrifuged at 5,000 x g at 4°C for 10 minutes. The supernatants were transferred to clean 1.7 ml polypropylene tubes, and these tubes were put on the SpeedVac for evaporation at medium heat (~40°C) for 1 hour and 45 minutes. After evaporation, to the first tube with the residue were added 40 µL of 20 mM ammonium formate, pH 3.8, which was pipetted up and down in the tube ~ 4 times, after which the solution in the first tube was transferred to the second tube, where the up and down pipetting technique was repeated, and so on, down to the fifth tube, so that the residues from all five tubes were pooled. To the tube with the solution of pooled residue were added 60 µL of acetonitrile, and the solution was analyzed using conditions similar to those used for the GSH/GSSG analysis in red blood cells described earlier in this chapter except that solvent B in the LC/MS/MS protocol consisted of 90% acetonitrile and 10% ethyl acetate (Optima brand, Fisher.
Scientific, p/n E196-4) and the dwell time was lowered from 50 ms to 5 ms. Four analysis replicates were completed (n=4).

Results and Discussion

The concentration of tGSH was 1.21 ± 0.7 µmol/g, and that of reduced GSH was 1.27 ± 0.2 µmol/g. The values for the tGSH and reduced GSH were, thus, statistically indistinguishable. GSSG was detected in two of the four runs, and its calculated concentration ranged from 1.60 nmol/g on the high end to ND (not detected). The chromatogram from one of these runs is shown in Figure 6. The peaks for the transitions of natural abundant GSSG (613 m/z → 355 m/z) and the spiked enriched GSSG (617 m/z → 359 m/z) were weak, as were the peaks for the “hybrid sandwich” (transition – 616 m/z → 358 m/z) and the “white bread sandwich” (transition – 619 m/z → 361 m/z). Although further research is needed to improve the sensitivity for GSSG detection, these results represent a step toward the goal of performing direct quantitation of GSSG, as well as GSH, in a whole blood sample from a nonlocal setting using SIDMS technology.
Researchers, such as Steghens, et al. have found the concentration of GSSG to be significantly lower (7–fold) in whole blood samples than in red blood cells from healthy adults, thereby making the detection of it in the former matrix more difficult than in the latter one (22). The Steghens research group analyzed blood samples from 20 healthy adults for reduced GSH and GSSG concentration using LC/MS and found the mean reduced GSH concentration to be $1.31 \pm 0.12$ mM and the mean GSSG concentration to be $0.64 \pm 0.22$ µM. They, too, found the analysis of GSSG in whole blood to be difficult.
as evidenced by the mean S/N ratio reported being between 5 and 6 for the analysis of GSSG in the 20 samples. Future work in our research group will involve further optimization of our method for analysis of the GSH/GSSG ratio in whole blood for our remote blood draw tube project.

As discussed, GSH forms conjugates with various species of mercury. That being the case, the development of methods for the analysis of such conjugates in the body would complement the analysis of GSH. An important step toward this end would be the initial development of methods for quantitatively (not just qualitatively) analyzing these conjugates in simple matrices so that those methods can be further modified for more complex matrices, such as blood, plasma, and urine. GSH holding the conjugate toxicants is one set of analytes for which quantitative analysis may aid in diagnosis and treatment of several conditions. Since GSH is a first line of defense in the immune system and is a major detoxification mechanism in the human body, investigating quantification of glutathione with the toxicant was another major scope of assessment in this research. Methods for these kinds of analyses in simple aqueous matrices are described in the next section.

**3D - Analysis of Conjugates of GSH with Mercury Species**

Positive mode ESI-MS analysis of a GSH conjugate with Hg$^{2+}$ by Burford, et al. showed that, because of the divalent nature of Hg$^{2+}$, two GSH molecules can combine with one Hg$^{2+}$ to produce a conjugate in which the most abundant peak appears at 815.1 m/z (23). Further research in this area by Feldmann, et al. also demonstrated that both Hg$^{2+}$ - GSH and CH$_3$Hg$^+$ - GSH conjugates, as well as conjugates of those two mercury
species with cysteine, could be detected by ESI-MS, with the characteristic mercury isotopic fingerprint being easily observed in all cases (24). This isotopic fingerprint can be observed in a GSH conjugate with C\textsubscript{2}H\textsubscript{5}Hg\textsuperscript{+} as well. An experiment carried out by our research group which demonstrates this idea is described next.

**Analysis of GSH Conjugates with three Mercury Species by nano-ESI-TOF-MS**

**Materials and Methods**

An aqueous solution containing 0.0015% GSH (Sigma-Aldrich, St. Louis, MO), 0.1% formic acid (Sigma-Aldrich), 50% Optima Acetonitrile (Fisher Scientific, Pittsburgh, PA), 10 ppm Hg\textsuperscript{2+}, 10 ppm CH\textsubscript{3}Hg\textsuperscript{+}, and 10 ppm C\textsubscript{2}H\textsubscript{5}Hg\textsuperscript{+} (Applied Isotope Technologies, Pittsburgh, PA) was prepared and analyzed by nano-ESI-TOF-MS. The system used for detection was an Agilent 6220 TOF-MS with Chipcube Technology. Analysis was carried out by direct infusion (Agilent infusion chip part number G4240-61002). The flow rate from the syringe pump (KD Scientific, Holliston, MS, model number KDS 100 CE) was 70 µL/hour. The capillary voltage was 1525 V, the fragmentor voltage was 225V, and the Nitrogen gas temperature was 325°C. The run time was 1 minute.

