I, Kevin Kubachka, hereby submit this work as part of the requirements for the degree of: Doctor of Philosophy in: Chemistry

It is entitled:
Elemental Speciation Analysis of Arsenic, Selenium and Phosphorus: Exploring Foods and Plants

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Elemental Speciation Analysis of Arsenic, Selenium, and Phosphorus: Exploring Foods and Plants

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ABSTRACT

Due to varying properties of an element, depending on its chemical form, total element concentration is often not sufficient information for accurate assessment. Therefore, the analysis of the distribution of an element amongst various chemical species (termed speciation) is critical. By implemented combinations of various separation techniques (namely high performance liquid chromatography (HPLC) and gas chromatography (GC)) with mass spectrometric techniques such as inductively coupled plasma mass spectrometry (ICP-MS) and molecular mass spectrometry techniques, a powerful speciation tool is created.

Elemental analysis using ICP-MS and speciation analysis can be used in complement to confirm species recovery and mass balance. When speciation information using HPLC/GC-ICP-MS does not provide enough information, molecular mass spectrometry techniques with softer ionization techniques (able to preserve structural characteristics) can enable confident identification of a given species.

Arsenic, selenium, and phosphorus are extremely important elements and are much concern in several fields of research. In specific, arsenic and selenium are of interest due to the drastic difference in toxicity among their respective species. The main goal of this dissertation is to examine the distribution of arsenic, selenium, and phosphorus among various species for assessment of toxicity, nutritional value, and environmental impact.

Wheat, potato, yam, and rice samples were examined to establish the impact of their consumption in regards to arsenic content. Selenium association with proteins was examined to further understand its biotransformation in mushrooms. The metabolism of
selenium in genetically modified *Brassica juncea* was examined to further understand the selenium flow of the plant in hopes of creating a more efficient phytoremediation tool. Organophosphorus chemical warfare agent degradation products were chromatographically separated from inorganic phosphate (a common interference) in several food samples using a combination of HPLC-ICP-MS and HPLC-ESI-MS in an effect to detect food contamination.
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CHAPTER ONE: 
Introduction to speciation analysis using chromatography techniques interfaced with mass spectrometry

1.1 Purpose of Work

Within the last 20 years, inductively coupled plasma mass spectrometry (ICP-MS) has evolved to be more than just an analysis method for metals. Due to several advances in instrument technology, ICP-MS has equipped researchers with the means to analyze non-metals for wide variety of applications. By combining ICP-MS with various forms of chromatography, a powerful tool is created, capable of exploring beyond total elemental content, and extending to speciation analysis. This work harnesses such combinations and complements them with molecular MS techniques, together to shed new light on previously unexplored areas of elemental speciation. Developing new methods and applications for speciation methods is the aim of this work as they are needed for many purposes including determining risk assessment, nutritional value, and environmental impact of foods and plants with regard to chemical species of arsenic, selenium, and phosphorus.

1.2 Elemental Speciation Analysis

There are several elements of high concern as they are occupational or residential exposure hazards. Often times, to monitor these elements they are analyzed with respect to their total element concentration. For example, in the US, as mandated by the Environmental Protection Agency (EPA) in 2001, arsenic (As) in drinking water is
limited to 10 parts per billion (ppb).[1] However, there are several elements in which more information is useful based on the fact that an element may have varying toxicities in different “forms.” One specific example is the toxicity of chromium (Cr), as Cr$^{VI}$ is considered carcinogenic and mutagenic and Cr$^{III}$ is an essential nutrient.[2] Research devoted to the investigation of various chemical forms or species of the same element has been termed “elemental speciation.” The research presented in this dissertation includes speciation analysis of arsenic (Chapter 2), selenium (Chapters 3 and 4 [3,4]), and phosphorus.

Speciation is necessary for a wide variety of applications in various sample matrices including environmental (water, soil, air, etc.), foods and beverages, and biological samples (hair, blood, urine, etc.). Due to the large variety of sample types, methods should be selective and able to resolve analytes from possible interferences. Qualitative analysis is important when identifying unknowns, while quantitation is necessary to establish toxic levels and enforce regulatory limits of various compounds. Also analysis at low levels is needed as many compounds are considered a hazard at, even part per billion levels (as in drinking water).

1.3 Instrumentation

Typical analyses of the previously mentioned elements are performed through the use of instruments with element specific detection capabilities, where these elements are used as “elemental tags.” One of the more common elemental analysis techniques includes atomic absorption spectroscopy (AAS) with flame ionization (FAAS) or electrothermal ionization (ETAAS). Optical emission spectrometry has used other
ionization sources, including as microwave induced plasma (MIP) and inductively coupled plasma (ICP). The inability of these spectroscopic methods to reach ultra-trace detection limits and presence of spectral interferences, increased the need for more sensitive and selective detectors.[5] The advent of ICP with mass spectrometry (ICP-MS) for element specific detection experiments, sensitivity and selectivity are significantly improved. Other methods such as molecular mass spectrometry (MS) with softer ionization methods, such as electrospray ionization (ESI), chemical ionization (CI), atmospheric pressure ionization (API), or electron impact ionization (EI) can also be used, however a decrease in both sensitivity and selectivity are typically observed compared to ICP-MS element specific detection.[5]

1.3.1 Inductively coupled plasma mass spectrometry (ICP-MS)

As previously mentioned, ICP-MS is an element specific detection method, with the ability to detect almost all elements in the periodic table virtually simultaneously depending upon the mass analyzer. The use of this harsh ionization technique in conjunction with a mass selective detector provides a tool for isotope specific experiments. ICP-MS is very sensitive with detection limits for some analytes at low part per trillion (ppt) levels. Quantitation is easily achieved using an external calibration curve, standard addition, or isotope dilution methods, with a dynamic range of up to 9 orders of magnitude.

What follows is a brief summary of ICP-MS from sample introduction to analyte detection as explained from left to right in accordance with Figure 1.1. Further information can be found several excellent reviews by Houk and Thompson [6] as well as
others.[7-10] The initial part is the sample introduction, typically involving nebulization of the liquid sample, in which gas forces the solvent out of end of a narrow inner diameter tube, thus forming a “mist” of sample via the so-called Bernoulli effect.[11]

The sample then is transported into the ionization source, which in most cases is an argon plasma, by the carrier gas, typically argon. Here, the sample is desolvated, ionized to a positive charge, and atomized by the plasma which is at temperatures from 6000-10,000 K. The ions then pass through the interface region consisting of both a sampler and skimmer cone (composed of either nickel or platinum depending upon the application). The interface region is the transition point from atmospheric pressure to the vacuum region of the instrument. This vacuum region consists of ion optics for focusing, collision/reaction cell for interference removal (described in section 1.3.1.1), mass analyzer (quadrupole, time of flight, sector field), and detector (electron multiplier).

Figure 1.1: Agilent ICP-MS Block Diagram. Reaction/collision cell is located after the Ion lens and before the mass analyzer (in this case, a quadrupole). From Agilent Technologies with permission.
1.3.1.1 Collision/reaction cell

When speciation analysis is required, it is easily interfaced with chromatographic techniques such as high performance liquid chromatography (HPLC), gas chromatography (GC), and capillary electrophoresis (CE). Further explanation of HPLC and GC in combination with ICP-MS for elemental specific detection is included in sections 1.2.2 & 1.2.3, respectively. As indicated above, ICP-MS is an element specific technique with the mass analyzer detecting an analyte based on its mass to charge ratio (m/z). However, interferences are present, including isobaric interferences ($^{54}$Cr and $^{54}$Fe) and polyatomic interferences ($^{40}$Ar$_2^+$ on $^{80}$Se$^+$). The $^{40}$Ar$_2^+$ polyatomic interference at 80 m/z is always present at the high levels in an argon plasma and interferes with the most abundant isotope of Se at 80 m/z (49.61%). To resolve this problem, the use of a collision or reaction gas in conjunction with an energy barrier (He, H$_2$), to collide/react with the interference or analyte was used. The collision/reaction gas system is commercially available on Agilent 7500ce ICP-MS (Agilent Technologies, Tokyo, Japan) and was utilized throughout the presented research. Further specifics on this topic can be found in the literature.[12-15] For example, the use of H$_2$ can reduce the spectral background at m/z 80 from 10,000,000 counts per second (cps) to 10 cps, producing a background equivalent concentration of approximately 1 part per trillion (ppt) for $^{80}$Se.[16]

There are other strategies of working around these interferences such as monitoring a less abundant isotope ($^{82}$Se) or calculating the contribution of possible interferences based isotopic information ($^{40}$Ar$^{35}$Cl$^+$ and $^{75}$As$^+$ by monitoring $^{40}$Ar$^{37}$Cl$^+$). For elements discussed in this chapter, the common interferences are shown in Table 1.1.
Table 1.1: Analytes of interest with their major isotopes. The relative isotopic abundance of each isotope and major corresponding polyatomic interferences are also listed.[17]

<table>
<thead>
<tr>
<th>Isotopes</th>
<th>Percent Abundance</th>
<th>Polyatomic Interferences</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{31}$P</td>
<td>100</td>
<td>$^{14}$N$^{16}$O$^1$H, $^{15}$N$^{16}$O</td>
</tr>
<tr>
<td>$^{75}$As</td>
<td>100</td>
<td>$^{40}$Ar$^{35}$Cl$^+$</td>
</tr>
<tr>
<td>$^{75}$Se, $^{80}$Se</td>
<td>23.5, 49.8</td>
<td>$^{40}$Ar$^+$, $^{38}$Ar$^{40}$Ar$^+$</td>
</tr>
</tbody>
</table>

1.3.2 High performance liquid chromatography (HPLC) with ICP-MS

With all the advantages of ICP-MS, one main disadvantage is that only elemental information is gained. Therefore, speciation was developed whereby we separate various compounds, based on its species specific properties using some type of chromatography, then pass the eluent into the ICP-MS for mass specific detection.

The most common separation technique coupled to ICP-MS is HPLC. More extensive information on HPLC-ICP-MS can be found in several reviews.[18-22] The interfacing is simple and well documented in the literature,[23] as just a PEEK tubing capillary from the end of the column to the nebulizer of the ICP-MS is typically all that is needed. The eluent is obviously in liquid form, which is ideal for conventional nebulizers. The main concern involving the nebulizer is to ensure that the chromatographic flow rate is compatible with the nebulizer.

Compositions of the mobile phases in HPLC are the most crucial of factors when interfacing with ICP-MS. Problems occur with high concentrations of organic modifiers such as methanol (MeOH) and acetonitrile (ACN). Typically these should be kept \( \leq 20\% \) v/v as higher levels can result in plasma instability, thus leading to a noisy baseline, and at high enough levels, the plasma will extinguish. Some research has cited that the addition of MeOH can actually increase the signal to noise of the analyte as it can
increase ionization efficiency of the plasma source. Due to this reason and greater plasma stability, with MeOH as compared to ACN, MeOH is typically the organic solvent of choice for liquid chromatographic methods applied to ICP-MS. This problem can be overcome by desolvation or dilution of the mobile phase prior to introduction into the ICP-MS [24] among other methods.[25,26] This premise also applies to solutions with high salt concentrations, as high enough levels will cause the similar problems and introduction of easily ionized elements such as sodium and potassium can lead to decreased ionization for analytes of interest. It is also important to note that exceeding any of these solution limitations could cause instrument components to dirty faster thus decreasing ion transmission and lowering sensitivity.

HPLC-ICP-MS has many advantages that make it very powerful for speciation analysis. HPLC is less time consuming and simple with regard to sample preparation in that no derivatization is needed either prior to analysis or post-column. The interfacing of the two techniques is user-friendly. ICP-MS is the ultimate in element specific detection as far as analyzing a wide range of elements with high sensitivity and selectivity. A plethora of HPLC separation schemes can be interfaced with ICP-MS, thus leading to a wide variety of applications.

1.3.3 Gas chromatography (GC) with ICP-MS

A second very popular speciation tool is GC-ICP-MS. The high resolving power of GC in combination with excellent sensitivity and selectivity of ICP-MS make for a great combination. More detailed information on GC-ICP-MS can be found in several reviews.[27-31] Capillary GC is commonly used for the separation of volatile
Compounds. Non-volatiles are possible, but require derivatization. Compounds analyzed in Chapter 4 were present in the headspace of the samples and sampled by solid phase microextraction (SPME), therefore no derivatization of the analytes was necessary. Compounds of interest are eluted based on interactions with the column stationary phase with their mobility being high dependent upon their boiling point. Upon exiting the GC oven, analytes are routed directly to the ICP-MS. This requires a special interface, as one designed and commercially available by Agilent Technologies was utilized in our laboratory. The interface is an external heated transfer line going from the GC oven to the ICP-MS torch. The basic requirement for an interface is to transport the analytes from the GC to the ICP in a manner that prevents condensation and species conversion.[30]

The setup in our laboratory utilized direct transfer line to torch interfacing providing the advantage of the absence of aerosol in the plasma which limits energy losses for desolvation and vaporization and results in a high sensitivity and reduces polyatomic interferences.[30] For volatile compounds GC-ICP-MS is an extremely powerful technique providing excellent separation in combination with low detection limits.

1.3.4 Complementary use of molecular MS

Similar to both separations, HPLC and GC, the various species exit the chromatograph and the ICP-MS detects only the specific element of interest. Therefore, if multiple analytes containing the element of interest elute at similar retention times they will appear as one peak, so chromatographic resolution is quite important. No structural
characteristics, other than those inferred from the given nature of the chromatography, are available. Species are therefore identified by retention time matching and/or fortification of the sample with the standards and monitoring increase in response. Identification using only (HPLC/GC)-ICP-MS becomes virtually impossible when standards cannot be used either due to lack of commercial availability or inability to synthesize the desired standard (often the case in selenium analysis).

Therefore complementary use of molecular MS to determine structural information is necessary. One approach is to use a chromatographic separation method that is compatible with ICP-MS and pair it with the desired molecular MS technique. When this approach is taken with GC analysis, conditions are easily transferable from GC-ICP-MS to GC-MS. The complementary use of GC-ICP-MS and GC-TOF-MS is explained in greater detail in Chapter 4. However, parameters for HPLC methods are not always compatible with ICP-MS and molecular MS. The buffer system and flow rate are critical when developing a method for uses with both ICP-MS and molecular MS. Complementary use of HPLC-ICP-MS and HPLC-ESI-MS is employed in Chapter 5 where buffer and flow rate compatibility issues are discussed in greater detail.

Another approach is to collect the unknown compound as it elutes from the HPLC, preconcentrate, and analyze with molecular MS.[32,33] Increasing the purity of the unknown compound simplifies the elucidation and confirmation of the structure. Therefore, sample cleanup frequently used to ensure compatibility with the desired MS method – such as removing previous buffers (namely salts) or other extraneous compounds. This can be achieved by using multiple chromatographic techniques, solid phase extraction, or other methods including molecular weight cut-off filters.
1.4 References


CHAPTER TWO:
Low level arsenic determination and speciation in wheat, potato, yam, and rice samples

2.1 Introduction

Arsenic is also under heavy surveillance as it is a common environmental pollutant. The main source of arsenic contamination for the aquatic environment is from geological sources, either from surface weathering or underground deposits.[1] Humans play a part in the problem with the use of pesticides and herbicides, pressure treated lumber, industrial manufacturing, and roxarsone (a growth promoter in chicken feed). The problem is especially prevalent in less developed countries (most infamously, Bangladesh), in which arsenic contamination of village water supplies is all too common.[2,3]

As previously mentioned, many regulatory limits on arsenic (including arsenic in drinking water) are based on total arsenic levels. However, different species of arsenic have varying levels of toxicity. Arsenic is most commonly found in these forms: Arsenite (As\textsuperscript{III}), Arsenate (As\textsuperscript{V}), Monomethylarsenic acid (MMA\textsuperscript{V}), and Dimethylarsenic acid (DMA\textsuperscript{V}). As\textsuperscript{III} and As\textsuperscript{V} are commonly believed to be acutely toxic species. MMA\textsuperscript{V} and DMA\textsuperscript{V} compounds are carcinogenic and research is currently underway to more fully understand their respective toxicities. However, it is generally accepted that the organic forms, arsenobetaine (AsB) and arsenocholine (AsC), are less toxic than the inorganic forms.[1]

Food and water are the primary sources of inorganic arsenic exposure for humans. Arsenic levels are highly monitored in the US, especially in drinking water; the
maximum allowable level of arsenic in drinking water was recently changed to 10 µg As L\(^{-1}\) [4] and the World Health Organization (WHO) has set the maximum tolerable daily intake (MTDI) of arsenic at 2 µg kg\(^{-1}\) bodyweight per day.[5] A second source of arsenic intake by humans is consumption of arsenic containing foods. In terrestrial foods, growing conditions highly affect arsenic intake as polluted soil and/or irrigation water are reflective of arsenic levels in the food. It typically enters the food as the soil it was grown on or irrigation water is contaminated with arsenic, whether it is from pesticides or other sources. Rice is the food of most concern regarding arsenic, as it generally contains higher levels of arsenic than most other terrestrial-based foods. Consumption rates of rice in the US have also increased over the past several years. Rice is more of a staple in ethnic groups such as Asians and Hispanics [6] compared to the typical American’s average intake (25 g day\(^{-1}\) of rice [7]). This stresses the importance for further understanding of the toxic contribution of rice pertaining to various arsenic species.