**Results and Discussion:**

Information pertaining to the abundances of the isotopes in mercury, as well as a visual display of the isotopic fingerprint, are shown in figure 7, which was taken from the webelements.com website (25).
Figure 7 – Mercury abundances and isotopic fingerprint (25)

The results from the simultaneous analysis of the three glutathione - mercury conjugates are shown in Figure 8:
Figure 8 – Simultaneous analysis of glutathione conjugated with three mercury species using nano-ESI-TOF-MS

The GSH conjugates of all three mercury species were detected by nano-ESI-TOF-MS with the characteristic mercury isotopic fingerprints clearly visible, just as with the Feldmann study.

Extending GSH and Conjugate Quantification to IDMS and SIDMS by MALDI

As helpful as it is to perform qualitative analysis of glutathione conjugates with these mercury species, quantitative analysis of these analytes would provide more
information regarding the extent of one’s toxic burden of these mercury species, if
detected in the body. Additionally, the development of a quantitative method for
analyzing these conjugates using Matrix Assisted Laser Desorption Ionization (MALDI)-
MS, along with nano-ESI-TOF-MS would be useful as well, as both MALDI and ESI are
soft ionization ionization techniques. An experiment demonstrating the quantitative
analysis of a Hg^{2+} - GSH conjugate using IDMS is described next.

3E - Quantitative Analysis of Hg^{2+} - GSH Conjugate by nano-ESI-TOF-MS and
MALDI-TOF-MS using IDMS

There was a time when quantitation using MALDI-MS did not have wide
popularity, likely, in part, due to the difficulty of obtaining accurate and precise and
accurate quantitative results with a significantly fluctuating signal, as well as
irregularities in the analyte distribution in the spots on the MALDI target. More recently,
however, quantitation with MALDI-MS has become more prevalent. One factor
contributing to this shift has been the use of internal standards to correct for signal
fluctuation (26), which is also an advantage that the use of IDMS has. Our research
group has achieved quantitation of GSH conjugated to Hg^{2+} by both nano-ESI-TOF-MS
and MALDI-TOF-MS using IDMS. This experiment is described next.
Materials and Methods

Quantitative IDMS with nano-ESI-TOF-MS

An aliquot of an aqueous 480.3 ppm natural abundant Hg\(^{2+}\) standard (~0.2000 g) was spiked with ~ 0.2000 g of isotopically enriched \(^{199}\)Hg\(^{2+}\) (Applied Isotope Technologies, Pittsburgh, PA) along with ~ 0.02000 g of ~50,000 ppm GSH, (Sigma-Aldrich, St. Louis, MO). ~1.6000 g of H\(_2\)O and ~1.6000 g of Optima methanol (Fisher Scientific, Pittsburgh, PA). This preparation of the IDMS sample was done in duplicate, as were the IDMS blanks, which were prepared like the IDMS samples were except that no natural abundant Hg\(^{2+}\) was added. These solutions were each analyzed six times (n=12) by nano-ESI-TOF-MS with Agilent chipcube technology using the conditions described for the analysis of the three mercury – GSH conjugates earlier in this chapter.

Quantitative IDMS with MALDI – TOF-MS

An aliquot of an aqueous 480.3 ppm natural abundant Hg\(^{2+}\) standard (~0.04800g) was spiked with ~0.10000g of isotopically enriched \(^{199}\)Hg\(^{2+}\), (Applied Isotope Technologies, Pittsburgh, PA) along with ~0.02000g of ~50,000 ppm GSH, ~0.05000g of H\(_2\)O, and ~0.07000g of ~5,000 ppm sinapic acid (Arcos Organics, Geel, Belgium) in Optima methanol. This preparation of the IDMS sample was done in duplicate, as were the IDMS blanks, which were prepared without natural abundant Hg\(^{2+}\). Each of these solutions were spotted (2 μL) on a gold MALDI target (Agilent p/n G1972-60025) twelve times (n = 24), and the spots were allowed to air dry at room temperature for three hours. The target was analyzed using Agilent MALDI-TOF-MS using the following conditions: wavelength of laser - 338 nm, Capillary Voltage - 2300 V, Fragmentor Voltage - 150 V,
Gas Temperature: 325 °C, run time for each spot - 1 minute (the laser was applied to several points on the spot over the course of 1 minute, and the average result over the course of this 1 minute was obtained).