Other commonly consumed foods such as wheat, yams, and potatoes are also of interest, since few studies have been carried out involving their toxic contribution to the diet as a result of As uptake. An FDA dietary intake study bases arsenic risk by assuming that all of the arsenic is inorganic.[8] Few attempts at speciation of terrestrial plants have included speciation analyses; those that have are inconclusive.[9] Therefore questions are still being raised about the species of arsenic in food crops.[10]

Due to the toxicity difference between inorganic and organic forms of arsenic, it is very important to not only evaluate total arsenic levels, but to understand what species are involved so that the risk of arsenic in the diet can be accurately assessed.
In previous work, inorganic arsenic, DMA, and small amounts of MMA were identified in six samples of rice.[11] The average inorganic content was $82 \pm 27$ ng g$^{-1}$ dry weight (DW) (ranging from 20-98 ng g$^{-1}$). In 2003, Lamont and coworkers analyzed 40 samples of white rice in the US for inorganic arsenic and found a mean concentration of $112 \pm 55$ ng g$^{-1}$ wet weight ranging from 25-271 ng g$^{-1}$).[12] In a study by Williams and coworkers, it was reported that US long grain rice contained 26 ng As g$^{-1}$ DW (n=7) of total arsenic – levels higher than European, Bangladeshi, and Indian rice.[13] Speciation analysis demonstrated that the average inorganic arsenic content was $85 \pm 32$ ng g$^{-1}$ DW (ranging from 20-140 ng g$^{-1}$).[13] In a more recent study of 134 samples, Williams et al., determined that rice from South Central US contained an average total arsenic level of 300 ng g$^{-1}$ DW while California rice contained 170 ng g$^{-1}$ DW; arsenic speciation was not performed.[7]

As wheat, potato, and yam samples have received less attention from the research world, little is known in terms of expected total arsenic levels as well as speciation levels. Findings include background levels of these foods in the part per billion range of total arsenic.[8] Previously, evaluating the individual arsenic species of samples containing such low levels would have been quite difficult, but the use of HPLC-ICP-MS, helps to alleviate this problem.

In this study, fifty-three rice samples from Arkansas, California, Louisiana and Texas; ten potato samples from Idaho, Maine, Washington and Wisconsin; and ten wheat samples were from North Dakota, Nebraska and Oklahoma were analyzed for total arsenic and arsenic speciation. Samples were collected in several of the main production areas of the US as part of a joint study by FDA, EPA and USDA in the early 1980s to
determine background levels of several elements (excluding arsenic) in raw agricultural crops.[14] Individual collection sites were chosen to minimize industrial contamination. Sites were >8km downwind from power plants and smelters, at least 200 meters from major roads, 100 meters from building sites and 50 meters from end rows and other areas where large quantities of fertilizers could have been deposited. Sample composites have been kept in frozen storage in Cincinnati, OH since the time of collection in 1980 and 1981. Results for each growing region are compared to each other and to seven samples of market rice collected in 2002. Three yam samples were obtained from a local market in 2003.

Arsenic speciation results obtained using ion exchange chromatography (IEC)-ICP-MS will be compared with total arsenic concentrations for several samples of long grain white rice as well as NIST SRM 1568a, rice flour. In addition, the use of a microwave evaporation accessory will be evaluated as a means of removing excess trifluoroacetic acid (TFA) from the samples allowing for greater detection capability via preconcentration.

This study hopes to shed more light on the content and nature of arsenic in US rice, potatoes, yams and wheat to assess the level of risk involved with their consumption.

2.2 Experimental

2.2.1 Chemicals and standards

All chemicals used were reagent grade or higher. Water used throughout the experiments was 18 mΩ doubly distilled, deionized water (DDW) using a Millipore filtration system (Bedford, MA). Ultrex II Ultrapure HNO₃ was purchased from J. T.
Baker (Phillipsburg, NJ). TFA was purchased from Aldrich (St. Louis, MO). Arsenic and germanium (internal standard) stock solutions of 1000 µg mL$^{-1}$ from Spex Certiprep (Metuchen, NJ) were diluted to the proper levels and used for total arsenic analysis. For arsenic speciation, individual standards of As$^{III}$ and As$^V$ were purchased from Spex Certiprep. Dimethylarsinic acid (DMA) and disodium methyl arsenate (MMA) were obtained from Chem Service (98% purity, West Chester, PA). The standard reference material 1568 and 1568a rice flour were purchased from NIST (Gaithersburg, MD,). Ammonium phosphate was obtained from GFS Chemicals (Columbus, OH) and ammonium nitrate was received from J. T. Baker.

2.2.2 Instrumentation

For total arsenic analysis, two different ICP-MS instruments were used: an Agilent 7500ce (Agilent Technologies, Palo Alto, CA) and a VG PQ2 (VG Elemental, Winsford, Cheshire, UK). For speciation analysis, two instrument combinations were used: 1) Agilent 1100 HPLC with an Agilent 7500ce ICP-MS and 2) Dionex GP40 pump (Dionex Corporation, Sunnyvale, CA) with Dionex AS3500 autosampler with VG PQ2 ICP-MS. The effluent from the column was directly sent to the ICP-MS via a 40 cm length 0.25 mm id PEEK tubing from the column switching valve to the ICP nebulizer.

For analysis of total arsenic levels, the instrument was set to the elements, Ge ($m/z$ 74 & 76), As ($m/z$ 75). The common polyatomic interference for As at $m/z$ 75, is $^{40}\text{Ar}^{35}\text{Cl}^+$, therefore $m/z$ 77 was monitored to assess the level of interference due to the presence of chloride. When using the Agilent 7500ce, it is possible to minimize the ArCl interference by introducing a collision gas. When analyzing samples a flow rate of 3-4
mL min\(^{-1}\) of He as the collision gas, has been shown to increase the signal to noise ratio and render detection limits in the low part per trillion level.[15] When analyzing samples for total arsenic, the Agilent 7500ce was programmed to analyze a sample under normal mode (no collision gas) followed immediately by analysis using He. No significant differences in results were observed. For speciation analysis, no collision cell gas was used, except when attempting to identify the unknown compound discussed later. In nearly all cases, the use of the collision cell was unnecessary as chlorine was not present at substantial levels in the samples.

2.2.2.1 Total arsenic analysis

To quantitate arsenic, external calibration was employed. Arsenic standards ranging from 0 – 100 ng As mL\(^{-1}\) were prepared in 2% HNO\(_3\) (v/v) in DDW and used to generate calibration curves. As previously mentioned, an internal standard of 10 ng Ge mL\(^{-1}\) was used to correct arsenic levels for instrument drift. After every 10 samples, arsenic standards were analyzed to assure accuracy. Standard additions and percent recoveries for sample fortifications were accurate (between 77 and 96%).

2.2.2.2 Arsenic Speciation Analysis

Chromatographic conditions for the separation of arsenic species are shown in Table 2.1. As an internal standard for chromatographic analysis, a flow injection peak of 2 ng As\(^V\) mL\(^{-1}\) was injected post column at ~2 min to correct chromatographic peak areas for instrument drift. Sample fortification using As\(^{III}\) or DMA at a concentration 100 ng
mL$^{-1}$ was performed to each sample type prior to extraction to confirm extraction efficiency and monitor inter-species conversion. Quantification was achieved using external calibration, employing standards ranging in concentration from 0 – 50 ng mL$^{-1}$ of each species (prepared in DDW). Calibration curves were generated by manually integrating individual peaks using the respective software for each ICP-MS. Due to the conversion of As$^V$ to As$^{III}$, total inorganic arsenic is reported rather than individual concentrations.[16]

Table 2.1: Chromatographic conditions for arsenic speciation

<table>
<thead>
<tr>
<th>System</th>
<th>Agilent 1100 HPLC system or Dionex GP40 pump, AS3500 autosampler</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>PRP-X100 (4.6 x 250 mm, 10 µm)</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>10 mM NH$_4$NO$_3$, 10 mM NH$_4$H$_2$PO$_4$, pH adjusted to 6.2 using NH$_4$OH</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>1.0 mL min$^{-1}$</td>
</tr>
<tr>
<td>Injection Volume</td>
<td>50 µL</td>
</tr>
<tr>
<td>Flow Injection Peak</td>
<td>2 ng As$^V$ mL$^{-1}$, postcolumn</td>
</tr>
</tbody>
</table>

2.2.3 Sample collection and preparation

Rice samples (~200 heads each) were collected directly from the field taking care to limit contamination. Samples were dried in a forced air oven at <50 °C. Rice kernels were separated from hulls, rinsed quickly with DDW, and dried in a clean-air environment. Samples were blender-ground to produce unpolished brown rice powder. Percent moisture was determined by oven drying a portion of the ground composite to constant weight. Wheat, yam, and potato samples were milled to ensure sample homogeneity. Store bought yams samples were peeled, chopped then lyophilized.
Samples were stored in sealed, opaque vials. All samples were then stored at 2 °C until used for analysis.

### 2.2.3.1 Sample digestion

All samples were prepared in triplicate. Rice samples (~0.25 g) were weighed into Teflon, PFA (Perfluoro (Alkoxy Alkane)) microwave digestion vessels and 7 mL of concentrated HNO₃ was added. Wheat, yam, and potato samples (~1 g) were digested similarly with 5 mL of concentrated HNO₃. Samples were left over night and subjected to microwave accelerated digestion using a CEM MARS Xpress (CEM, Matthew, NC). During microwave digestion, sample vessels were ramped to 200 °C (350 psi) over 10 min, held for 15 min, allowed to cool, then vented. The digested solution was diluted to 25 g with DDW, a 1 mL aliquot was diluted to 10 g with DDW, and prior to analysis and an internal standard of Ge was added at a final concentration of 10 ng of Ge mL⁻¹. Standard additions were prepared for each replicate by adding 50 µL of 500 ng As mL⁻¹ standard for a final solution concentration of 5 ng As mL⁻¹. Sample fortifications were performed for each sample type by adding 50 µL of 10 µg As mL⁻¹ standard prior to digestion for a concentration of 20 ng As mL⁻¹. One method blank was prepared for every 12 samples. NIST SRM 1568 and 1568a rice flour were prepared as mentioned above to ensure method validation.
2.2.3.2 Sample extraction

All sample extractions were performed in triplicate. The conditions for TFA extraction were optimized and are further explained in the Results and Discussion section. Samples (~1 g) were weighed out in microwave vessels and 7 mL of 0.5 M TFA was added. Each sample was placed on platform shaker (Innova 2100, New Brunswick Scientific, Edison, NJ) operated at 200 rpm for one hour. The samples were then subjected to microwave assisted extraction using a CEM MARS Xpress. During the extraction procedure, the temperature was ramped to 85 °C (200 psi) over 20 min, held for 10 min at 80 °C, allowed to cool, and vented. Extracted solutions were transferred to 25 mL volumetric flasks and diluted to 25 mL using DDW. They were then transferred to 50 mL centrifuge tubes and centrifuged at 4000 rpm for 10 min to draw particulate to the bottom. The supernatant was then filtered through 0.45 µm nylon syringe with polypropylene prefilter prior to analysis. When necessary, samples of very low arsenic levels (as determined by total arsenic analysis), were pre-concentrated by evaporating a 5 mL aliquot to dryness using a LabConco RapidVap (Labconco Corporation, Kansas City, MI) at a pressure of 80 mbar and 40 °C. Extracts were reconstituted ≤ 2.5 mL DDW (dilution factor chosen to reach desired pre-concentration level). Reconstitution of samples in this manner had exhibited no inter species conversion or effect on recovery. NIST SRM 1568 and 1568a rice flour were prepared as mentioned above to ensure method validation.
2.3 Results and Discussion

2.3.1 Total and speciated arsenic analysis of rice

The total arsenic concentration for each sample was determined. Figures of merit were calculated using the averages for five separate analysis days and are as follows: instrument detection limits were 0.012 ng As mL\(^{-1}\) and method quantitation limits were 42.1 ng g\(^{-1}\) for the total As analysis. Sample fortification recoveries were reasonable and standard addition of each sample confirmed no matrix suppression on arsenic levels. As a quality assurance measure, the standard reference material (SRM) 1568 was analyzed in parallel with the rice samples. The total arsenic concentration was found to be 396 ± 20 ng As g\(^{-1}\) (n=7), which agrees very well with the certified value of 410 ± 50 ng As g\(^{-1}\). SRM 1568a was also analyzed in the same manner with our findings to be 298 ± 10 ng As g\(^{-1}\) and the certified values is 290 ± 30 ng As g\(^{-1}\). Results for the total arsenic concentrations of the SRMs are shown in Table 2.2. In addition, the total arsenic levels of each sample served as a mass balance record, such that the sum of the concentrations of each arsenic species should equal the total arsenic concentration.

After determining total arsenic levels in each sample, the sample was then subjected to speciation analysis to determine the arsenic distribution among the various arsenic species. The instrumental limits of detection ranged from 0.05-0.1 ng As mL\(^{-1}\) for each of the arsenic species of interest. Figure 2.1 shows a typical HPLC-ICP-MS chromatogram of a mixture of the four common arsenic standards. Method quantitation limits ranged from 4-10 ng As g\(^{-1}\) Recoveries of method spikes of As\(^{\text{III}}\) and DMA (n = 6) at the 100 ng As g\(^{-1}\) level averaged 89% and 108%, respectively. To ensure mass balance (extraction
efficiency) the sum of all species was compared to the total arsenic levels (denoted by * in Tables 2.2-7). Table 2.2 shows the arsenic speciation results for two rice flour standard reference materials and Figure 2.2 shows a typical HPLC-ICP-MS chromatogram of an SRM. The sum of the arsenic species found compared well with the certified total arsenic. These results ensure that the method is precise and accurate.

![Figure 2.1: Arsenic standard mixture As$^{III}$, DMA, MMA, As$^{V}$ at 5 ng mL$^{-1}$ in 0.2M TFA for each species](image)

![Figure 2.2: Speciation of SRM 1568 (rice flour), typical for all SRM 1568s analyzed.](image)
Table 2.2: Arsenic speciation in SRM rice flour samples

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Inorganic As</th>
<th>DMA</th>
<th>MMA</th>
<th>Sum of Species</th>
<th>Certified As</th>
<th>Sum / Certified</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRM 1568†</td>
<td>116 ± 9</td>
<td>285 ± 6</td>
<td>22 ± 2</td>
<td>423 ± 8</td>
<td>410 ± 50</td>
<td>103%</td>
</tr>
<tr>
<td>(n=12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRM 1568‡</td>
<td>123 ± 19</td>
<td>279 ± 10</td>
<td>ND</td>
<td>402 ± 20</td>
<td>410 ± 50</td>
<td>98%</td>
</tr>
<tr>
<td>(n=3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRM 1568a‡</td>
<td>77 ± 11</td>
<td>176 ± 3</td>
<td>ND</td>
<td>253 ± 11</td>
<td>290 ± 30</td>
<td>87%</td>
</tr>
<tr>
<td>(n=3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

† Data collected with 7500ce ICP-MS
‡ Data collected with PQ2 ICP-MS

Total and speciated arsenic results obtained for each state are shown in Tables 2.3-6. The three digit number in each sample name identifies the county from which the samples were obtained. Total arsenic concentrations in the sixty rice samples analyzed in this study ranged from 26 to 1000 ng As g⁻¹. Results for total arsenic show significant variation by growing location. California as a group had the lowest total arsenic concentrations ranging from 26 to 89 ng As g⁻¹. Louisiana and Arkansas had the next lowest average total arsenic concentrations at 167 and 184 ng As g⁻¹, respectively. Of the samples analyzed in this study, Texas had the highest total arsenic levels ranging from 119 to 1000 ng As g⁻¹.
### Table 2.3: Arsenic speciation in rice from Louisiana (n=20)