Additional steps

Along with both the nano-ESI and the MALDI runs were analyzed an additional solution of natural abundant Hg$^{2+}$ to determine the preliminary mass bias correction. When GSH conjugates with Hg$^{2+}$, two GSH molecules bind to one Hg$^{2+}$, such that, in positive mode ESI and MALDI, the most intense peak appears at ~815 m/z and the fourth most intense peak appears at ~812 m/z. These peak intensities approximately (but not exactly) quantitatively parallel the most abundant isotope in elemental Hg (the $^{202}$Hg isotope) and the fourth most abundant isotope in elemental Hg (the $^{199}$Hg isotope), respectively, in terms of isotope abundance. The 812/815 ratio was used for the IDMS calculation. The preliminary mass bias correction was done with the understanding that the initial answer obtained from it represented only an approximation of what the correct Hg$^{2+}$ value should be. An additional correction was made by analyzing a known 940.5 ppm Hg$^{2+}$ solution using the nano-ESI method described earlier, and calculating the answer by an IDMS algorithm developed in our laboratory. The actual known value (940.5 ppm) was divided by the experimental value (1350 ppm) to obtain a supplemental correction factor of 0.697. This supplemental correction factor was multiplied by the initial experimental values from the nano-ESI and the MALDI analysis of the 480.3 ppm Hg$^{2+}$ solution to obtain the final experimental value.
Results and Discussion

The results from the quantitative analysis of Hg$^{2+}$ in GSH conjugate are shown in Table 2.

<table>
<thead>
<tr>
<th>Method</th>
<th>Concentration of Hg$^{2+}$ in ppm in GSH conjugate (reported to 95% C.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nano-ESI-TOF-MS with IDMS</td>
<td>485 ± 18</td>
</tr>
<tr>
<td>MALDI-TOF-MS with IDMS</td>
<td>474 ± 73</td>
</tr>
<tr>
<td>Actual value</td>
<td>480.3</td>
</tr>
</tbody>
</table>

Table 2 – Comparison results from analysis of Hg$^{2+}$ in glutathione conjugate conducted by IDMS-nano-ESI-TOF-MS and by IDMS MALDI-TOF-MS as compared to the actual value.

The results for the concentration of Hg$^{2+}$ from analysis of the Hg$^{2+}$ - GSH conjugate in the MALDI IDMS studies and the nano-ESI IDMS studies were in agreement with the correct value within the 95% C.I. The precision achieved from both methods was better than ± 20%. These results demonstrate the potential of performing quantitative analysis of mercury-GSH conjugates using IDMS with both MALDI-MS and nano-ESI-MS.

3F - Conclusion

This chapter has described several applications involving of EPA Method 6800A for the quantitation of analytes which are important to GSH metabolism. Of particular importance was the finding, discovered by using EPA Method 6800A, that in the red blood cell study, the autistic patients had higher red blood cell tGSH concentrations than the healthy control patients did (p = 0.012), suggesting the possibility that the autistic patients are making more GSH in response to possible elevated body burden of toxic heavy metals, such as lead, antimony, aluminum, and cadmium. Results also showed that
EPA Method 6800A could be used to quantitatively analyze a toxic heavy metal species (Hg$^{2+}$) with GSH in aqueous laboratory – prepared samples, indicating the potential for performing such an analysis in a more complex matrix, such as blood or plasma. EPA Method 6800A can be applied to monitor other analytes of metabolic importance as well, such as substrates for enzymes for the purpose of assessing enzyme activity. An experiment in which this different type of application was executed is described in the next chapter.
References:


5. Sigma Glutathione Assay Kit, product code CS0260.


14. EPA Method 6800a – website:


16. PBS “Ghost in Your Genes” website with transcripts from the video production:
   http://www.pbs.org/wgbh/nova/transcripts/3413_genes.html


19. Personal communication with Dr. Andrew Alpert from Poly LC, Inc., at the 2011 Pittsburgh Conference in Atlanta, GA.

20. Personal communication with Brian Rappold from Labcorp at the 2011 American Society for Mass Spectrometry (ASMS) Conference in Denver, CO.

21. Personal communication with Dr. Richard King from Pharmacadence Analytical Services, LLC, during a training session at Duquesne University, September, 2011.


25. website: www.webelements.com

Chapter 4 - IDMS Analysis of GLA Activity in Tissue Lysates from Mice

Fabry’s disease, also called *alpha-galactosidase-A (GLA) deficiency*, is caused by a lack of or faulty enzyme needed to metabolize lipids. It is one of a group of 44 lysosomal storage diseases (LSDs) characterized by the lack of or deficiency of enzyme production. GLA deficiency results in the accumulation of glycosphingolipids with terminal alpha-galactosyl residues, mainly globotriaosylceramide (Gb3) (1). Symptoms include chronic pain, kidney impairment, and cardiac lipid build up (1). One in 117,000 people have this particular LSD disease (2). The GLA mechanism is shown below in Figure 1(3):

![GLA Mechanism Diagram](image)

**Figure 1 – Human GLA Mechanism, taken from reference 3**

In the above mechanism, Aspartate–170 functions as a nucleophile, and Aspartate-231 functions as an acid in the first step, then as a base in the second step.

Treatment options for Fabry’s disease include enzyme replacement therapy and pain medication (4). Gene therapy for Fabry’s disease represents a potential alternative
that can be less expensive over the long term than other treatments. An initial attempt to develop a GLA gene therapy animal model was made using fluorescence equipment at Allegheny Singer Research Institute with limited success due to the background fluorescence from the tissue lysates (5). A collaboration between the Kingston Research Group and the Allegheny Hospital (AH) was formed in 2009 and mass spectrometry was introduced to take the place of the fluorescence measurements that were less sensitive and accurate. The same samples used for the fluorescence experiments were reanalyzed in our Center of Excellence by mass spectrometry and by our research group using IDMS with nano-ESI-TOF-MS-MS, and the sensitivity and clarity of the results improved significantly over the fluorescence data previously obtained by the Allegheny research collaborators. These quantitative methods developed to analytically improve the quantitative assessment of a mouse model and new salivary gland gene therapy alternative may eventually advance the research into treatment of humans. The development and application of these MS measurements is described next and lead to an important contribution in medical research published in Human Gene Therapy (6).

**Materials and Methods:**

**Initial work at Allegheny Singer Institute**

Details about the mice that were used in the study, how the gene therapy was administered, and how the tissues from the mice were processed are described in detail in the referenced literature paper that was published in 2011 in Human Gene Therapy (6). Briefly, control and knock out mice were obtained from Jackson Labs and brought to
Allegheny Singer Institute. The mice were divided into four categories: 1. wild type – normal mice (N = 8), 2. GLA gene deficient – lacked GLA gene (N = 14), 3. GLA gene deficient, exposed to low dose of adenovirus particles (1 x 10^9 particles) with the GLA gene (N = 8), and 4. GLA gene deficient, exposed to high dose of adenovirus particles (2 x 10^{10} particles) with the GLA gene (N = 14). Four days after initial exposure of the treatment mice to the adenovirus particles, the mice were sacrificed, and their liver, kidney, and brain tissues were harvested, after which these tissues were homogenized and subjected to a GLA activity assay using fluorescence. Differences between gene deficient and wild type samples were noticeable, but limited due to the background fluorescence from tissue lysates. Because of this limitation, the initial manuscript of the paper describing this project was rejected for publication. Over 100 mouse tissue samples obtained by AH researchers were reanalyzed by IDMS in the Mass Spectroscopy Center of Excellence and the paper was then rewritten around these higher quality analytical measurements and resubmitted. New significance was emphasized and several landmark findings were discovered that were new contributions to the research.

Quantitative Mass Spectrometry Development and Analysis at Duquesne University

In an attempt to increase the clarity of the GLA activity in the control and treated mice analysis in the mouse tissue samples by IDMS eliminated the background fluorescence problems experienced by the AG collaborators. Over 100 tissue samples were reprocessed with the IDMS method, after the GLA assay was completed, subjected to an SPE protocol, then analyzed by nano-ESI-Quadrupole time-of-flight Q(TOF)-MS-MS.
Details about the GLA assay and sample preparation for nano-ESI-QTOF-MS-MS analysis are mentioned in the paper describing this research project, which was published in Human Gene Therapy earlier this year with greater clarity of result significance over the previous fluorescence data previously submitted and rejected for publication (6). Briefly, the IDMS method was a modification of a procedure from Gelb et al. (7) originally designed for blood spot GLA analysis. An outline for this procedure is depicted in Figure 1 below:

![Figure 1 – GLA assay described by Gelb, et al. (7)](image)

In the procedure displayed in Figure 1, the sample is spiked with both natural abundant synthetic substrate (referred to as “Fabry Substrate” in the figure) and isotopically enriched synthetic standard (referred to as “Fabry Internal Standard” in the figure). If the GLA enzyme is working properly, the ratio of the natural abundant product (referred to as “Fabry Product” in the figure) to the isotopically enriched standard should be higher than it would be if the enzyme were either absent or deficient. When performing ESI-MS-MS on the natural abundant product and isotopically enriched standard, the tertiary butoxycarbonyl (t-BOC) group can be fragmented off in the
collision cell, resulting in the formation of the natural abundant product ion (384 m/z) and the isotopically enriched product ion (389 m/z).

In the collaborative project between Allegheny Research Institute and our research group, for the GLA assay, 15 µL of the assay solution (3.33 mmol/L of GLA-S (Fabry substrate in figure 1), 6.67 µmol/L of GLA-IS (Fabry Internal Standard in figure 1), 160 mmol/L ,N- Acetylgalactosamine, and 0.142 mol/L sodium acetate, pH 4.6) and 10 µL of tissue homogenate were added to a 1.7 mL centrifuge tube, centrifuged at 1350 x g for 1 minute, and then incubated at 37°C for 20 minutes. After incubation, the tube was briefly centrifuged, then the reaction was quenched by adding 100 µL of ethyl acetate/methanol 1:1. 400 µL of ethyl acetate, followed by 400 µL of H₂O were added and mixed with up and down aspiration in the pipette tip. The tube was then centrifuged at 1350 x g for 5 minutes to get the two phase separation. 300 µL of the top organic layer was transferred to a clean 1.7 mL microcentrifuge tube.