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>As^{III} + As^{V}</th>
<th>DMA</th>
<th>MMA</th>
<th>Sum of Species</th>
<th>Percent Inorganic</th>
<th>As Totals</th>
<th>Mass Balance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>S81-LA-007-35</td>
<td>95</td>
<td>46</td>
<td>7</td>
<td>143</td>
<td>66%</td>
<td>111</td>
<td>129%</td>
</tr>
<tr>
<td>S81-LA-003-22</td>
<td>121</td>
<td>146</td>
<td>8</td>
<td>276</td>
<td>44%</td>
<td>259</td>
<td>106%</td>
</tr>
<tr>
<td>S81-LA-003-33</td>
<td>56</td>
<td>19</td>
<td>ND</td>
<td>75</td>
<td>75%</td>
<td>75</td>
<td>96%</td>
</tr>
<tr>
<td>S81-LA-003-11</td>
<td>131</td>
<td>123</td>
<td>ND</td>
<td>254</td>
<td>52%</td>
<td>243</td>
<td>104%</td>
</tr>
<tr>
<td>S81-LA-003-5</td>
<td>116</td>
<td>96</td>
<td>8</td>
<td>214</td>
<td>54%</td>
<td>241</td>
<td>89%</td>
</tr>
<tr>
<td>S81-LA-003-13</td>
<td>142</td>
<td>177</td>
<td>7</td>
<td>322</td>
<td>44%</td>
<td>340</td>
<td>95%</td>
</tr>
<tr>
<td>S81-LA-003-3</td>
<td>44</td>
<td>23</td>
<td>ND</td>
<td>67</td>
<td>66%</td>
<td>115</td>
<td>58%</td>
</tr>
<tr>
<td>S81-LA-003-2</td>
<td>103</td>
<td>44</td>
<td>ND</td>
<td>129</td>
<td>80%</td>
<td>138</td>
<td>93%</td>
</tr>
<tr>
<td>S81-LA-009-1</td>
<td>101</td>
<td>37</td>
<td>ND</td>
<td>133</td>
<td>76%</td>
<td>96</td>
<td>139%</td>
</tr>
<tr>
<td>S81-LA-009-2</td>
<td>119</td>
<td>85</td>
<td>ND</td>
<td>198</td>
<td>60%</td>
<td>174</td>
<td>114%</td>
</tr>
<tr>
<td>S81-LA-009-1</td>
<td>151</td>
<td>142</td>
<td>ND</td>
<td>204</td>
<td>52%</td>
<td>245</td>
<td>120%</td>
</tr>
<tr>
<td>S81-LA-003-10</td>
<td>110</td>
<td>178</td>
<td>8</td>
<td>266</td>
<td>37%</td>
<td>267</td>
<td>111%</td>
</tr>
<tr>
<td>S81-LA-007-34</td>
<td>134</td>
<td>63</td>
<td>ND</td>
<td>197</td>
<td>68%</td>
<td>142</td>
<td>139%</td>
</tr>
<tr>
<td>S81-LA-003-1</td>
<td>157</td>
<td>81</td>
<td>ND</td>
<td>238</td>
<td>66%</td>
<td>199</td>
<td>119%</td>
</tr>
<tr>
<td>S81-LA-009-3</td>
<td>123</td>
<td>82</td>
<td>ND</td>
<td>206</td>
<td>60%</td>
<td>165</td>
<td>125%</td>
</tr>
<tr>
<td>S81-LA-1</td>
<td>82</td>
<td>12</td>
<td>ND</td>
<td>94</td>
<td>87%</td>
<td>122</td>
<td>77%</td>
</tr>
<tr>
<td>S81-LA-9</td>
<td>91</td>
<td>32</td>
<td>ND</td>
<td>123</td>
<td>74%</td>
<td>138</td>
<td>89%</td>
</tr>
<tr>
<td>S81-LA-007-10</td>
<td>68</td>
<td>50</td>
<td>ND</td>
<td>118</td>
<td>57%</td>
<td>130</td>
<td>91%</td>
</tr>
<tr>
<td>S81-LA-007-11</td>
<td>95</td>
<td>31</td>
<td>ND</td>
<td>127</td>
<td>75%</td>
<td>99</td>
<td>126%</td>
</tr>
<tr>
<td>S81-LA-007-13</td>
<td>63</td>
<td>6</td>
<td>ND</td>
<td>69</td>
<td>91%</td>
<td>46</td>
<td>150%</td>
</tr>
</tbody>
</table>

Average ± 1σ: 105 ± 32 74 ± 54 2 ± 7 178 ± 82 64% 167 ± 78 109% ± 23%

### Table 2.4: Arsenic speciation in rice from Arkansas (n=12)

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>As^{III} + As^{V}</th>
<th>DMA</th>
<th>MMA</th>
<th>Sum of Species</th>
<th>Percent Inorganic</th>
<th>As Totals</th>
<th>Mass Balance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>S81-AR-001-1</td>
<td>53</td>
<td>22</td>
<td>ND</td>
<td>75</td>
<td>70%</td>
<td>65</td>
<td>115%</td>
</tr>
<tr>
<td>S81-AR-001-2</td>
<td>88</td>
<td>26</td>
<td>5</td>
<td>119</td>
<td>74%</td>
<td>90</td>
<td>132%</td>
</tr>
<tr>
<td>S81-AR-001-3</td>
<td>73</td>
<td>132</td>
<td>ND</td>
<td>205</td>
<td>36%</td>
<td>214</td>
<td>96%</td>
</tr>
<tr>
<td>S81-AR-001-4</td>
<td>74</td>
<td>23</td>
<td>ND</td>
<td>97</td>
<td>76%</td>
<td>99</td>
<td>98%</td>
</tr>
<tr>
<td>S81-AR-001-6</td>
<td>78</td>
<td>20</td>
<td>ND</td>
<td>98</td>
<td>73%</td>
<td>76</td>
<td>126%</td>
</tr>
<tr>
<td>S81-AR-001-7</td>
<td>98</td>
<td>162</td>
<td>ND</td>
<td>261</td>
<td>38%</td>
<td>240</td>
<td>109%</td>
</tr>
<tr>
<td>S81-AR-001-9</td>
<td>123</td>
<td>385</td>
<td>ND</td>
<td>508</td>
<td>24%</td>
<td>425</td>
<td>117%</td>
</tr>
<tr>
<td>S81-AR-001-10</td>
<td>78</td>
<td>28</td>
<td>ND</td>
<td>106</td>
<td>74%</td>
<td>80</td>
<td>133%</td>
</tr>
<tr>
<td>S81-AR-001-16</td>
<td>101</td>
<td>72</td>
<td>7</td>
<td>176</td>
<td>57%</td>
<td>185</td>
<td>106%</td>
</tr>
<tr>
<td>S81-AR-001-21</td>
<td>50</td>
<td>108</td>
<td>ND</td>
<td>156</td>
<td>32%</td>
<td>187</td>
<td>93%</td>
</tr>
<tr>
<td>S81-AR-001-22</td>
<td>99</td>
<td>89</td>
<td>ND</td>
<td>158</td>
<td>44%</td>
<td>159</td>
<td>100%</td>
</tr>
<tr>
<td>S81-AR-001-27</td>
<td>88</td>
<td>381</td>
<td>ND</td>
<td>449</td>
<td>15%</td>
<td>421</td>
<td>107%</td>
</tr>
</tbody>
</table>

Average ± 1σ: 79 ± 21 121 ± 131 1 ± 5 201 ± 140 52% 184 111% ± 14%
Table 2.5: Arsenic speciation in rice from Texas (n=9)

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>AsIII + AsV</th>
<th>DMA</th>
<th>MMA</th>
<th>Sum of Species</th>
<th>Percent Inorganic</th>
<th>As Totals</th>
<th>Mass Balance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>S81-TX-039-22</td>
<td>131</td>
<td>34</td>
<td>ND</td>
<td>165</td>
<td>80%</td>
<td>188</td>
<td>88%</td>
</tr>
<tr>
<td>S81-TX-039-2</td>
<td>97</td>
<td>294</td>
<td>8</td>
<td>339</td>
<td>29%</td>
<td>337</td>
<td>101%</td>
</tr>
<tr>
<td>S81-TX-039-19</td>
<td>96</td>
<td>43</td>
<td>ND</td>
<td>140</td>
<td>70%</td>
<td>119</td>
<td>113%</td>
</tr>
<tr>
<td>S81-TX-157-1</td>
<td>141</td>
<td>695</td>
<td>21</td>
<td>796</td>
<td>13%</td>
<td>1000</td>
<td>73%</td>
</tr>
<tr>
<td>S81-TX-157-2</td>
<td>120</td>
<td>605</td>
<td>9</td>
<td>724</td>
<td>10%</td>
<td>751</td>
<td>98%</td>
</tr>
<tr>
<td>S81-TX-157-3</td>
<td>116</td>
<td>900</td>
<td>20</td>
<td>1030</td>
<td>11%</td>
<td>801</td>
<td>118%</td>
</tr>
<tr>
<td>S81-TX-157-6</td>
<td>107</td>
<td>201</td>
<td>3</td>
<td>316</td>
<td>34%</td>
<td>332</td>
<td>95%</td>
</tr>
<tr>
<td>S81-TX-481-2</td>
<td>113</td>
<td>170</td>
<td>3</td>
<td>288</td>
<td>39%</td>
<td>261</td>
<td>110%</td>
</tr>
<tr>
<td>S81-TX-481-4</td>
<td>87</td>
<td>52</td>
<td>ND</td>
<td>140</td>
<td>63%</td>
<td>124</td>
<td>113%</td>
</tr>
<tr>
<td><strong>Average ± 1σ</strong></td>
<td><strong>111 ± 17</strong></td>
<td><strong>319 ± 315</strong></td>
<td><strong>8 ± 8</strong></td>
<td><strong>438 ± 184</strong></td>
<td><strong>40%</strong></td>
<td><strong>445 ± 342</strong></td>
<td><strong>102% ± 13%</strong></td>
</tr>
</tbody>
</table>

Table 2.6: Arsenic speciation in rice from California (n=12), samples S80-CA-2, 6, and 20 were analyzed in a previous study and total arsenic levels were not available

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>AsIII + AsV</th>
<th>DMA</th>
<th>MMA</th>
<th>Sum of Species</th>
<th>Percent Inorganic</th>
<th>As Totals</th>
<th>Mass Balance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>S80-CA-101-1</td>
<td>52</td>
<td>ND</td>
<td>ND</td>
<td>52</td>
<td>100%</td>
<td>41</td>
<td>128%</td>
</tr>
<tr>
<td>S80-CA-2</td>
<td>68</td>
<td>28</td>
<td>ND</td>
<td>97</td>
<td>70%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S80-CA-101-3</td>
<td>86</td>
<td>15</td>
<td>ND</td>
<td>112</td>
<td>86%</td>
<td>74</td>
<td>151%</td>
</tr>
<tr>
<td>S80-CA-6</td>
<td>83</td>
<td>15</td>
<td>ND</td>
<td>98</td>
<td>85%</td>
<td>72</td>
<td>139%</td>
</tr>
<tr>
<td>S80-CA-101-7</td>
<td>98</td>
<td>18</td>
<td>ND</td>
<td>116</td>
<td>84%</td>
<td>89</td>
<td>130%</td>
</tr>
<tr>
<td>S80-CA-101-9</td>
<td>58</td>
<td>ND</td>
<td>ND</td>
<td>58</td>
<td>100%</td>
<td>44</td>
<td>131%</td>
</tr>
<tr>
<td>S80-CA-101-10</td>
<td>63</td>
<td>13</td>
<td>ND</td>
<td>81</td>
<td>84%</td>
<td>63</td>
<td>129%</td>
</tr>
<tr>
<td>S80-CA-12</td>
<td>37</td>
<td>90</td>
<td>ND</td>
<td>117</td>
<td>32%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S80-CA-101-13</td>
<td>61</td>
<td>39</td>
<td>ND</td>
<td>100</td>
<td>61%</td>
<td>63</td>
<td>121%</td>
</tr>
<tr>
<td>S80-CA-101-14</td>
<td>33</td>
<td>10</td>
<td>ND</td>
<td>43</td>
<td>77%</td>
<td>26</td>
<td>165%</td>
</tr>
<tr>
<td>S80-CA-011-15</td>
<td>53</td>
<td>18</td>
<td>ND</td>
<td>71</td>
<td>75%</td>
<td>60</td>
<td>109%</td>
</tr>
<tr>
<td>S80-CA-20</td>
<td>25</td>
<td>ND</td>
<td>ND</td>
<td>25</td>
<td>100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Average ± 1σ</strong></td>
<td><strong>61 ± 23</strong></td>
<td><strong>20 ± 22</strong></td>
<td><strong>ND</strong></td>
<td><strong>81 ± 31</strong></td>
<td><strong>80%</strong></td>
<td><strong>62 ± 21</strong></td>
<td><strong>133% ± 16%</strong></td>
</tr>
</tbody>
</table>

Similar to the total arsenic concentrations, DMA concentrations varied widely in the rice samples. California samples clearly had the lowest levels of DMA with an average of 20 ng of DMA g⁻¹. Average DMA concentrations in Louisiana, Arkansas, and Texas samples were 74, 121, and 319 ng of DMA g⁻¹, respectively. However, the range of values was very large within each state. An example HPLC-ICP-MS chromatogram of rice sample S81-LA-099-2 is shown Figure 2.3.
Due to its toxicity, inorganic arsenic levels are the largest concern in regards to arsenic. In comparison to the total arsenic and DMA concentrations, inorganic arsenic concentrations in the samples were relatively consistent. California samples appear to have the lowest concentration of inorganic arsenic at 61 ± 23 ng g\(^{-1}\). Average inorganic concentrations in Louisiana, Arkansas, and Texas samples were 105 ± 32, 79 ± 21, and 111 ± 17 ng g\(^{-1}\), respectively. However, the significance of these differences has not yet been established. This value compares well with the 112 ng g\(^{-1}\) mean inorganic arsenic level reported by Lamont et al.[12] Typical chromatograms of SRM

### 2.3.2 Total and speciated arsenic analysis of store-bought rice

Because these samples are approximately 25 years old, seven samples of US grown rice obtained more recently at local markets were also analyzed using the same methodology. The growing location of these samples is unknown. The results for the
market rice samples compare well with the crop study samples as shown in Table 2.7. The average for the market rice samples is $98 \pm 36$ ng As g$^{-1}$. The overall mean inorganic arsenic concentration for all 60 samples of rice analyzed in this study was $91 \pm 32$ ng As g$^{-1}$.

Table 2.7: Summary of Results: Arsenic Speciation in Market Rice Samples

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>$\text{As}^{\text{III}} + \text{As}^{\text{V}}$ (ng g$^{-1}$)</th>
<th>DMA (ng g$^{-1}$)</th>
<th>MMA (ng g$^{-1}$)</th>
<th>Sum of Species (ng g$^{-1}$)</th>
<th>Percent Inorganic (%)</th>
<th>As Totals (ng g$^{-1}$)</th>
<th>Mass Balance* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brand R01</td>
<td>90</td>
<td>239</td>
<td>ND</td>
<td>329</td>
<td>27%</td>
<td>340</td>
<td>97%</td>
</tr>
<tr>
<td>Brand R02</td>
<td>110</td>
<td>61</td>
<td>ND</td>
<td>171</td>
<td>64%</td>
<td>160</td>
<td>107%</td>
</tr>
<tr>
<td>Brand R03</td>
<td>36</td>
<td>192</td>
<td>ND</td>
<td>228</td>
<td>18%</td>
<td>210</td>
<td>108%</td>
</tr>
<tr>
<td>Brand R201</td>
<td>129</td>
<td>235</td>
<td>ND</td>
<td>363</td>
<td>36%</td>
<td>381</td>
<td>93%</td>
</tr>
<tr>
<td>Brand R202</td>
<td>95</td>
<td>150</td>
<td>ND</td>
<td>245</td>
<td>39%</td>
<td>255</td>
<td>99%</td>
</tr>
<tr>
<td>Brand R203</td>
<td>148</td>
<td>87</td>
<td>ND</td>
<td>215</td>
<td>89%</td>
<td>225</td>
<td>99%</td>
</tr>
<tr>
<td>Brand R204</td>
<td>77</td>
<td>158</td>
<td>ND</td>
<td>235</td>
<td>33%</td>
<td>267</td>
<td>99%</td>
</tr>
<tr>
<td>Average ± 1σ</td>
<td>$98 \pm 36$</td>
<td>$157 \pm 72$</td>
<td>ND</td>
<td>$255 \pm 67$</td>
<td>40%</td>
<td>$264 \pm 79$</td>
<td>$98% \pm 7%$</td>
</tr>
</tbody>
</table>

2.3.3 Total and speciated arsenic analysis of wheat, potato, and yams samples

The total arsenic content of the wheat, potato, and yam samples are shown in Table 2.8. The levels are quite low as only one sample had total arsenic levels higher that 100 ng As g$^{-1}$. The average arsenic content of the wheat samples was $32 \pm 39$ ng As g$^{-1}$ with only 4 samples contain >20 ng As g$^{-1}$. The potato samples had the lowest average of those analyzed at $15 \pm 21$ ng As g$^{-1}$. The yam samples contained an average level of $32 \pm 39$ ng As g$^{-1}$.

Due to the very low total arsenic levels in the wheat, potato, and yam samples, it is quite difficult to speciate as the low total arsenic levels equate to even lower levels of the individual arsenic species. Samples of higher arsenic content were chosen for initial speciation analysis, with results shown in Table 2.9. A chromatogram of the separation
of arsenic standards of $\text{As}^{\text{III}}$, DMA, MMA, $\text{As}^{\text{V}}$ and a chromatogram of wheat sample ND-017-17 is shown in Figure 2.4. As can be seen from Table 2.9 and Figure 2.4, all the detectable arsenic in the samples is present as inorganic arsenic.

One main conclusion of this analysis is that US grown terrestrial plants that up take arsenic in low levels (<100 ng As g$^{-1}$), accumulate it as inorganic arsenic, thus confirming the previous assumption.[8] The remaining speciation data obtained by coworkers (not included in this dissertation), confirms that the vast majority of arsenic in the wheat, potato, and yam samples surveyed, is inorganic in nature. This trend is also exhibited in rice samples and is discussed further in section 2.3.5.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Arsenic concentration per dry weight (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wheat</strong></td>
<td></td>
</tr>
<tr>
<td>S79-ND-009-1</td>
<td>5</td>
</tr>
<tr>
<td>S79-ND-101-7</td>
<td>2</td>
</tr>
<tr>
<td>S82-ND-017-8</td>
<td>40</td>
</tr>
<tr>
<td>S82-ND-017-17</td>
<td>111</td>
</tr>
<tr>
<td>S82-ND-017-22</td>
<td>94</td>
</tr>
<tr>
<td>S79-NE-105-10</td>
<td>24</td>
</tr>
<tr>
<td>S79-NE-105-12</td>
<td>11</td>
</tr>
<tr>
<td>S80-OK-139-1</td>
<td>14</td>
</tr>
<tr>
<td>S80-OK-139-7</td>
<td>18</td>
</tr>
<tr>
<td>S80-OK-039-12</td>
<td>4</td>
</tr>
<tr>
<td><strong>Average ± 1σ</strong></td>
<td><strong>32 ± 39</strong></td>
</tr>
<tr>
<td><strong>Potato</strong></td>
<td></td>
</tr>
<tr>
<td>S79-ID-011-9</td>
<td>5</td>
</tr>
<tr>
<td>S79-ID-011-10</td>
<td>5</td>
</tr>
<tr>
<td>S79-ID-011-11</td>
<td>7</td>
</tr>
<tr>
<td>S79-ID-011-13</td>
<td>12</td>
</tr>
<tr>
<td>S79-ME-003-4</td>
<td>70</td>
</tr>
<tr>
<td>S79-WA-025-4</td>
<td>14</td>
</tr>
<tr>
<td>S79-WA-025-9</td>
<td>2</td>
</tr>
<tr>
<td>S79-WA-005-22</td>
<td>1</td>
</tr>
<tr>
<td>S80-WI-135-3</td>
<td>3</td>
</tr>
<tr>
<td>S80-WI-067-205</td>
<td>30</td>
</tr>
<tr>
<td><strong>Average ± 1σ</strong></td>
<td><strong>15 ± 21</strong></td>
</tr>
<tr>
<td><strong>Yam</strong></td>
<td></td>
</tr>
<tr>
<td>RY</td>
<td>96</td>
</tr>
<tr>
<td>GY</td>
<td>22</td>
</tr>
<tr>
<td>WY</td>
<td>20</td>
</tr>
<tr>
<td><strong>Average ± 1σ</strong></td>
<td><strong>46 ± 43</strong></td>
</tr>
</tbody>
</table>
2.3.4 Unknown arsenic compound in rice samples

The chromatograms for approximately 10 samples from LA, AR, and TX showed the presence of an unidentified peak eluting between DMA and MMA. The previously mentioned unknown peak was not detected in any of the market rice samples. Figure 2.5 shows the chromatogram obtained for a sample containing this unknown peak (Unk). In the majority of the samples, this peak is significantly less than in this example. After 7 days the unknown is significantly reduced in size while increases in As\textsuperscript{III} and MMA are
noted. Several attempts were made to further examine the compound. Including the unknown in the summation of species, relates well to the mass balance. The peak is not a chlorine induced interference as no signal is observed at \( m/z \ 77 \) and the peak remains even after the use of the collision cell to remove interferences. Several known standards were added to the sample in attempt to match retention time, but no matches were determined. The peak only occurs in samples with high DMA content, and thus maybe associated with DMA. Attempts to identify this peak are ongoing.

![HPLC-ICP-MS chromatogram for rice samples S81-TX-157-1](image)

**Figure 2.5: HPLC-ICP-MS chromatogram for rice samples S81-TX-157-1**

### 2.3.5 Trends in arsenic content in rice

The impact of these findings is better understood when taking into account the average rice consumption trends of humans. USDA’s Economic Research Service currently estimates per capita rice consumption is roughly equivalent to 33 g rice day\(^{-1}\) DW.[17]
Hispanic consumers (but not Mexican-Americans) consumed 34% higher than average rice consumption.[6] Asian, Pacific and Native American ethnic groups consumed >115 g rice d\(^{-1}\) DW.[6] Using the overall mean inorganic arsenic concentration determined in this study (90 ng As g\(^{-1}\)), the average daily intake of inorganic arsenic from rice in the US would be approximately 3 µg. Ethnic groups which consume higher amounts (~115 g) of rice daily would have a daily intake of approximately 10 µg of inorganic arsenic.

There are a wide range of reported values in the literature for inorganic arsenic content in rice. In this study, the percent inorganic ranged from 11-100%. However, a visible trend can be send according to Figure 2.6. The plot shows data from: the samples collected from 1980-1982 survey (×), the samples bought from local markets (♦), from Heitkemper et al. [11] (■) and from Williams et al. [13] (□). As the total arsenic content increases, the levels of inorganic arsenic increase to approximately 150 ng As g\(^{-1}\) then levels off. In the presented data, there are no instances of accumulation of inorganic arsenic levels >160 ng g\(^{-1}\). However, Lamont et al. showed data in which several samples accumulated >160 ng g\(^{-1}\). As the total arsenic levels increases so does the levels of DMA. Figure 2.7 demonstrates that as total arsenic levels increase, the % of DMA as total arsenic increases. The correlation is somewhat linear until the total arsenic levels reach approximately 400 ng As g\(^{-1}\) and then the % DMA increases such that samples with extremely high levels of total arsenic contain almost 90% DMA. This lends cause to the fact that US rice may uptake arsenic as inorganic arsenic up to approximately 100-150 ng As g\(^{-1}\), then accumulates arsenic as DMA. This maybe means of detoxifying the arsenic as some type of self protection mechanism.
2.4 Conclusions

The purpose of this work was to establish the risk of rice, wheat, potato, and yam consumption in terms of arsenic. The main finding in regards to wheat, potato, and yam
samples was that low levels, typically >100 ng g\(^{-1}\) are accumulated and the large majority of the arsenic is inorganic arsenic. More attention has been drawn to arsenic in rice due to the recent publication by Williams et al. [7] in which the authors discussed the possible danger of rice consumption due to its high level of inorganic arsenic. The authors speculated that total arsenic levels are proportional to the inorganic levels based on an average of 42% of total arsenic is inorganic (based on averages of Heitkemper et al. [11] and Williams et al. [13]). Using this assumption, samples analyzed for total arsenic in their study, could contain as much as 280 ng g\(^{-1}\) of inorganic arsenic. Applying this assumption to data from this study could show levels as high as 420 ng g\(^{-1}\) (TX sample S81-TX-157-1). However, as discussed previously, this assumption of % inorganic arsenic being the same in low and high total arsenic levels is incorrect (Figures 2.6 & 2.7). Therefore our results somewhat contradict assumptions and conclusions drawn by Williams et al. in terms of the risk of US rice contribution to inorganic arsenic in the diet, thus further supporting that total arsenic analysis is insufficient and complete speciation analysis is necessary.

Arsenic levels in this study showed diversity based on growing location when total arsenic levels were concerned. The average total arsenic content is 202 ± 200 ng As g\(^{-1}\) and the inorganic arsenic level is 91 ± 32 ng g\(^{-1}\) (67% are below 100 ng g\(^{-1}\)). The main fluctuation involved DMA levels, which were often indicative of high overall levels (Figure 2.7). Inorganic arsenic levels were quite consistent throughout all the samples, including recently purchased market brands. The levels of inorganic arsenic have seemed to remain constant – according to our study and that of Lamont,[12] Williams,[13] and Heitkemper.[11] As the risk of US rice due to inorganic arsenic may not be as serious as
Williams et al. [18] reported, concern could increase as the toxicity of DMA is further explored. If DMA is determined to be of more concern than previously thought, especially as a cancer promoter, then arsenic contamination in rice should be reevaluated.

2.5 Acknowledgements

This project was supported in part through an Interagency Agreement between the US FDA and US EPA and by an appointment to the Research Fellowship Program at the FDA administered by Oak Ridge Associated Universities through a contract with the US FDA.

2.6 References


3.1 Introduction

High interest involving selenium centers on its dual personality in forming toxic/beneficial compounds. Most recently it was discovered that Se has anti-cancer properties as shown in research by Clark et al.[1] Selenium has also been investigated in slowing progression from AIDS and HIV along with proper immune functioning.[2] Levels below the daily recommended amount can lead to increased aging, increased risk of cardiovascular disease as well as other degenerative diseases.[3] Too much selenium can lead to hair and nail brittleness and loss, gastrointestinal disturbances, skin rash, garlic breath, fatigue, irritability, and nervous system abnormalities. The problem with selenium is that the toxic and the beneficial range is quite small; in general it is considered to be between 40-100 µg kg\(^{-1}\) of body weight per day.[4] Due to this narrow toxicity range, accurate and precise methods are needed to accurately assess selenium levels.

Selenium reaches the environment mainly from coal mining and irrigation water that extracts the selenium from underground shale. It is generally accepted that the inorganic forms, selenate and selenite, are considered toxic while the organic forms are non-toxic or even beneficial. Many supplements are now sold containing the most common beneficial Se compound, selenomethionine (SeMet). Other compounds, such as methylselenocysteine (MeSeCys), are also thought to have anti-cancer properties.(cite) Recently, several selenosugars have been reported.[5,6] Other commonly analyzed forms
of selenium include methylselenomethionine, selenocystine (SeCys$_2$), and volatile forms such as dimethselenide and dimethylselenide. Structures of some commonly analyzed organic selenium species is shown in Figure 3.1.

![Figure 3.1: Some commonly analyzed organic selenium compounds.](image)

The nature of selenium depends heavily on the system. In mammals selenium can be incorporated into proteins as it is present in a number of glutathione peroxidases (GPx) including cellular GPx (GPx1) and phospholipid hydroperoxide GPx (PHGPx; GPx4), iodothyronine 50-deiodinases (IDI), sperm capsule selenoprotein and thioredoxin reductase.[7] Selenium enters proteins by specifically being coded as in selenocysteine via the UGA codon.[8] Also selenium can be non-specifically incorporated into proteins as selenomethionine as Se replaces the sulfur of methionine. Proteins in which selenium is incorporated are termed selenoproteins and approximately 35 selenoproteins that have been found in mammals,[9] while others have been found in yeast.[10] To date, no proteins containing selenocysteine have been found in plants; only those containing
SeMet have been identified. It is also possible for selenium to bind to proteins, but not incorporated; such proteins are referred to as selenium-binding proteins.[11,12]

Mushrooms have been a part of the human diet for thousands of years. They also have been used frequently in homeopathic medicine. Mushroom consumption has been markedly increasing throughout the world. *Lentinula edodes* (*L. edodes*), commonly known as Shiitake mushrooms, are quite popular in several cultures as a food. Typically mushrooms are consumed for enjoyment as well as their health benefits such as containing relatively few calories and relatively high amounts of vegetable proteins. Their fruiting bodies, on a dry weight basis, contain about 39.9% carbohydrate, 17.5% protein and 2.9% fats, with the rest consisting of minerals.[13] It has been suggested that fungi possess an effective mechanism that enables them to take up some trace elements from the growth medium more readily.[13] Edible mushrooms are known to be selenium accumulators.[14-16] Because mushrooms contain relatively high protein levels and they can accumulate large amounts of selenium, it is reasonable to expect that selenium could be incorporated into the proteins or contained in proteins. Therefore selenium supplemented *L. edodes* samples offer great potential for studying selenium and its interactions with proteins.

There are few reports on selenium speciation in the literature in mushrooms samples. One such study examined the water extract of supplemented *L. edodes* in which they identified SeMet as the major component, by use of HPLC-ICP-MS and HPLC-ESI-MS.[16] Due to its ability to specifically monitor selenium and improved sensitivity for selenium upon the utilization of the collision/reaction cell, ICP-MS is the unrivalled choice for the analysis of selenium. This combination of chromatography and ICP-MS
element-specific detection for selenium speciation has been successfully applied to various matrices such as soybeans [17] and nuts.[18]

Typically to analyze large molecules such as proteins and peptides, reversed-phase or ion-exchange chromatography is used to separate proteins. However the high organic concentrations associated with reversed phase (RP) separations and the high salt concentrations associated with ion-exchange, leave them incompatible with ICP-MS. Size exclusion chromatography (SEC) is often used in speciation studies to screen for metalloproteins or metals bound to biomolecules.[19,20] Also it can provide crucial information regarding the association of specific elements with various molecular weight fractions in the sample. It does not require organic solvents that might denature the biological molecules or otherwise remove the metals of interest.[21]

Therefore, in the present study, SEC with on-line UV-Vis detection (to monitor possible proteins via UV absorbance) plus ICP-MS is the preferred method for the assessment of the protein molecular weight distribution of selenium. Once a distribution of selenium among various molecular weight ranges of proteins is established, it is necessary to convert the high molecular weight fractions into more manageable portions appropriate for alternate separations including reversed phase ion pairing chromatography. This allows previously developed methods of speciation to be preformed to establish the identity of smaller pieces deriving from the high molecular weight fraction.

Identification of selenium compounds was performed by matching the retention times of a standard mixture of different selenium species to the retention times of the
sample. Also the standards were fortified into the samples to identify the species based on peak growth.

3.2 Experimental

3.2.1 Reagents and standards

All reagents were of analytical reagent grade and the presence of selenium was not detected in the working range of these experiments. HPLC-grade methanol (MeOH) from Fisher (Fisher Scientific, Pittsburgh, PA) was used. All the solutions were prepared in 18 MΩ cm⁻¹ doubly distilled, deionized water (DDW) (Sybron Barnstead, Boston, MA, Boston). Acetone was obtained from Pharmco (Pharmco Products Inc. Brookfield, CT). D, L-selenomethionine (SeMet), selenocystine (SeCys₂) and sodium selenite were purchased from Aldrich (Milwaukee, WI). Methylselenocysteine (MeSeCys) was purchased from Sigma (St Louis, MO). Working solutions were prepared daily by appropriate dilution. For the determination of total selenium, working solutions were prepared daily by appropriate dilution of 1 mg Se mL⁻¹ standard solution. Nitric acid, 68% (Suprapure) from Pharmaco and hydrogen peroxide, 30% from Fisher Scientific were used for sample digestion. The chromatographic mobile phases (Table 3.1) were prepared from Sigma reagents. Solutions of the following Sigma reagents were used: Hepta-fluorobutyric acid (HFBA), pyridine formate, Tris-hydroxymethyl aminomethane, and sodium hydroxide. Protein standards: albumin, $M = 66$ kDa; myoglobin, $M = 16$ kDa; cytochrome c, $M = 12.5$ kDa; aprotinin, $M = 6.5$ kDa; and methylcobalamin, $M = 1.34$ kDa were purchased from Sigma-Aldrich (Sigma- Aldrich Co, St-Louis, MO, USA). The
enzymes: proteinase K (from *Tritirachium album*), protease XIV (from *Streptomyces griseus*), trypsin (from bovine pancreas), driselase (from *Basidiomycetes* sp.) were also purchased from Sigma.

### 3.2.2 Instrumentation

Chromatographic separations were carried out with an Agilent 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a binary HPLC pump, an autosampler, a vacuum degasser system, a temperature column compartment and a diode array detector. The ICP-MS detector was an Agilent 7500ce (Agilent Technologies, Tokyo, Japan). The ICP-MS is equipped with an octopole reaction cell and can be operated with or without the collision/reaction gas. Both detectors were used on-line allowing the monitoring of the species. A conventional Micromist nebulizer (Glass Expansion, Pocasset, MA), a Peltier-cooled spray chamber (2 °C) and a shielded torch constitute the sample introduction system under standard plasma conditions.

For size exclusion chromatography a Superdex 75 10/300 GL (10 mm × 300 mm) and a Superdex 200 10/300 GL (10 mm × 300 mm) (Amersham Pharmacia Biotech, Uppsala, Sweden) were used. Reversed phase chromatography was performed using a C₈ Alltima (Alltech, Deerfield, IL) column (250 mm × 4.6 mm, 5 µm particle size) equipped with a guard column.