Additional Sample Cleanup

Further sample preparation was carried out by our research group at Duquesne to optimize detection of the analytes by nano-ESI-TOF-MS-MS. This protocol involved SPE using hydrophilic interaction liquid chromatography (HILIC). The SPE column used was a UCT (Bristol, PA) diol column, with 200 mg of sorbent and a 1 mL volume. The steps used in the SPE procedure are described next:

Step 1 - Condition SPE column with 1.8 mL of acetonitrile/H₂O 80/20
Step 2 - Add 150 µL from the 300 µL ethyl acetate solution to the column
Step 3 - Run 1 mL of acetonitrile/H₂O through the column and collect eluent

Step 4 - Add 2 µL of 90% formic acid to the eluent to help with ionization

**nano-ESI-QTOF-MS-MS conditions**

The conditions used for the analysis were as follows: system – Agilent chipcube with infusion chip (Agilent part number G4240-61002) and 6530 QTOF, syringe pump flow rate – 70 µL/hr, capillary voltage – 1450V, fragmentor voltage – 150V, collision cell – 20V, gas temperature – 325°C, drying gas flow – 4L/min, run time – 1 minute. Analytical runs were completed in triplicate.

**Results and Discussion:**

An example MS/MS spectrum from a gene deficient sample is shown below in Figure 2:

- Figure 2 - The peak at ~384.2279 m/z is the product ion from the natural abundant enzyme-substrate reaction product (theoretical m/z = 384.2282); the one at ~389.2584 m/z is the product ion from the isotopically enriched form of the analyte (theoretical m/z = 389.2595)
An example MS/MS spectrum from a wild type sample is shown below in Figure 3:

- Figure 3 - The peak at ~384.2283 m/z is the product ion from the natural abundant enzyme-substrate reaction product (theoretical m/z = 384.2282); the one at ~389.2584 m/z is the product ion from the isotopically enriched form of the analyte (theoretical m/z = 389.2595)

Figure 4 on the next page shows the comparison between the results from the fluorescent assay and those from the isotope dilution - MS/MS analysis:
Figure 4 – Results from both the fluorescent assay and the MS/MS assay.
The florescence spectroscopy assay does not have the fidelity to distinguish between the low dose mice, and a lack of quantitative fidelity diminished the significance of the tissue samples. In direct comparison, the IDMS data was able to show the differences in the animal model.

The clarity of the results using the isotope dilution – MS/MS analytical method demonstrated that the fluorescence method lacked the fidelity and quantitative accuracy to dramatically emphasize the significance of the animal model. As a result of these improved analytical results being obtained, the paper for this project was resubmitted for publication with the MS/MS results, and the publication was accepted (6).

For future work, research is ongoing to develop a gene therapy method without having to use a viral delivery system. The method involves sonication through the animal’s salivary gland and will be evaluated using the MS/MS method described in this chapter.

**Conclusion**

This chapter described experiments involving the application of IDMS TOF-MS for quantitative analysis of enzyme activity. Results showed that this method provided superior methodology for enzyme activity analysis compared to more conventional methods, such as the fluorescence spectroscopy assay mentioned earlier.

The use of TOF-MS, being a high resolution method, with mass accuracy usually within ± 5 ppm, can also be used to confirm results for the identification of compounds obtained from other analytical methods, such as $^1$HNMR. GC/MS, which uses electron
ionization at 70 eV and yields mass spectrometry data with virtually compound-unique fragmentation patterns, can also be used to complement $^1$H NMR results. This application of TOF-MS and GC/MS is described in the next chapter.
References:


4. National Institute of Neurological Disorders and Stroke website link: 

5. Personal communication with Dr. Michael Passineau from the Allegheny Singer Institute at Allegheny General Hospital, Pittsburgh, PA.


For organic and medicinal chemists to get their research published, they need to demonstrate that the synthetic schemes for their compounds of interest are successful by confirming the identity of the final products in those synthetic schemes by using at least two independent analytical methods. If one wants to publish in the Journal of Organic Chemistry (JOC), for example, one needs to provide both $^1$HNMR and $^{13}$CNMR results, along with results from either HRMS analysis or elemental analysis (1). For HRMS analysis of compounds below 1,000 G/mol, this journal considers results within 0.003 m/z of the theoretical value to be considered acceptable. This journal also allows for theoretical values to include ones in which atoms are added to the analytes’ ions, such as when sodium adducts are formed.

At Duquesne University (DU), first a walk up use of the Agilent TOF was attempted for groups wishing to use the mass spectrometer to do their own HRMS data for their publications. This experiment proved to be unsuccessful as the students did not take the care to prepare the sample properly, filter them, or dilute them, and the instrument frequently had to be repaired as a result. This practice was abandoned. It was then reformulated to have these graduate students from these synthetic laboratories submit their samples to an experienced analytical chemist who, initially, was Dr. Stephanie Wetzel from the Chemistry and Biochemistry Department at Duquesne University and, later on, after receiving training from Dr. Wetzel, was the author of this dissertation. The analyst would manage the final preparation and the instrument oversight and analyses. This proved to be successful for over three years, and an experiment was
commenced to see how the support of other laboratories would proceed under these conditions and what would be the use and need and talent needed to successfully create an HRMS service laboratory system. This was done for free as an experiment in the Department of Chemistry and Biochemistry on the NSF MRI granted instrumentation that had been received and completed in its construction in 2006 through 2011.