Collision/reaction cell conditions were optimized by introducing H₂ as collision/reaction gas at flow rates between 0-6 mL min⁻¹ while monitoring ⁷⁷Se, ⁷⁸Se, ⁸⁰Se and ⁸²Se isotopes to achieve maximum signal to noise ratio. The optimum H₂ flow rates were typically between 3-5 mL min⁻¹.
Table 3.1: Instrument conditions for ICP-MS (similar for both total selenium determination and speciation) and chromatographic conditions for SEC, and RP-HPIPC

<table>
<thead>
<tr>
<th>ICP-MS Parameters</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward Power</td>
<td>1300 W</td>
</tr>
<tr>
<td>Plasma gas flow rate</td>
<td>15.0 L min⁻¹</td>
</tr>
<tr>
<td>Carrier gas flow rate</td>
<td>1.13 L min⁻¹</td>
</tr>
<tr>
<td>Dwell time</td>
<td>0.1 s per isotope</td>
</tr>
<tr>
<td>Isotopes monitored</td>
<td>$^{77}\text{Se}$, $^{78}\text{Se}$, $^{80}\text{Se}$, &amp; $^{82}\text{Se}$</td>
</tr>
<tr>
<td>Collision gas</td>
<td>$\text{H}_2$ @ 4 mL min⁻¹</td>
</tr>
<tr>
<td>Quadrupole bias</td>
<td>-16 V</td>
</tr>
<tr>
<td>Octopole bias</td>
<td>-18 V</td>
</tr>
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<table>
<thead>
<tr>
<th>SEC parameters</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Columns</td>
<td>Superdex 200 10/300 GL</td>
</tr>
<tr>
<td></td>
<td>Superdex 75 10/300 GL</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>30 mM Tris HCl buffer, pH 7.5 in DDW</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.7 mL min⁻¹</td>
</tr>
<tr>
<td>Injection volume</td>
<td>100 µL</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>RP-HPIPC parameters</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Alltima C₈ (250 mm × 4.6 mm, 5 µm)</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>0.1% HFBA , 5% MeOH, pH 2.5 in DDW</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.0 mL min⁻¹</td>
</tr>
<tr>
<td>Injection volume</td>
<td>100 µL</td>
</tr>
</tbody>
</table>

3.2.3 Sample Growth

A commercially available “kit” was used to grow the *Lentinula edodes*, Shiitake mushrooms. The shiitake mushroom patch was purchased from Fungi Perfecti (Olympia, WA, USA). The manufacturer’s growing instructions were followed. For selenium enriched mushrooms, 10 µg Na₂SeO₃ mL⁻¹ of de-chlorinated, non-distilled tap water was used. The mushrooms were harvested in one crop within a week of enriching the patch. They were then lyophilized and ground into a consistent powder. The mushroom powder was stored in a desiccator until needed.
3.2.4 Sample preparation for total selenium determination

For determination of total selenium by ICP-MS, an Agilent 7500ce was employed. Approximately 0.01 g of mushroom powder was digested by using 10 mL HNO₃ (50%) and 1 mL H₂O₂ (30%). Samples were covered with a watch glass and heated at reflux on a hot plate for ~18 h. The final solutions were diluted with deionized water to 50 mL. Three replicates of each sample were prepared and analyzed. Reagent blanks were digested in the same manner. Residues from extraction procedures were digested using 5 mL of 50% HNO₃ and 1 mL 30% H₂O₂. The final residue solutions were diluted with deionized water to 10 mL. The method of external calibration was applied for the quantification of the selenium as standards ranging from 0-100 ng Se mL⁻¹ were used to generate a calibration curve. ICP-MS parameters for total selenium concentration determination are shown in Table 3.1. Total selenium of selenized mushroom powder was compared to total selenium content in subsequent extraction residues to determine extraction efficiency and assure mass balance.

3.2.5 Sample preparation for analysis of water soluble proteins by SEC-ICP-MS

Two extraction protocols (A & B) were investigated to develop an approach providing the optimal extraction of intact selenoproteins and/or selenium binding proteins from the mushroom matrix. Extraction protocols C & D were used to extract selenopeptides or selenoamino acids present in the mushroom matrix.
Sodium hydroxide extraction has been shown to extract high molecular weight fractions containing selenium.[22] Tris-HCl extraction has been shown to selectively extract water soluble proteins.[23] Extraction protocol (Ex-Prot) A uses NaOH extraction and Ex-Prot B uses Tris-HCl for extraction. Extraction of selenium compounds supposedly bound to protein present in the sample was performed according to Wrobel et al.[24] Ex-Prot C uses proteinase K and protease XIV without driselase, Ex-Prot D uses the same enzymes but with driselase. The importance of driselase is explained in more detail in section 3.3.2.3.

**Extraction Protocol A:** ~0.05 g of sample was placed in a plastic tube and 3 mL of sodium hydroxide solution (0.1 M) was added. The mixture was shaken on a Vortex system for 15 min and then centrifuged at 5000 rpm for 20 min. 100 µL of the supernatant was then introduced into the SEC-ICP-MS system.

**Extraction Protocol B:** 25 mL of 30 mM Tris-HCl (pH=7.5) solution was added to ~0.25 g of sample. The sample was extracted for 24 h with a magnetic stirrer at room temperature. The extract was centrifuged for 20 min at 5000 rpm. The supernatant was transferred to another vial. Acetone was added to the supernatant until the final concentration of acetone reached 80% (v/v) (optimum for the precipitation of high MW and low MW proteins).[25] The sample was placed at 5°C for 24 h. The solution was then centrifuged for 20 min at 5000 rpm at 5°C. The supernatant was removed. This fraction may contain low molecular weight selenium compounds, but they would not be associated with the proteins. Therefore the acetone supernatant was not analyzed. The protein precipitate was dried under steam of pure nitrogen gas and re-solubilized with 1
mL 30 mM Tris-HCl (pH=7.5) buffer for analysis by SEC-ICP-MS as 100 µL was used for injection.

**Extraction Protocol C**: 5 mL 30mM Tris-HCl buffer (pH= 7.5) containing 1 mM CaCl$_2$ was added to 0.2 g of sample and 0.03 g of proteinase K enzyme. The solution was kept at constant temperature of 50 °C and constantly stirred for 18 h. Then, 0.03 g of protease XIV enzyme was added to the above mixture and kept at 50 °C with constantly stirring for 12 h. The final mixture was centrifuged for 20 min at 5000 rpm and 100 µL of the supernatant was injected into the SEC-ICP-MS system.

**Extraction Protocol D**: 5 mL 30mM Tris-HCl buffer (pH= 7.5) containing 1 mM CaCl$_2$ was added to 0.2 g of sample and 0.03 g of driselase enzyme. The solution was kept at constant temperature of 50 °C and constantly stirred for 18 h. After that, 0.03 g of proteinase K enzyme was added to the above mixture and kept at 50 °C with constantly stirring for 18 h. And finally 0.03 g protease XIV was added to the sample and kept at 50 °C with constantly stirring for 12 h. The final mixture was centrifuged for 20 min at 5000 rpm and 100 µL of the supernatant was injected to the SEC-ICP-MS system.

A summary of Ex-Prots A-D are shown in flow chart format in Figure 3.2.
3.2.6 Sample preparation of water soluble proteins for RP-HPIPC-ICP-MS

To identify Se species (selenoamino acids and selenopeptides) either incorporated in proteins or bond to proteins or peptides, the most frequently applied method is the enzymatic treatment based on the use of proteolytic enzymes.[28,29] The application of proteinase K,[26] protease XIV and trypsin [27] in Se speciation analysis have been reported in several papers.

After carrying out protein precipitation according to Protocol B (determined to be optimum extraction conditions, explained in more detail in section 3.3.2), three different enzymatic digestions (I, II, III) were utilized. These processes were used to breakdown
the protein into its respective selenium species, which were then analyzed by RP-HPIPC-ICP-MS to identify the selenium species in the protein fraction. Each enzymatic digestion (Enz-Dig) uses a different combination of specific (trypsin) and non-specific proteases (proteinase K) and details are explained in the following paragraphs.

**Enzymatic digestion I:** 0.03 g of trypsin was added to the sample. The solution was kept at constant temperature of 50 °C and constantly stirred for 24 h. The final mixture was centrifuged for 20 min at 5000 rpm and the supernatant was analyzed by RP-HPIPC-ICP-MS.

**Enzymatic digestion II:** 0.03 g of proteinase K was added to the sample. The solution was kept at constant temperature of 50 °C and constantly stirred for 24 h. After that, 0.03 g of proteinase XIV enzyme was added to the above mixture and kept at 50°C with constant stirring for 24 h. The final mixture was centrifuged for 20 min at 5000 rpm and the supernatant was injected to the RP-HPIPC-ICP-MS system.

**Enzymatic digestion III:** 0.03 g of trypsin was added to the sample. The solution was kept at constant temperature of 50°C and constantly stirred for 24 h. After that, 0.03 g of proteinase K enzyme was added to the above mixture and kept at 50°C with constant stirring for 24h. The final mixture was centrifuged for 20 min at 5000 rpm and the supernatant was injected to the RP-HPIPC-ICP-MS system.

A summary of Enz-Digs I-III are shown in flow chart format in **Figure 3.3**.
3.3 Results and Discussion

3.3.1 Total selenium determination

Total selenium analysis was performed on the enriched *Lentinula edodes* which was found to contain 46 ± 1 µg per g of dried sample. The method detection limit of total selenium was calculated as three times the standard deviation of the background signal.
(3σ) divided by the slope of the calibration curve and was found to be 12 ng of Se g⁻¹ of sample (dry weight).

3.3.2 SEC-UV-ICP-MS of selenium associated proteins

The Superdex 200 10/300 GL and 75 10/300 GL size exclusion columns were calibrated with a standard protein mixture of ferritin ($M = 474$ kDa), albumin ($M = 66$ kDa), myoglobin ($M = 16$ kDa), methylcobalamin ($M = 1.34$ kDa). These standards were chosen to cover the separation range of the columns and serve as approximate molecular weight markers. Detection of the protein standards was performed with UV detection at a wavelength of 280 nm. It is important to note that absorption at 280 nm is characteristic of proteins (due to Trp, Tyr, and cystine) [28], but is not unique to proteins. The separation of the SEC columns is based on hydrodynamic volume (usually indicative of molecular weight) as a logarithmic relationship between MW and retention time. For the Superdex 200, compounds with MW >660 kDa eluted with the void volume of the column, whereas compounds with MWs <70 kDa eluted after approximately 1700 sec. For the Superdex 75, compounds with MW >70 kDa eluted with the dead volume of the column, whereas compounds with MWs <7 kDa eluted after approximately 1600 sec. Typical ICP-MS and UV chromatographic profiles resulting from different extraction protocols (NaOH, Tris-HCl, and enzymes) are shown in Figure 3.4. The chromatograms presented in Figure 3.4 are using the Superdex 75 as data from this column was sufficient to determine the most effective extraction procedures and Superdex 200 data is not shown although selenium species extend into the 600-70 kDa range. Despite low resolution and only approximate molecular weight information, the SEC-UV-ICP-MS
method is very effective for screening for selenium association with various molecular weight fractions of the mushroom extracts offering non-denaturing conditions, relatively quick analysis, and 100% column recovery occurs so that all species can be seen. This method enables quick and accurate assessment of extractions procedures as the range of species extracted, relative amount of species extracted, and selenium levels among those species can be easily determined.
Figure 3.4: SEC-ICP-MS and SEC-UV of *L. edodes*. Ex-Prots A, B, & C. SEC chromatograms for Ex-Prot D were very similar to Ex-Prot C and not shown.
3.3.2.1 Extraction protocol A

For extraction protocol A, sodium hydroxide (0.1 M) was used for protein solubilization and extraction. In Figure 3.4A a typical SEC-UV-ICP-MS chromatogram obtained with the Superdex 75 column of the mushroom extract is presented. As can be seen in the fractionation profile shows a predominant selenium-containing fraction at a molecular weight of ~12 kDa and another at <7 kDa. The absence of a UV signal at 280 nm throughout the separation range shows that little to no protein fractions with MW greater than 7 kDa were extracted using this method.

3.3.2.2 Extraction protocol B

For leaching of water soluble proteins, 30 mM Tris-HCl extraction media with pH close to 7.5 to mimic physiological pH and minimize protein degradation. After the protein precipitate was re-dissolved in 30 mM Tris-HCl buffer, SEC-UV-ICP-MS was performed and a typical chromatogram is shown in Figure 3.4B. There are possible selenium associated protein fractions throughout the separation range of the column as well as selenium containing fractions >70 kDa and lower than <7 kDa. Figure 3.4B demonstrates that Ex-Prot B is more efficient for extracting high and low molecular weight fractions from the L. edodes sample matrix when compared to Ex-Prot A.

3.3.2.3 Extraction protocols C and D

In order to release selenium bound to proteins, enzymatic hydrolysis was implemented. This method will also extract selenium just from the water soluble fraction,
as no prior protein precipitation was utilized. This was achieved by using an enzyme mixture of the general proteases - proteinase K and protease XIV. One main objective of comparing Ex-Prot C & D, was the exploration of intracellular proteins as a digestion with a mixture of cellulase and hemicellulase enzymes referred to as driselase was used to solubilize the mushroom cell walls [8] in Ex-Prot C, but omitted in Ex-Prot D.

Both samples were treated with the respective extraction protocols and were then analyzed by SEC-UV-ICP-MS. The chromatographic fractionation profiles revealed a complete conversion of the high molecular weight (HMW) peaks in Figure 3.4B to much lower MW selenium-containing fractions shown in Figure 3.4C. The shift in MW confirms that the suspected proteins were broken down by the enzyme cocktails. SEC-UV-ICP-MS chromatograms for Ex-Prot D were virtually identical that of Ex-Prot C. Therefore, cell wall degradation with driselase did not cause significantly increases the extraction of selenium of the mushroom samples. This shows that selenium is not bound to proteins in a high concentration within the cell walls. Extraction efficiencies of the different sample preparation procedures are given in Table 3.2.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Extraction Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. edodes</td>
<td>Extraction yield (%) of total Se</td>
</tr>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>65%</td>
</tr>
</tbody>
</table>

3.3.2.4 SEC-UV-ICP-MS of extraction protocol B followed by enzymatic digestions

After extracting the water soluble protein fractions containing selenium associated with various molecular weight compounds, these extracts were enzymatically digested to reduce their molecular weight and make it more adept to analysis by other
chromatographic methods. Figure 3.5 shows the original sample extract using protocol B, then a subsequent digestion using Enz-Digs II & III. The initial separation was performed on the Superdex 200 so establish the distribution of selenium above 70 kDa. After digestion, virtually all the selenium was converted to lower molecular weight portions, (less that 70 kDa) thus verify the effectiveness of the enzymatic digestions. Enz-Dig I only used trypsin and little breakdown to lower molecular weight peaks were shown. Both Enz-Digs II & III were virtually the same and further separation on Enz-Dig III was performed on the Superdex 75 to separate the lower fractions (as all species below 70 kDa will coelute using the Superdex 200) and determine if the new species were broken down below the 70 kDa range. As shown in Figure 3.6, the majority of the selenium is in the lower molecular weight are around 7 kDa or less. Some selenium remained in higher molecular weight portions, perhaps signifying that the selenium was not attached to proteins but rather another high molecular weight moiety unable to be broken down by the utilized enzymes.
3.3.3 RP-HPIPC-ICP-MS of proteolytic digest of water soluble proteins fraction

Reversed-phase high performance ion-pairing chromatography (RP-HPIPC) has been previously used for the speciation of selenium compounds in plant samples.[29,30] In the present work, the separation of the selenium species present in water soluble
protein fraction treated by different enzymatic digestions (I, II, and III described previously) and then the solutions were analyzed using RP-HPIPC-ICP-MS. The chromatographic conditions are listed in Table 3.1. The use of methanol concentration lower than 20% (v/v) in the mobile phase avoided instability effects on the plasma permitting the sensitive detection of selenium by ICP-MS. Higher concentrations of MeOH typically needed to elute more hydrophobic proteins/peptides could not be used as they would cause problems with the plasma. Separation of four commercially available selenium standards was achieved within 20 min and is shown in Figure 3.7. The elution order is: (1) inorganic selenium ($t_R = \sim 200$ sec); (2) SeCys$_2$ ($t_R = \sim 370$ sec); (3) MeSeCys ($t_R = \sim 500$ sec); and (4) SeMet ($t_R = \sim 1200$ sec).

![Figure 3.7: RP-HPIPC-ICP-MS chromatogram of selenium standards: Se$^{IV}$, SeCys$_2$, MeSeCys, and SeMet in DDW at a concentration of 100 ng mL$^{-1}$ for each standard. Chromatographic conditions for RP-HPIPC chromatography are shown in Table 3.1](image)

In order to investigate the selenium species released by means of proteolysis from the water-soluble proteins fraction, proteins extracted (as in protocol B) from the dried sample were re-solubilized in Tris-HCl buffer and three different enzymatic digestions (I,
II, and III) were utilized. Enzymatic extracts of the proteins were introduced into RP-HPIPC-ICP-MS system for selenium speciation. The chromatographic profiles of the three different enzymatic digestion procedures are shown in Figure 3.8. The highest degradation of the protein fraction to its respective selenium species was achieved with the Enz-Digs II & III. Both digestions eluted similar selenium species. Attempts were made to identify eluted species fortification of the sample with selenium standards.

The chromatographic profile shown in Figure 3.9 it can be seen that just three selenium species were identified and all of these were eluted earlier than the standards. The lack of commercially available standards limits the number of species that can be identified using only ICP-MS for detection. SeCys$_2$ eluted at ~200 sec in the standard mix, but eluted earlier in the sample matrix at ~130 sec. MeSeCys eluted at ~150 min, which is more than 120 sec earlier than the standard elution. This shift in retention time is most likely due to the high matrix of the samples. There is a peak on the Figure 3.9
chromatogram at ~400 sec which was identified as SeMet. Two unknown selenium species were also detected in the protein extract at $t_R = \sim 1625$ and $\sim 1700$ sec. Column recovery of Se through the C$_8$ Alltimax column (with guard column) for this sample matrix was 91%. The remaining 9% of the selenium most likely remained on the column as 5% MeOH as not enough to elute high hydrophobic compounds.

![Figure 3.9: RP-HPIPC-ICP-MS chromatogram of Ex-Prot III with peak identities from sample fortification](image)

3.4 Conclusions

This main goal of this investigation was to study the possible association of selenium with water soluble proteins extracted from *L. edodes*. The selenium distribution in the protein fraction was successfully analyzed using SEC-UV-ICP-MS. The Tris-HCl extraction (ExP B) proved to be the most efficient means of extraction. By digesting the extract from ExP B with enzyme cocktails the HMW selenium compounds were successfully cleaved into smaller portions. The highest degradation efficiency was
achieved with the combination of enzymes of trypsin and proteinase XIV or proteinase XIV and protease K (Enz-Digs II & III). Analyzing the low molecular weight fractions by RP-HPIPC-ICP-MS led to the confirmation of SeCys$_2$, MeSeCys, and SeMet and their association with proteins in the digested extract. The non-proteinogenic amino acid MeSeCys, is water soluble and was most likely coextracted with the proteins. Therefore it should be made clear that the presence of MeSeCys in this fraction does not imply that the MeSeCys is incorporated into the mushroom proteins. *L. edodes* is a source of SeMet and MeSeCys which have been shown to have beneficial health properties. Specifically, SeMet is commonly known as an antioxidant with chemopreventive properties as MeSeCys has been shown to induce apoptosis through caspase activation in human promyelocytic leukemia (HL-60) cells.[55] Also given the fact that the mushrooms were supplemented with selenite but metabolized the selenium into various less toxic organo-selenium compounds lends fact to the use of mushrooms as possible phytoremediation tool.

Future studies will be undertaken to determine identities of selenium associated proteins the unknown selenium species present in the digested mushroom extract.

### 3.5 Acknowledgements

The authors would like to acknowledge Pam Bishop (Department of Biology, University of Cincinnati, Cincinnati USA) for helping to grow the *Lentinula edodes* in the greenhouse. The research was partially supported by NIEHS grant ES04908.
3.6 References


CHAPTER FOUR:
Selenium volatiles as proxy to the metabolic pathways of selenium in genetically modified *Brassica juncea*

4.1 Introduction

Selenate and selenite are major contaminants in soils especially in the Central Valley region of California where Se has been shown to be a factor in high rates of mortality and embryonic deformities of resident birds of the Kesterson Reservoir.[1] The selenium originates from the erosion of natural cretaceous shale and travels via the irrigation water which eventually evaporates, causing the concentration of Se to reach levels >12 mg Se kg\(^{-1}\) soil.[2] To control the problem, some attempts have been made, such as pooling the drainage water into evaporation ponds, but via evapo-concentration, the Se levels ultimately increase and the cleanup of these polluted areas becomes a necessary objective.

The use of selenium accumulator plants to uptake and detoxify contaminants (phytoremediation) is an attractive solution for cleaning such sites. Certain plants extract selenium from the contaminated soil and ultimately convert it to virtually nontoxic volatile organic forms like dimethyl selenide. Selenium hyper-accumulator plants, such as *Astragalus bisulcatus*, have been examined for this purpose, but slow growth rate and small biomass limit their effectiveness for phytoremediation.[3]

An attractive solution to this problem is the genetic engineering of fast growing Se accumulator plants, such as Indian mustard (*Brassica juncea*) as the efficiency of Se phytoremediation, a naturally occurring process, can be dramatically enhanced this way.[4-7] One strategy for genetically engineering plants for improved phytoremediation
is to increase the levels of rate-limiting enzymes involved in the uptake and/or detoxification of selenium. An example of this strategy is the overexpression of the APS1 gene (encodes for ATP-sulfurylase\(^1\), EC 2.7.7.4) from \textit{Arabidopsis thaliana} that overcomes the rate-limiting step of reducing selenate to selenite.\([6-8]\) A newer strategy for genetically engineering plants takes advantage of the increasing information about the Se-hyperaccumulation mechanism. An example of this approach involves the overexpression of a gene isolated from the Se-hyperaccumulator, \textit{A. bisulcatus}, which encodes for selenocysteine methyltransferase\(^2\) (EC 2.1.1.9).\([5]\) Overexpressing SMT increases levels of selenocysteine methyltransferase which methylates SeCys to form Se-(methyl)-selenocysteine (MeSeCys), a non-proteinogenic amino acid, thus diverting the Se flow away from SeCys and subsequent production of SeMet (see \textbf{Figure 4.1}), both of which can be mis-incorporated into proteins, altering their native structure and function.

\(^1\) The ATP-sulfurylase overexpressing transgenic plants will be abbreviated as APS.

\(^2\) The selenocysteine methyltransferase overexpressing transgenic plants will be abbreviated as SMT.
Figure 4.1: Outline of the selenium metabolic pathway in *B. juncea* shown as uncontrolled wild-type pathway (→ dimethyl selenide) and genetically modified pathway (→ dimethyl diselenide).[9]

Decreasing SeCys levels represent an important Se tolerance strategy. Recently, the two approaches were combined by developing *B. juncea* overexpressing both APS1 and SMT.[10] The double transgenic plants (APS×SMT) appear to have the combined the benefits of overexpressing both genes as they were able to accumulate significantly more Se than the single transgenics (APS and SMT) when grown on selenate-containing media.

In genetically modifying a plant, the transgene is introduced with the intent it will integrate into the genome. To determine whether this process occurred successfully, several approaches are practiced. Polymerase chain reaction (PCR) analysis is often the first step. In this process a DNA fragment specific for the used primers will be amplified from DNA extracted from genetically modified (GM) cultures, whereas no such DNA
can be amplified from wild type (WT). Using gel electrophoresis to separate DNA fragments, a positive result can be visualized as an ethidium bromide stained band.

A second test is based on the fact that the over expression of the gene results in increased production of the desired protein. Western or immuno-blotting is typically used to detect the protein of interest using an antibody that will selectively interact with the desired protein. In some cases, this can add practical complications since obtaining appropriate antibodies can be difficult and expensive. Also, there is the possibility that the antibody will hybridize with endogenous proteins (as is the case with the SMT antibody) thus leading to the false positives. In most cases, however, this method (like PCR) is highly specific and presence of the modification can be detected as a stained protein band at the expected molecular weight.

The aim of this study is to investigate alternative elemental and molecular mass spectrometric methods for rapidly detecting molecular level changes produced by genetic modifications, thus differentiating wild type *B. juncea* from genetically modified *B. juncea* similar to the conventional electrophoresis methods.

4.2 Experimental

4.2.1 Chemicals and standards

All reagents were of analytical grade and were used without any further purification. Dimethyl selenide (DMeSe), dimethyl sulfide (DMeS), and dimethyl disulfide (DMeDS) were purchased from Fluka (Milwaukee, WI, USA). Dimethyl diselenide (DMeDSe) and diethyl disulfide (DEtDS) were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Diethyl diselenide (DEtDSe) was purchased from Strem
Chemicals (Newburyport, MA, USA). The stock solutions of 1000 µg mL\(^{-1}\) were prepared by dilution of 2.5 µL of compound with 2500 µL HPLC grade methanol (Fisher Scientific; Fair Lawn, NJ, USA). \(\text{CH}_3\text{SeSCH}_3\) was prepared via chalcogenide exchange reaction as described elsewhere. [19] The liquid Ar used to run the ICP-MS as well as the Ar:N\(_2\) optional gas (50:50) were obtained from Wright Brothers (Cincinnati, OH, USA).

D,L-selenomethionine (SeMet), selenocystine (SeCys\(_2\)) and sodium selenite (Na\(_2\)SeO\(_3\)) were purchased from Aldrich (Milwaukee, WI, USA). Se-(methyl)-selenocysteine (MeSeCys) was purchased from Sigma (St Louis, MO, USA). Se-(methyl)-selenomethionine (MeSeMet) was synthesized from SeMet and methyl iodide according to Fan et al.[11] All the solutions were prepared daily in 18 MΩ cm distilled deionized water (Sybron Barnstead, Boston, MA, USA). Heptafluorobutyric acid (HFBA) was obtained from Sigma and HPLC-grade methanol (Fisher Scientific, Pittsburgh, PA, USA) was used to prepare mobile phases.

For the determination of total Se, solutions were prepared by appropriate dilutions of 1000 µg mL\(^{-1}\) Se(IV) standard solution (Spectrum Chemical, Gardena, CA, USA). For digestion of samples, nitric acid (HNO\(_3\), 68%, supra-pure) and hydrochloric acid (HCl, 36.5%, supra-pure) were purchased from Pharmco (Brookfield, CT, USA).

4.2.2 Instrumentation

An Agilent Technologies (Agilent Technologies; Palo Alto, CA, USA) model 6890 series gas chromatograph (GC) was used and was coupled to an Agilent 7500ce inductively coupled plasma mass spectrometer (ICP-MS) equipped with a reaction/collision cell (Agilent Technologies; Tokyo, Japan). The GC was interfaced to
the ICP-MS through a heated transfer line (Agilent Technologies; Tokyo, Japan). A second Agilent 6890 GC was used with a quadrupole mass analyzer. For additional structural conformation, a third Agilent 6890 GC coupled to a Micromass (Manchester, UK) GCT orthogonal time-of-flight mass spectrometer (TOF-MS) was used with standard operating conditions (electron impact ionization at 180 °C, trap current 400 µA, 1 s scan time) using heptacosa(fluorotributylamine) as a calibration and as the lock mass compound (m/z 218.9856 ion). Average mass accuracy usually was less than 0.001 Da. Chromatographic settings are shown in Table 4.1.

The operating conditions for GC are listed in Table 4.1. ICP-MS was operated with the collision/reaction cell using the following standard conditions: 1150 W plasma power, 1.0 L Ar min⁻¹ carrier gas, 2.0 mL H₂ min⁻¹ collision cell gas, quadrupole bias –16 V, octoploe bias –18 V and 100 ms dwell time for each monitored isotope (³²S, ³⁴S, ⁷⁷Se, ⁷⁸Se, ⁸⁰Se, and ⁸²Se). 50:50 Ar/N₂ mixture was used as an optional gas at 5% carrier gas flow.

For total Se analysis, the Agilent Integrated Autosampler was used for continuous flow sample introduction. ICP-MS was operated with the collision/reaction cell using the following standard conditions: 1500 W plasma power, 1.21 L Ar min⁻¹ carrier gas, 3.5 mL H₂ min⁻¹ collision cell gas, quadrupole bias –16 V, octoploe bias –18 V and 100 ms dwell time for each monitored isotope (⁷⁷Se, ⁷⁸Se, ⁸⁰Se, and ⁸²Se).

Table 4.1: GC conditions for molecular and atomic mass spectrometric analysis

<table>
<thead>
<tr>
<th></th>
<th>GC-ICP-MS</th>
<th>GC-MS</th>
<th>GC-TOF-MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>DB-5; 30 m × 320 µm i.d. × 0.25 µm</td>
<td>HP-5; 60 m × 320 µm i.d. × 1.0 µm</td>
<td>HP-5; 30 m × 320 µm i.d. × 0.25 µm</td>
</tr>
<tr>
<td>Purge time</td>
<td>0.5 min</td>
<td>0.5 min</td>
<td>0.5 min</td>
</tr>
<tr>
<td>Purge Flow</td>
<td>5 mL He min⁻¹</td>
<td>5 mL He min⁻¹</td>
<td>1 mL He min⁻¹</td>
</tr>
</tbody>
</table>
Volatile were sampled from the headspace by using a 75 µm Carboxen/poly(dimethyl siloxane) solid phase microextraction (SPME) fiber (Supelco; Bellefonte, PA, USA). The fiber was cleaned every day by putting the fiber in the injection port for the entirety of the GC analysis.

Separation of selenium compounds extracted from plant material was carried out using an Agilent 1100 liquid chromatograph equipped with a binary HPLC pump, an autosampler, a vacuum degasser system, and temperature column compartment. The detector used was the Agilent 7500ce (conditions same as for total Se analysis). Altech Alltima C8 column was used for separation (250 mm × 4.6 mm, 5µm). The mobile phase contained 0.1% heptafluorobutyric acid and 5% methanol (v/v) in water (pH ~ 2.5). The flow rate was 1 mL min⁻¹ and 50 µL of sample was injected.

<table>
<thead>
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<th>250 °C</th>
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<th>220 °C</th>
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<td>n/a</td>
</tr>
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</table>
| Temp Program        | 40 °C for 4 min
40-125 °C @ 15 °C min⁻¹
Hold 5 min
125-300 °C @ 35 °C min⁻¹
Hold 1 min | 40 °C for 4 min
40-125 °C @ 15 °C min⁻¹
Hold 5 min
125-300 °C @ 35 °C min⁻¹
Hold 1 min | 50-220 °C @ 10 °C min⁻¹
Hold 7.5 min |
| Carrier Gas         | 2.4 mL He min⁻¹ | 3.0 mL He min⁻¹ | 1.0 mL He min⁻¹ |
| Auxiliary 1 & 2     | 250 °C | 250 °C | 250 °C |

4.2.3 Plant growth

Indian mustard (*Brassica juncea*; Accession no.173874) seeds were initially obtained from the North Central Regional Plant Introduction Station (Ames, IA, USA) and propagated. Transgenic APS plants were obtained as described by Pilon-Smits *et al*.,[6] by expression of the *Arabidopsis thaliana* APS1 gene (encoding ATP sulfurylase)
and transgenic SMT plants were obtained as described by LeDuc et al.,[5] by expression of the *Astragalus bisulcatus* SMT gene (encoding Se-cysteine methyltransferase). Double transgenic APS×SMT plants were obtained by crossing homozygous APS plants with homozygous SMT plants as described in LeDuc et al.[10]

**GC-ICP-MS experiments.** Indian mustard seeds (WT, SMT and APS×SMT) were sterilized and sown on media without adding any Se-compounds as described above. After two days of cold treatment (4 °C) and three days incubation in a growth chamber kept at 25 °C under continuous light, approximately 7-10 seedlings were transferred to each sterile glass bottle (Wheaton) containing 5 mL of liquid media, described above, without the phytagar, capped with aluminum foil, and returned to the 25 °C growth chamber for a one day incubation. Following that, some of the bottles were capped with a Teflon seal and crimped shut without addition of Se-compounds (controls). To other bottles, sodium selenate or sodium selenite was added to a final concentration of either 200 or 500 µM, the bottles capped with a Teflon seal, and then crimped shut. The seedlings were returned to the 25 °C growth chamber for 7-10 days and frozen at –80 °C upon first indications of plant deterioration. The samples were frozen at –20 °C until analysis. Following GC-ICP-MS analysis, the plant material was retrieved from the bottles and dried in a 50 °C oven in order to determine plant dry mass.

**HPLC-ICP-MS experiments.** Indian mustard seeds (WT, SMT and APS×SMT) were sterilized by rinsing in 95% ethanol for 30 s, in 0.65% hypochlorite solution for 30 min, and in sterile deionized water for 5×10 min, on a rocking platform. 25 sterilized seeds were sown in a grid pattern in Magenta boxes (Sigma) on half-strength MS medium with 10 g L⁻¹ sucrose and 5 g L⁻¹ phytagar (Sigma), with or without 100 µM selenite.
After two days at 4 °C, the boxes were moved to a growth chamber kept at 25 °C under continuous light. On day 7, individual seedlings (both control and selenite-treated) were harvested, washed in running de-ionized water to remove any Se externally bound to the roots, roots and shoots separated, flash-frozen in liquid nitrogen, and ground to fine powder.

**4.2.4 Sample analysis and optimization**

**GC-ICP-MS.** The headspace of the standards and the plants was sampled by exposing the SPME fiber through the sample vial septum for 10 min and then injecting it into the GC using an injection port designed for SPME (0.75 mm i.d. injection liner). A mixture of Se and S volatile standards was used to optimize the GC separation. Optimized GC-ICP-MS conditions (from Tables 4.1) were employed, noting that pulsed/splitless injection was used, to minimize peak broadening of later eluting compounds. After optimization, standards of DMeSe and DMeDSe (ranging from 2.6 – 520 nmol) were used to create a calibration curve based on integrated peak area from the $^{78}$Se signal of the ICP-MS. Previous data from Meija et al.[12] were also compiled and analyzed to determine the correlation between the concentration ratio of DMeSe to DMeDSe and genetic modification.

**HPLC-ICP-MS.** According to the proposed Se pathway in *B. juncea*, the precursors to the prevalent volatile compounds DMeSe and DMeDSe are MeSeMet and MeSeCys, respectively.[9] These amino acids were extracted using a previous protocol by Montes-Bayón et al.[13] For the extraction, a ratio of 1 mL of 0.1 M HCl per 75 mg of sample material was used. The mixture was stirred for 24 h and centrifuged at 4000 rpm for 10
min. The supernatant was collected and diluted 1:1 with the mobile phase. The sample (50 µL) was injected and Se levels in eluates were monitored by the ICP-MS.

### 4.3 Results and Discussion

#### 4.3.1 Evaluating Se compounds in relationship to genetic modification

In 1968 Evans et al. showed that the headspace of a Se-accumulator plant *A. bisulcatus* contained dimethyl diselenide and no traces of dimethyl selenide.[14] In contrast to that, cabbage plants (Se non-accumulators) contained only CH$_3$SeCH$_3$ as the only [major] Se-containing volatile.[15] The marked differences in the observed Se-volatile profiles served as a tool to distinguish between the accumulator and non-accumulator plants even at the early stages of research in this area.[16] In this study we expand on the use of the volatile Se profiles to follow the genetic modifications that increase Se accumulation thus making *B. juncea* more apt for Se phytoremediation. Since the genetic modifications of wild type *B. juncea* converts it from a Se-accumulator plant into a Se-hyper-accumulator plant, the resulting changes can be monitored from the headspace Se volatiles (see Figure 4.1).