For these years, the Kingston Research Group has been supporting the chemistry faculty and the pharmacy faculty by providing these services using the NSF granted instrument. The author of this dissertation has been the analyst in recent years performing these analyses and has been on a Research Assistant (RA) funded in the Professor Kingston performing these analyses as an experiment of service to the DU synthetic chemists. These in-house HRMS analyses have been a free service for research groups in both the Chemistry and Pharmacy departments and the use of the facility has grown to over 200 HRMS samples per year. The monetary value of this service can be seen as considerable given the fact that other universities, such as the University of Iowa, charge their within-university staff for HRMS analysis. The rate at the University of Iowa for HRMS analysis, for example, is $59 per sample (2). At Duquesne University, just in 2010 alone, approximately 200 HRMS samples were analyzed for no charge for research groups in the Chemistry and Pharmacy departments. Had the pricing system of the University of Iowa been in place at Duquesne, the HRMS samples analyzed in 2010 for the Duquesne research groups would have cost over $11,000. This represents a significant savings for the university.

When samples are submitted to the analyst at Duquesne for HRMS analysis, the samples come with a submission form which gives the analytical chemist information as
to what analyte m/z values to look for. Included in this submission form is also a section where the proposed structure of the compound is to be given. This helps the analyst because it provides information regarding what functional groups are present and how polar the compound is, which, in turn, enables the analytical chemist to decide how to best analyze the sample. This chapter has been prepared to both document the development of this capability and to permit this service should this service be continued.

A description of the structure the organic molecule including polarity is essential for the analyst to determine how best to analyze the sample. For example, polar compounds with amines are good candidates for positive mode ESI-MS analysis, whereas compounds of relatively low molecular weight (~1,000 Dalton or below) and moderately low to intermediate polarity would be good candidates for Atmospheric Pressure Chemical Ionization (APCI)-MS analysis (3). According to Dr. John Greaves, who runs the HRMS laboratory at the University of California at Irvine, there are some compounds that are so nonpolar that even APCI-MS will not work (4). An example of a case study involving the successful analysis of such a compound by the analyst at Duquesne will be discussed later in this chapter.

Most (about 90%) of the compounds submitted thus far to the analyst at Duquesne can be analyzed using positive mode nano-ESI-TOF-MS by direct infusion using the Agilent equipment described in chapters 3 and 4. Typically, the compounds are dissolved in either Acetonitrile or Methanol (high purity brand, such as Optima from Fisher Scientific, Pittsburgh, PA), and, often, the sodium adduct is the most abundant ion detected due to the presence of sodium in the glassware used by the synthetic chemists. Although a reasonable analyte concentration for the HRMS analysis would be about 10
ppm, usually, when compounds are submitted, no information whatsoever is given to the analyst as to what the concentration is. This renders it necessary for the analytical chemist, as a precautionary measure, to dilute the sample at least 100-fold to minimize the likelihood of overloading the instrument with sample. If it turns out that the concentration of the diluted sample is too low, all one has to do is use a higher concentration, and only a few minutes are lost. If, however, this precautionary measure is not followed, one runs the risk of overloading the instrument with a solution with a concentration that is too high (usually over 100 ppm), after which can take several hours to flush the compound out of the system.

If the analyte is not detected during the HRMS analysis, there can be several possible reasons. Some of these possibilities are given below:

- The concentration was too low.
- The container and/or HRMS form was mislabeled.
- The solvent that the compound was dissolved in reacted with the compound.
- The proper NMR analysis was not completed before sample submission.
- The compound decomposed while waiting to be analyzed.
- The appropriate source was not used (e.g. ESI vs. APCI)
- The appropriate mode was not used (positive vs. negative)

If the analyte is not detected by the analyst, the analytical chemist who performed the HRMS analysis can communicate with the synthetic chemist who submitted the sample to determine what, if any, alternative methods can be used. The following three case studies represent real examples of situations in which the initial HRMS analysis of the submitted samples with the usual positive mode nano-ESI-TOF-MS was not successful,
but eventually the desired results were obtained by the analytical chemist. No chemical structures will be shown due to the proprietary nature of the compounds that were submitted, but general descriptions of the compounds will be given.

Case # 1

A sample was submitted by a person from Dr. Fleming’s research group. The compound seemed to be present based upon NMR analysis, but the person who submitted the sample also mentioned that it was sensitive to oxidation. According to the proposed structure that was given to the analytical chemist, the sample was a low molecular weight (below 500 amu) compound with a sulfur atom. When the sample was initially analyzed using positive mode nano-ESI-TOF-MS, the analyte of interest was not detected. Upon further examination however, it was noticed that there were three peaks detected which were about 16 m/z units higher than their corresponding expected [M+H]⁺, [M+Na]⁺, and [M+K]⁺ peaks. When the proposed structure was modified to include an additional oxygen atom, the experimental m/z values were in agreement with the theoretical m/z values to within 10 ppm error of the theoretical mass. The successful detection of the modified compound enabled the person from Dr. Fleming’s group who submitted the compound to publish his work in the literature.

Case # 2

A sample was submitted by a person from Dr. Basu’s research group which was of moderately low polarity. An initial attempt to analyze the sample by positive mode nano-ESI-TOF-MS was not successful. It was hypothesized that the polarity of the
sample was too low for ESI-MS analysis. Positive mode APCI-TOF-MS was used instead. As a result, the moderately nonpolar compound was successfully detected.

**Case # 3**

A compound with a long hydrocarbon chain, an oxygen atom, and several fluorine atoms was submitted to the analytical chemistry staff by a person from Dr. Janjic’s group from the Pharmacy Department. The compound seemed to be present according to the NMR results, but attempts to analyze the sample by both positive and negative mode nano-ESI-TOF-MS were not successful (according to Dr. Schilling from the University of Illinois at Chicago, negative mode can be used with the Agilent Chipcube system provided that the capillary voltage remains at or below 1600V (5)). Dr. Janjic sent the sample to outside laboratories for analysis, and the sample was still not detected by any of the commercial laboratories. At the 2010 ASMS meeting in Salt Lake City, Dr. John Greaves, who runs the HRMS laboratory at the University of California at Irvine, after being given a general description of the compound without revealing the structure, surmised that the compound was so nonpolar that even APCI-MS would not ionize the compound. He mentioned that his laboratory had a high resolution GC/MS system and that the sample could be sent to him for analysis, but he suggested that the analyst at Duquesne analyze it first on low mass resolution GC/MS at Duquesne. After successfully detecting it with the low resolution GC/MS at Duquesne, the sample could be sent to him for high mass resolution GC/MS analysis. This analysis was carried out by Becky Wagner, one of the analytical chemistry graduate students at Duquesne. The following conditions were used for the analysis:
- System – Agilent 6890N GC coupled with Agilent 5973 MS

- Sample dissolved in 2-propanol (concentration – 60 ppm)

- Molecular weight: 432 g/mol

- 1 µL injection volume

- Injector: 250°C

- Start oven → 75°C, hold 1 minute

- 10°C/min up to 275°C, hold for 4 minutes

- Column → HP-5 MS

- Transfer line temperature: 280°C

- 4 minute solvent delay

- Range: 40 m/z – 500 m/z

The GC chromatogram is shown in Figure 1 below. The analyte peak of interest was detected at ~ 10.5 minutes:
The MS fragmentation pattern from the peak at ~ 10.5 minutes in the chromatogram (from 240 m/z upward) is shown in Figure 2 below:

Figure 2 – MS fragmentation pattern from 240 m/z upward from peak detected at ~ 10.5 minutes in the GC chromatogram.
In the fragmentation pattern in Figure 2, the parent ion was successfully detected at 432 m/z. The peak at 403 m/z represents the loss of an ethyl group (C₂H₅ → 29 m/z). There are several other peaks about 14 m/z units apart, representing the loss of successive methylene groups (CH₂), which is common in the GC/MS analysis of compounds with long-chain hydrocarbon groups. The continuation of this fragmentation pattern with the loss of CH₂ groups can be seen from 40 m/z to 240 m/z shown in Figure 3, which is shown below:

Figure 3 - MS fragmentation pattern from 40 m/z – 240 m/z from peak detected at ~ 10.5 minutes in the GC chromatogram
The results from the GC/MS analysis were consistent with what was expected for the compound submitted by the person from Dr. Janjic’s group.

In order for successful HRMS analysis to occur, proper communication between the person submitting the sample and the person analyzing the sample must exist. The tools available to the analytical chemist at Duquesne are many, but no one instrumental method consisting of a single ionization source is sufficiently robust to detect every type of analyte submitted, as the case studies in this chapter demonstrate. Skill and experience several ionization systems are necessary for the analyst. Success comes as a result of teamwork, and it is a requirement for the analytical chemist performing the HRMS analysis and the organic/medicinal chemist submitting the samples for HRMS analysis to exchange necessary information with each other for the program to work properly. The analyst can not do the job if the synthetic/medicinal chemist does not provide the necessary information, such as what solvent the sample is dissolved in, whether or not the compound of interest has been successfully analyzed by $^1$HNMR and/or $^{13}$C NMR, and how to best store the sample until analysis. To assist with this submission process, an updated submission form has been developed that has areas where the synthetic chemist can submit the necessary information. It was found that safety information was especially necessary, and appropriate other information and instructions were necessary as well. This form was developed as a result of this several years of experiment and iteration, and is attached as an appendix to the dissertation after this chapter.
References:


2. The University of Iowa High Resolution Mass Spectrometry Facility website link: https://research.uiowa.edu/vpr/units/hrmsf/rates.html


4. Personal communication with Dr. John Greaves at the 2010 ASMS Conference in Salt Lake City, UT.

5. Personal communication with Dr. Alex Schilling from the University of Illinois at Chicago, 2010.
Appendix – Updated draft of Official HRMS submission form

(Begin form)

Updated Official Submission Form for HRMS Analysis

Faculty member’s name (printed) _____________________________
Faculty member’s signature _____________________________
Student’s name (printed) _______________________________
Student’s signature _______________________________
Compound name ________________________________
Date submitted ________________________________
Molecular weight ________________________________
[M + H]^+ theoretical m/z value ______________________
[M + Na]^+ theoretical m/z value ______________________
[M + K]^+ theoretical m/z value ______________________
Solvent that the sample is dissolved in if submitted in solution form (use either acetonitrile or methanol) ________________
Concentration of sample in units of parts per million (ppm) ________________
Molecular formula of compound __________________________
Safety precautions that need to be taken with the sample:
Structure of compound (draw below):