Altering the expression of enzymes along the selenium metabolism pathway will, in most cases, lead to a detectable change in the relative amounts of different Se metabolites. As seen in Figure 4.1, the modified pathway versus the unmodified pathway can result in the following trends: (1) by modifying the pathway to increase SMT expression, more Se is routed towards MeSeCys and then volatilized as dimethyl diselenide (DMeDSe), (2) if the *B. juncea* is left unmodified, the Se pathway should result in the formation of more MeSeMet that will ultimately be volatilized as dimethyl...
selenide (DMeSe). As such, Se-amino acids and Se-volatiles can be used as proxies to determine the overall effects of the genetic modification attempted. Accordingly, WT samples should have higher MeSeMet/MeSeCys and DMeSe/DMeDSe ratios whereas GM types will exhibit lower ratios, with the decrease in the ratio proportional to the extent of the enzyme expression. This also varies depending on the position in the genome - where the transgene integrates.

### 4.3.2 DMeSe and DMeDSe in relationship to genetic modification

The DMeSe/DMeDSe ratios of the WT, SMT, and APS×SMT *B. juncea* samples were analyzed using GC-ICP-MS and the average results for each sample type are shown in Figure 4.2. For the WT cultures the DMeSe/DMeDSe ratio is independent of supplementation type; neither Se enrichment form nor concentration significantly altered the ratio of DMeSe/DMeDSe. Though cultures SMT1 and SMT2 were both SMT modified, when grown under the same supplementation conditions, differences in DMeSe/DMeDSe ratio were exhibited. This is because the transgene, which is inserted randomly, could be in a more advantageous location in the SMT1 culture than the SMT2 culture, thus explaining the lower ratio for SMT1 when supplemented with selenite and selenate. This also agrees with earlier findings that SMT expression at the mRNA and protein level is lower in the SMT2 plants than in the SMT1.[5]
Another interesting point is that in the SMT1 and SMT2 cultures DMeSe/DMeDSe ratios are maintained whether supplemented with selenite or selenate. This is likely due to the fact that the SMT modification only affects the methylation of SeCys and does not directly affect the reduction of selenate to selenite, thus making its effects independent of the upstream selenium form added.

When the plant is doubly genetically modified (APS×SMT) and supplemented with selenite, the DMeSe/DMeDSe ratios were even lower than the SMT modified cultures. This trend is rather unexpected, since the APS modification should affect only the uptake of selenate, not selenite. However, since ATP sulfurylase is an early enzyme in the sulfur/selenium assimilation pathway this remains an open question as the exact mechanism of the APS modification is not fully understood. It is also possible that the
SMT modification is simply in a more advantageous location in the both APS×SMT cultures than in the SMT cultures.

Note that only APS×SMT plant cultures show difference between the DMeSe/DMeDSe ratios when supplemented with selenate vs. selenite. This is expected as overexpression with APS directly discriminates between the selenate and selenite: in our opinion, a rather elegant way of detecting the presence of the APS gene.

Though it was established that DMeSe/DMeDSe ratios for both WT and SMT overexpressing plants were independent of selenium source (selenate or selenite), the overall quantities of the DMeSe and DMeDSe produced were drastically different. Approximately 10 times more DMeSe and DMeDSe (normalized to sample weight) is produced in all plant types when the plants were supplemented with selenite than with selenate.[8]

4.3.3 Minor Se and S headspace volatiles

It is well documented that there are two main selenium-containing volatiles emitted from Se-accumulating plants: dimethyl selenide, $\text{CH}_3\text{SeCH}_3$, and dimethyl diselenide, $\text{CH}_3\text{SeSeCH}_3$.[17,18] A third major Se volatile that is found in the headspace of $B.\text{ juncea}$ plants is dimethyl selenosulfenate, $\text{CH}_3\text{SeSCH}_3$.[12, 20-22] In this study multiple steps were taken to identify as many of the minor Se compounds as possible. The first step towards this is the GC-ICP-MS retention time comparison with available alkyl selenide standards. In the second step, selected $B.\text{ juncea}$ samples were re-analyzed using GC-MS and high resolution GC-TOF-MS, in order to gather structural information and
confirm the identities of those tentatively established by GC-ICP-MS. Analysis of selenium compounds is less selective and sensitive when using GC-MS, but the distinct Se isotope pattern makes interpreting the mass spectra easier.

A second basis for low intensity peak identification using GC-MS is retention time when applied to an aliphatic series. When using a constant temperature ramp, the retention time of unbranched dialkyl selenides increases linearly with increasing alkyl length, shown in Figure 4.3. When using these data in combination with molecular ion peaks derived from mass spectra, Se isotope pattern, and fragmentation patterns, relatively confident identifications of several dialkyl selenides could be made. Additional information is presented in Table 4.2 while the compound eluting at 10.63 min deserves more discussion. The molecular ion of this species is present at $m/z = 138$ ($^{75}$Se) and initially was thought to be EtSeEt; however, the commercial standard of EtSeEt elutes at 9.21 min under the same conditions. Therefore, this was tentatively identified as PrSeMe (further fragment ion analysis was not possible due to its low levels).
Figure 4.3: (A) SPME-GC-ICP-MS chromatogram and (B) SPME-GC-MS chromatogram (Ion 93 = SeCH\(^+\)) of an APS×SMT1 modified B. juncea headspace. Identities of various alkyl selenides found in B. juncea are labeled between chromatograms A and B, showing that retention time increases linearly with increasing alkyl length (retention time of CH\(_3\)CH\(_2\)SeCH\(_3\)CH\(_3\) is from a standard as it was not detected in the headspace of any samples).

The third step of the analysis was to use the exact mass capability and the higher sensitivity of the GC-TOF-MS to further confirm the structures of the minor Se volatiles. The most intense of the minor Se-volatiles is ethylmethyl selenide, with the measured exact mass of 123.981 Da. The mass spectrum of this compound shows the presence of CH\(_3\)SeH\(^+\) (measured at 95.9490 Da, \(^{80}\)Se) and CH\(_3\)CH\(_2\)Se\(^+\) (measured at 108.9565 Da, \(^{80}\)Se) and is shown in Figure 4.4.
By searching the GC-TOF-MS for exact masses of various dimethyl polychalcogenides, two trichalcogenides were observed. The first compound was of the molecular formula C$_2$H$_6$Se$_2$S (measured exact mass 221.853 Da, $^{79}$Se). Such elemental composition corresponds to two isomers, the asymmetric MeSeSeSMe and the symmetric MeSeSSeMe. Since both synthesized isomers are available[19] the retention time and mass spectral comparison of these two compounds allowed us to establish the presence of MeSeSSeMe in the headspace of the genetically modified *B. juncea* plants (see Figure 4.5). Among the mass spectral differences of the two isomers, most notable is the loss of the central chalcogen atom (S for MeSeSSeMe and Se for MeSeSeSMe) leading to the abundant $m/z$ 190 cluster for MeSeSSeMe and $m/z$ 142 cluster for MeSeSeSMe.
A second trichalcogenide was discovered at the exact mass of 267.8032 Da. This mass (along with the isotopic pattern) corresponds to C$_2$H$_6$Se$_3$. As shown previously, the most abundant m/z peak is not representative of $^{80}$Se$^{80}$Se$^{80}$Se, rather it is from $^{78}$Se$^{80}$Se$^{80}$Se.\[19\] The mass spectral fragmentation and the retention time comparisons agree with the dimethyl triselenide, MeSeSeSeMe (Figure 4.6). In recent work, other trichalcogenides such as, MeSSSeMe, MeSSeSMe and MeSeSeSMe, were reported [23-24]. To the best of our knowledge, this is the first report of MeSeSSeMe and MeSeSeSeMe in plants.
Figure 4.6 Mass spectrum of dimethyl triselenide, CH$_3$SeSeSeCH$_3$, from GC-TOF-MS analysis of APS×SMT1 B. juncea supplemented with selenite.

Table 4.2: Summary of the Se-containing volatiles identified from the headspace of APS×SMT1 B. juncea supplemented with selenite.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Retention Time (min)$^b$</th>
<th>Relative abundance$^c$</th>
<th>Method of identification$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH$_3$SeCH$_3$ (110 Da)</td>
<td>5.35</td>
<td>32.1%</td>
<td>RT, MS, EC, AS</td>
</tr>
<tr>
<td>CH$_3$SeCH$_2$CH$_3$ (124 Da)</td>
<td>7.42</td>
<td>2.1%</td>
<td>MS, EC, AS</td>
</tr>
<tr>
<td>CH$_3$SeCH$_2$CH$_2$CH$_3$ (138 Da)</td>
<td>10.63</td>
<td>1.4%</td>
<td>MS</td>
</tr>
<tr>
<td>CH$_3$SeCH$_2$CH$_2$CH$_2$CH$_3$ (152 Da)</td>
<td>11.83</td>
<td>0.7%</td>
<td>MS, AS</td>
</tr>
<tr>
<td>CH$_3$CH$_2$SeCH$_2$CH$_2$CH$_3$ (166 Da)</td>
<td>13.72</td>
<td>0.6%</td>
<td>MS, AS</td>
</tr>
<tr>
<td>CH$_3$SeCH$_3$ (142 Da)</td>
<td>10.20</td>
<td>10.7%</td>
<td>RT, MS, EC</td>
</tr>
<tr>
<td>CH$_3$SeCH$_2$CH$_3$ (156 Da)</td>
<td>12.10</td>
<td>0.7%</td>
<td>MS</td>
</tr>
<tr>
<td>CH$_3$SeCH$_2$ (190 Da)</td>
<td>11.65</td>
<td>51.4%</td>
<td>RT, MS, EC</td>
</tr>
<tr>
<td>CH$_3$SeSeCH$_3$ (222 Da)</td>
<td>19.4$^e$</td>
<td>0.1%</td>
<td>RT, MS, EC</td>
</tr>
<tr>
<td>CH$_3$SeSeSeCH$_3$ (270 Da)</td>
<td>21.5$^e$</td>
<td>&lt;0.1%</td>
<td>MS, EC</td>
</tr>
</tbody>
</table>

$^a$Monoisotopic molecular weight based on $^{80}$Se.
$^b$Retention time based on GC-MS conditions from Table 4.1, unless otherwise noted.
$^c$Based on the molecular ion peak intensity of all volatile Se species present.
$^d$RT: retention time matching with standards as performed on a 30 m and 60 m DB-5 (0.25 µm and 1 µm film thickness respectively) capillary columns. MS: aliphatic series with retention time vs. alkyl chain length. AS: electron impact mass spectra obtained from plants and compared to mass spectra of the matching standards. EC: elemental composition and isotope patterns verified with high resolution GC-TOF-MS.
$^e$Calculated based on GC-TOF-MS conditions from Table 4.1
4.3.4 Se amino acids in relationship to genetic modification

From all the Se-containing metabolites, to date only MeSeCys has been used as a proxy to *B. juncea* genetic modifications.[13] While the MeSeCys levels in leaves remain unchanged with the extent of genetic modification (data not shown), the amount of MeSeCys in the shoot material exhibits strong correlation, as shown in Figure 4.7. In the wild type plants MeSeCys represents 23-41% of the total extracted Se while in the SMT and APS×SMT cultures MeSeCys represents 45-51% and 60-71% of the total Se extracted, respectively. The increase in MeSeCys shoot content from wild type to APS×SMT cultures are also in good agreement with other recent findings.[10] As previously discussed in the Se volatile section, the increase in MeSeCys production from SMT to APS×SMT is unexpected and further supports the conclusion that the APS and SMT interaction is not as simple as portrayed in Figure 4.1.

![Figure 4.7: HPLC-ICP-MS chromatogram of WT shoots supplemented with 100 µM selenite compared to SMT and APS×SMT modified shoots.](image-url)
There have been reports of MeSeMet presence in WT *B. juncea* shoots when supplemented with selenite[25] or SeMet.[26] When supplemented with selenite, MeSeMet comprises ~10% of the total Se, while when supplemented with SeMet, it makes up ~60% of the total Se. In our study the plants were supplemented with selenite, and MeSeMet (HPLC-ICP-MS analyses) represented less than 6% of the total extracted Se in the WT plants. Furthermore, there was no noticeable change in the MeSeMet content from WT to GM types. Therefore, it seems that MeSeMet levels are inadequate to follow the genetic modifications of selenium.

For comparison, the total selenium content of the various plants determined in previous experiments[5,6] is also assembled in Figure 4.8 along with total MeSeCys content and DMeSe/DMeDSe ratios.

![Figure 4.8](image)

**Figure 4.8:** Overall summary of Se uptake and metabolism related to the various genetic modifications (WT, SMT, APS×SMT) of *B. juncea* treated with selenite. The bar graphs are: A) total Se content (µg Se g⁻¹ of sample (dry weight)), B) total MeSeCys content (µg g⁻¹ of sample) in plant extract from the shoot material, and C) DMeSe/DMeDSe ratio from the headspace of each plant type.
4.4 Conclusions

In this study we have shown that the ratio of the two major headspace Se-volatiles, DMeSe and DMeDSe, seems a better proxy to the genetic modification than conventional total selenium content and plant tissue levels of Se-amino acids, such as MeSeMet and MeSeCys. Also, as seen in Figure 4.8, the DMeSe/DMeDSe ratios are more sensitive to change between modifications than total selenium and MeSeCys levels. Besides, the complementary use of GC-ICP-MS and GC-TOF-MS proved very useful in identification and confirmation of commonly neglected minor selenium volatile present in the headspace of *B. juncea*. As a result, previously unreported minor Se-containing volatiles, such as MeSeEt, MeSeSSeMe and MeSeSeSeMe were discovered.

4.5 Acknowledgements

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4.6 References


CHAPTER FIVE:
Chemical warfare agent degradation product detection in foods using a combination of LC-ICP-MS and LC-ESI-MS

5.1 Introduction

In 1997, the Chemical Weapons Convention (CWC) banned the use, production, development, stockpiling, acquisition, or transfer of chemical weapons.[1] There are several categories of chemical warfare agents (CWAs) including nerve, blister, choking, vomiting, blood, tear, and incapacitating agents.[2] Of these, nerve agents are the most notorious. Nerve agents are alkylphosphonic acid esters and are categorized into two main classes: ‘G’ agents and ‘V’ agents.[3,4] In general G-type agents are more volatile and less persistent than V-type agents.

On March 20, 1995, members of Aum Shinrikyo released sarin (GB) at a Tokyo subway killing 12 and injuring more.[5] Due to this and other recent terrorist activity the threat of rogue regimes acquiring CWAs for implementation is becoming a pressing matter for all nations.[6] Substantial resources have been spent to improve all facets of response to the perceived terrorist threat.[7]

As G-type and V-type agents enter the environment, they rapidly hydrolyze into organophosphorus acid degradation products generally thought to be less toxic. The hydrolysis pathways of sarin, soman, tabun, and VX are shown in Figure 5.1. The presence of the parent agent will be accompanied by its respective chemical warfare agent degradation products (CWADPs). The combination of the existence of several CWAs, (within V and G agent classes) each with multiple environmentally dependent degradation pathways leads to a large number of possible CWADPs. In general CWAs
degrade from alkyl alkyl phosphonic acids (ethyl methylphosphonic acid) to alkyl phosphonic acids (methylphosphonic acid) with varying alkyl chain lengths and conformations), eventually degrading to dihydrogen phosphate.[8] A list of the CWADPs analyzed in this study, their structures, and pK$_A$ values are shown in Figure 5.2. Many of the hydrolysis products are specific to their respective parent compound (EMPA originates specifically from VX). Other breakdown products are specific to CWAs, but may derive from multiple parent agents (MPA is from VX, sarin, and soman, among others). Others are degradation products (DMHP and DEHP) deriving from both CWAs and pesticides. However, those with the C-P bond are unique to CWADPs, as this bond is not present in organophosphate pesticides.[4] CWADPs are considerably less volatile and more polar than the parent agents.[9]

Although the toxicities of CWADPs are thought to be significantly lower than their parent agents, exact health risk is relatively unknown.[10] According to a 2005, Center for Disease Control report to Congress, EMPA and MPA are slightly toxic.[10] The large number of possible CWADPs lends to incomplete knowledge regarding toxicity including long term exposure risks.

The existence of CWADPs in a given sample can help to confirm the previous use of CWAs. Several techniques for the analysis of CWADPs have been published utilizing a wide variety of methods. Screening methods include spot tests [11] and matrix assisted laser desorption ionization mass spectrometry.[3] Separation of CWADPs prior to analysis by various detection methods is necessary to determine individual CWADPs; consequently there are several gas chromatography (GC) (cite), liquid chromatography (LC) (cite) and capillary electrophoresis (CE) (cite) separations reported in the literature.
Gas chromatography (GC) with various detection methods including atomic emission detection,[7,12] flame photometric detection (FPD),[13] inductively coupled plasma mass spectrometry (ICP-MS),[14] and most commonly, mass spectrometry (MS)[15-18] and tandem MS (MS/MS),[19,20] have been employed for analysis of CWADPs. Sharp peaks, good resolution, lower background and subsequently lower detection limits [14] make GC methods very attractive for the analysis of parent CWAs because of their high volatility; the low volatility of CWADPs requires time-consuming derivatization. Considerable variability in the apparent recovery when large amounts of extraneous materials are co-extracted has been encountered.[9] Divalent metals such as calcium can cause interferences with derivatization steps [16] resulting in poor quantitation. Other less labor intensive methods are desired.