Questions about sample (response for each must be “yes” before submission for HRMS analysis)
1. Has the sample been successfully identified by $^1$H NMR (and, if necessary, $^{13}$C NMR) ? (yes/no)
2. Has the sample been purified to the point where ONLY the analyte peaks show up in the NMR (in other words, no peaks from, for example, excess starting material) (yes/no)
3. Is HRMS analysis necessary as the final step before publication? (yes/no)

General comments:

Samples submitted merely to monitor the progress of reactions will not be accepted unless, in extraordinarily rare cases, all other means to do so, such as normal phase thin layer chromatography, reversed phase thin layer chromatography, NMR, GC/MS, or other methods, have been exhausted. Providing the structure will help the person performing the HRMS analysis to determine how best to analyze the sample and will, thus, speed up the process. Generally, for compounds of relatively high polarity to intermediate polarity, nano-ESI-TOF-MS will be used. For samples that are of intermediate polarity or are relatively nonpolar, APCI-TOF-MS will be used.

(End of form)
Chapter 6 – Conclusion

This dissertation has described research projects involving applications of EPA 6800A to new areas, such as toxicology, biomarker discovery, and enzyme activity analysis. In closing, taking a step back and looking at the big picture, one can see how these three applications fit together. The speciation of toxic heavy metals, such as mercury, in biological matrices, such as blood, is related to the analysis of possible autism biomarker, such as GSH, in those same biological matrices, as the concentration of GSH in red blood cells appears to be affected by the body burden of toxic heavy metals (1). Toxic heavy metals can also have an impact on enzyme activity via inhibition, and it may be that some of the enzymes being inhibited are ones which contribute to GSH synthesis, as it has been suggested that GSH synthesis can be inhibited by mercury; conversely, toxic metals other than mercury may stimulate GSH synthesis (1).

The process of answering one question leads to the formation of more questions such that, no matter how many questions one answers, there will always be further questions left unanswered. This dissertation is no exception to that rule. One question that needs to be answered pertains to why the autistic patients in our red blood cell study have higher red blood cell tGSH values than their corresponding controls. It could be that their bodies are making more GSH in response to a greater body burden of toxic heavy metals other than mercury, but further research is needed to determine this, and EPA Method 6800A can be used to address this issue by using it for the analysis of heavy metals in the red blood cell patients. This work is currently being carried out by my colleague, Greg Zinn, a graduate student at Duquesne University. Another question that needs to be
addressed more thoroughly pertains to what the metabolic fate of Thimerosal in the body is. C₂H₅Hg⁺, a component of Thimerosal, can interconvert with other mercury species during sample preparation and, as was presented in this dissertation, EPA Method 6800A can be used to correct for these interconversions; hence, EPA Method 6800A would be the ideal method of choice to address this question as well. An additional question that needs to be addressed concerns which toxic heavy metals are being bound to which biomolecules and in what amounts. The work described in this dissertation pertaining to the analysis (qualitative and quantitative) of GSH conjugates with mercury species in aqueous solutions provides a starting point, but further work is needed in this area. Ideally, one would like to detect the heavy metal bound to the biomolecule by mass spectrometry using a soft ionization method, such as ESI or MALDI, and it would be best for one to obtain quantitative, rather than just qualitative results. EPA Method 6800A can also be used for this area of research.

In closing, it is essential for scientists to be open to new ideas. Over the centuries, many scientific ideas that were initially met with rejection were eventually accepted. One such example occurred with an astrophysicist by the name of Subrahmanyan Chandrasekhar. In the 1930s, as a young scientist, he proposed that, if a collapsing star has a mass above a certain limit (known today as the Chandrasekhar limit), the star would collapse down to what is today known as a black hole. His supervisor at the time, Sir Arthur Eddington, strongly rejected this idea, as did Albert Einstein; nevertheless, Chandrasekhar’s idea was eventually shown to be correct, and his work in this area contributed to his winning the Nobel Prize in 1983 (2). Another example occurred with a rocket scientist by the name of Dr. Robert Goddard. In 1920, he proposed that it would
one day be possible to send a rocket to the moon. This idea was met with rejection by many in the scientific community, as well the popular culture. The New York Times newspaper ridiculed this idea. In 1969, 49 years later, a day after Apollo 11 left for the moon, the New York Times retracted its statement (3). An additional example occurred with my research advisor at Duquesne, Dr. H.M. “Skip” Kingston, when he proposed that it would be possible to perform chemistry experiments (extraction, digestion, synthesis, etc.) in the microwave which would prove to be more efficient than the more classical methods. This idea was initially met with rejection. Some of his colleagues jokingly referred to him as “Betty Crocker.” (4). Despite this, the microwave chemistry concept eventually gained acceptance in the scientific community, with many publications describing the successful use of this methodology being made, including this one.

In short, a good scientist has the humility to recognize that, no matter how much one learns, there is always more to learn. No matter how much one grows, there is always room for further growth.

St. Albert the Great, patron saint of scientists, pray for us.
References:


4. Personal communication with Dr. H.M. “Skip” Kingston from Duquesne University, 2005.