Due to the low volatility and high solubility in water, CWADPs are model samples for LC separation methods. Typically these compounds are extracted from the media using water, with sample pretreatment dependent on the complexity of the sample.[21] LC is the most commonly used separation for analysis of CWADPs. For CWADPs analysis, LC has been used in combination with FPD,[22] indirect UV,[23] conductivity,[23,24] evaporative light scattering detection,[25] ICP-MS,[26] and MS[2,9,27-31] as well as MS/MS [7,31].

LC-MS and LC-MS/MS are the most commonly used techniques. The selectivity, sensitivity, and structural identification capabilities of MS and MS/MS analyses are among the advantages of these methods. However, ionization involving conventional MS sources such as atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI) can experience deleterious effects of constituents of various mobile
phases and extraneous compounds in the sample. Matrix problems and ionization suppression have also been indicated in some papers.[2] For these reasons, a more robust ionization source, such as ICP, has great potential. For the most part, ICP-MS is more tolerant of high matrices, and can be used with mobile phases not suitable for APCI or ESI-MS. ICP-MS is an element specific analyzer capable of qualitative and quantitative determination, has a large dynamic range, high sensitivity, (often at low part per trillion levels for some analytes) and is easily interfaced with liquid chromatography. [26]

Due to the harsh nature of ICP ionization in the 6000-10000 K argon plasma, no structural information is possible; only elemental data is available. In order to detect an analyte using ICP-MS, the analytes must have an “elemental tag” such as $^{31}$P. Recent applications involving LC-ICP-MS analysis using $^{31}$P, typically use He as a collision cell gas to lower the background of polyatomic interferences such as $^{14}$N$^{16}$O$^+$ and $^{15}$N$^{16}$O$^+$ and report very low detection limits (<1 ng mL$^{-1}$); such was implemented in this analysis.[26,32]

Though ICP-MS can detect $^{31}$P specifically in complicated matrices, its inability to differentiate various species of $^{31}$P requires chromatographic separation prior to detection. Analytes are subsequently identified by retention time matching and sample fortification with standards. This places crucial dependence on the prior chromatographic method as coelution of common interferences and standards can cause false positives. The preferred LC separation for CWADPs is reversed phase (RP) with molecular MS for detection. Without the use of ion pairing agents, MPA and H$_2$PO$_4^-$ coelute in the void volume of a RP separation. This problem is often avoided or not discussed in molecular MS applications as H$_2$PO$_4^-$ is virtually invisible in positive ion mode MS, but at high
levels, it can decrease the ionization of other analytes. However, when using a phosphorus specific detection method such as ICP-MS, $\text{H}_2\text{PO}_4^-$ is detectable and should consequently be treated as an additional analyte. In the only LC-ICP-MS method in the literature for CWADPs analysis,[26] $\text{H}_2\text{PO}_4^-$ coeluted with EMPA when using myristyl trimethylammonium bromide as an ion pairing agent. Therefore, when using ICP-MS for detection, chromatographic methods should be developed to resolve $\text{H}_2\text{PO}_4^-$ from analytes of interest, since $\text{H}_2\text{PO}_4^-$ is present at various levels in practically all real world samples. This problem becomes increasing inherent when dealing with more complicated matrices such as foods which often contain high levels of $\text{H}_2\text{PO}_4^-$ (~100-3,000 ppm of total phosphorus).

As previously discussed, because of the lack of structural information from ICP-MS, peaks must be identified by retention time matching and sample fortification. It would be beneficial to obtain structural information in combination to ICP-MS identification; therefore effort was taken to develop a LC method compatible with both ICP-MS and ESI-MS. Choosing mobile phases and other separation conditions was not trivial and will be further discussed in the section 5.3.1.4.

The aim of this work is to establish a method to analyze several CWADPs in the presence of $\text{H}_2\text{PO}_4^-$. Four LC separation schemes were evaluated each using ICP-MS for detection. Upon optimization, two methods were applied to food samples containing varying levels of phosphate. Separations 3 & 4 are the first methods, to our knowledge, to be applied to food, as a method is needed to respond in case of a food tampering/contamination involving CWAs. To our knowledge, Separation 4 is the first
LC separation that is compatible with ICP-MS and ESI-MS for the analysis of CWADPs and $\text{H}_2\text{PO}_4^-$.

**Figure 5.1: Hydrolysis pathways of some common CWAs from Munro et al. [4]**

### 5.2 Experimental

#### 5.2.1 Reagents

All chemicals used were reagent grade or higher. Water used throughout the experiments was 18 MΩ cm$^{-1}$ doubly distilled, deionized water (DDW) using a Millipore filtration system (Bedford, MA). Acetic acid, sodium acetate, formic acid (FA) and
ammonium formate were purchased from Sigma-Aldrich (St. Louis, MO). Methanol was acquired from Fisher Scientific (Fair Lawn, NJ). Nitric acid and ammonium carbonate were supplied by J.T. Baker (Phillipsburg, NJ). Ammonium phosphate was obtained from GFS Chemicals (Columbus, OH). A 1000 µg P mL⁻¹ tune standard from Leeman Labs (Lowell, MA) was diluted and used for ICP-MS optimization for ³¹P.

5.2.2 Standards

Stock solutions of methylphosphonic acid (MPA), ethyl methylphosphonic acid (EMPA), dimethyl hydrogen phosphate (DMHP), diethyl hydrogen phosphate (DEHP), ethyl hydrogen dimethylamidophosphate, sodium salt (EHDAP), isopropyl methylphosphonic acid (IMPA), isopropyl hydroden ethylphosphonate (IPHEP), isobutyl hydrogen methylphosphonate (IBHMP), diisopropyl methyl phosphonate (DIPMP), and cyclohexyl methylphosphonic acid (CMPA), in MeOH at 1000 µg mL⁻¹ of each standard, were purchased from Cerilliant (Round Rock, TX). Ethylphosphonic acid (EPA) and propylphosphonic acid (PPA) were obtained from Fisher Scientific in powder form, and 1000 µg mL⁻¹ standards in MeOH were created. Abbreviations of CWADPs tend to vary among publications; abbreviations in this work were partially adopted from Black et al.[9] All standards were kept in amber vials at -20 °C. Food samples were purchased at local markets. Stock solutions were diluted to appropriate concentrations prior to analysis.
Figure 5.2: Structures and pKₐ values of CWADPs analyzed in this study. H₃PO₄⁻ is the last breakdown product of many CWAs, but is considered an interference in this study as it is prevalent in many sample matrices. CWADPs 12 & 13 were only successfully analyzed in Separation 1 & 2. The designated number listed next to each compound will be used in chromatograms throughout this chapter for identify each peak.

5.2.3 Instrumentation

An Agilent 1100 (Agilent Technologies, Palo Alto, California) high-performance liquid chromatograph (HPLC) equipped with a binary pump, autosampler, vacuum degasser, thermostated column compartment, and diode array detector was used for all chromatography. Four chromatographic methods were developed. Optimum conditions for Separation 1-4 are shown in Table 5.1. Separation 1 used a Dionex IonPac AS7 column (Sunnyvale, CA) containing alkyl quaternary ammonium as the functional group. Separation 2 was carried out using a Thermo Hypercarb (Waltham, MA) with porous graphitic carbon as a stationary phase. Both Separation 3 & 4 utilized the same type of column, the Hamilton PRP-X100 (Reno, NV), which has a stationary phase of styrene–divinylbenzene substrate that has been derivatized chemically so as to introduce
trimethylammonium quaternary sites.[33] The stationary phases for each column have anionic exchange properties as well as hydrophobic characteristics.

An Agilent 7500ce (Agilent Technologies, Tokyo, Japan) ICP-MS equipped with shielded torch and collision/reaction cell technology was used for the element specific detection of $^{31}$P throughout this experiment. The effluent from the column was directly sent to the ICP-MS via a 40 cm length 0.25 mm ID PEEK tubing from the column outlet to the ICP nebulizer.

The collision cell using helium gas as the collision gas was implemented. The initial flow rate of 1 mL min$^{-1}$ provided a small increase in signal to noise ratio. Signal to noise ratios were virtually unaffected from 1-4 mL min$^{-1}$ of He. The main function was to lower to background, not to increase the signal to noise ratio. The instrument parameters for the ICP-MS were optimized using a solution of 1 $\mu$g $^{31}$P mL$^{-1}$ solution in the Buffer A prior to analysis. A description of typical ICP-MS operating conditions is provided in Table 5.2.

The LC-ESI-MS system used was an Agilent 1100 Series LC/MSD Trap SL with an ESI interface (Agilent Technologies). The effluent from the column was sent directly, via a 40 cm length 0.25 mm ID PEEK tubing from the column outlet, to the ESI interface with a high-flow spacer installed. The ESI-MS spectra were acquired in positive ion mode. The parameters of the ESI-MS were optimized for lower masses characteristic of CWADPs by directly infusing an EMPA (10 $\mu$g mL$^{-1}$) in MeOH standard solution at 350 $\mu$L hr$^{-1}$ using a syringe pump (KD Scientific, Holliston, MA). The conditions are shown in Table 5.2.

Table 5.1: Chromatographic conditions for Separation 1 & 2 including gradient program.
### Separation 1

Column: IonPac® AS7 4.1x 250 mm with guard column
A: 0.5 mM CH₃COOH/CH₃COO⁻Na⁺ in DDW
B: 25 mM HNO₃ in DDW
Flow Rate: 1.0 mL min⁻¹
Inj Vol: 25 µL

### Separation 2

Column: Hypercarb 2.1 x 100 mm 5 µm
Mobile Phase: A = 0.1% CH₃COOH, in DDW
B = 1% CH₃COOH, 0.5% ACN in DDW
Flow Rate: 0.3 mL min⁻¹
Inj Vol: 10 µL

### Separation 3

Column: PRP-X100 4.6 x 100mm 5 µm
Mobile Phase: A = 0.5% FA (pH ~2.3), 5% MeOH in DDW
B = 0.3M NH₄HCO₃ (pH ~2.3), 22% MeOH in DDW
Flow Rate: 1.0 mL min⁻¹
Inj Vol: 100 µL

### Separation 4

Column: PRP-X100 2.1 x 150mm 5µm
Mobile Phase: A = 10mM (NH₄)₂CO₃ (pH ~8.5) in DDW
B = 50mM (NH₄)₂CO₃ (pH ~8.5) in DDW
Flow Rate: 1.0 mL min⁻¹
Inj Vol: 100 µL

### Gradient Profile

None – Isocratic at 15% B

### Table 5.2: Typical conditions for ICP-MS and ESI-MS

<table>
<thead>
<tr>
<th>ICP-MS Conditions</th>
<th>ESI-MS Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward Power</td>
<td>1500 W</td>
</tr>
<tr>
<td>QP Focus</td>
<td>-7 V</td>
</tr>
<tr>
<td>Octopole Bias</td>
<td>-18 V</td>
</tr>
<tr>
<td>Quadrupole Bias</td>
<td>-15 V</td>
</tr>
<tr>
<td>He gas flow</td>
<td>2.0 mL min⁻¹</td>
</tr>
<tr>
<td>QP Focus</td>
<td>-8 V</td>
</tr>
<tr>
<td>Octopole Bias</td>
<td>-16 V</td>
</tr>
<tr>
<td>Quadrupole Bias</td>
<td>-14 V</td>
</tr>
<tr>
<td>He gas flow</td>
<td>2.0 mL min⁻¹</td>
</tr>
<tr>
<td>Ion Mode</td>
<td>Positive</td>
</tr>
<tr>
<td>Skimmer</td>
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</tr>
<tr>
<td>Trap Drive</td>
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<tr>
<td>Capillary Exit</td>
<td>98.5 V</td>
</tr>
<tr>
<td>Skimmer</td>
<td>45.7 V</td>
</tr>
</tbody>
</table>

### 5.3 Results and Discussion
5.3.1 Chromatography

Structurally, CWADPs are quite similar, often only differing by one methyl group. Reversed phase separations are plagued with coelution problems which can be overcome with molecular MS detection. To elute the more hydrophobic CWADPs, high organic levels are used, which are not compatible with analytical scale LC-ICP-MS. The pK_A values of organophosphorus CWADPs are typically 2-2.5. This narrow range complicates separation based on anionic properties alone. The use of a multimodal separation utilizing a column with both hydrophobic interactions with anion exchange (PRP-X100) is an attractive solution. One particular emphasis was to resolve H_2PO_4^- and MPA due to their similar structure and pK_A values.

5.3.1.1 Optimization of Separation 1

Separation 1 was derived from work investigating the separation of arsenic compounds such as As^V and MMA, [34] which are similar in pK_A and structure to H_2PO_4^- and MPA, respectively. Previous separations using ion exchange capabilities of a Dionex AS4A-SC were used to separate H_2PO_4^-, MPA and EPA. [25] Typically separations using an AS7 column are carried out using mM concentrations of HNO_3. [35] HNO_3 is commonly used with ICP-MS at concentrations <2%, therefore mobile phase compatibility using an AS7 column is not an issue. Separation conditions were optimized using the mobile phases listed in Table 5.1 for Separation 1. Initial optimization was carried out using the major degradation products: MPA, EMPA, and IMPA in the presence of H_2PO_4^- and changing the percentage of mobile phase B. The optimum separation was achieved at 20% B, which equates to 0.4 mM CH_3COOH/CH_3COO^-Na^+.
and 5 mM HNO₃ in DDW. At lower %B, EMPA and IMPA approach and merge with MPA as resolution between H₂PO₄⁻ and MPA increases in conjunction with separation time. At higher %B levels, EMPA and IMPA moved closer together and coelute with H₂PO₄⁻ as separation times shortened. High levels (>100 µg mL⁻¹) of H₂PO₄⁻ were resolved from MPA, EMPA, and IMPA and shown in Figure 5.3B. Because H₂PO₄⁻ is the latest eluting peak, even higher levels of H₂PO₄⁻ are unlikely to interfere significantly with the separation, thus making this separation adept to real world samples with high H₂PO₄⁻ content. Optimum separation, achieved in less than 5 min, is shown in Figure 5.3A.

![Figure 5.3: Separation 1 of A) 10 µg mL⁻¹ MPA, EMPA, IMPA, H₂PO₄⁻ at 20% B (optimum separation) and B) 10 µg mL⁻¹ MPA, EMPA, IMPA with >100 µg mL⁻¹ H₂PO₄⁻ at 15% B.](image)

Additional CWADPs were added to the mixture, but coelution plagued the separation as several of the other CWADPs coeluted. Of the CWADPs tested, only IBHMP overlapped with H₂PO₄⁻. Retention times of some CWADPs are plotted on Figure 5.4. It is possible that this method could be used for a quick, preliminary
screening method or in cases which MPA and H$_2$PO$_4^-$ overlap as this separation offers the best resolution between the two. The coelution of multiple CWADPs is most likely due to the overwhelming anionic interactions as compared to hydrophobic interactions as even the most hydrophobic compounds of CMPA were eluted within 6 minutes. Further work regarding this separation is ongoing.

![Figure 5.4: Retention times of various CWADPs using Separation 1 at 20% B. The ••• represents the length of the peak and the number to the left of each segment represents the designated CWADP number.](image)

5.3.1.2 Optimization of Separation 2

Separation 2 explored the implementation of the Thermo Hypercarb stationary phase consisting of porous graphitic carbon. The overall retention on Hypercarb is a combination of two mechanisms: 1) the dispersive interactions between analyte – mobile phase and analyte – graphite surface, by which retention increases as the hydrophobicity of the molecule increases 2) charge induced interactions of a polar analyte with the polarizable surface of graphite.[36] Previous work was done by Mercier et al.[29] using
the PGC stationary phase with ESLD and MS detection for the analysis of CWADPs. However, the gradient separation conditions, in which 99.9% acetonitrile (ACN) was used, are incompatible with ICP-MS. In the study by Mercier et al., the authors were able to demonstrate that retention can be controlled by competition of electronic interactions in addition to hydrophobic interactions.

As the retention mechanism is somewhat similar in nature to reversed phase, the initial goal was to separate H$_2$PO$_4^-$ from MPA, as this is quite difficult to accomplish using RP separations (without ion pairing). The use of 0.1% acetic acid (AA) in DDW was determined to give the best resolution between H$_2$PO$_4^-$ and MPA even at 10X more H$_2$PO$_4^-$ than MPA, as shown in Figure 5.5A. Higher concentrations of AA or similar concentrations of stronger electron interaction competitors (formic acid, tri-fluoroacetic acid, etc.) resulted in shorter elution time and increased peak overlap. Lower concentrations of AA resulted in broader peaks, longer separation time, but increased resolution.

Additional CWADPs were added to the mixture, but required increasing levels of AA and ACN were needed to elute the more strongly retained CWADPs. Increasing both the AA and ACN concentrations had similar effects on reducing retention time as the displacement of 5% AA was comparable to that of 4% ACN. Initial gradient optimization resulted in the separation shown in Figure 5.5B. By using a buffer of 0.1% AA and 2% ACN it was possible to elute IBHMP, DIPMP, and CMPA, which are the three most hydrophobic the CWADPs analyzed in this study; the chromatogram is shown in Figure 5.5C. Therefore, this separation method has potential to separate several CWADPs in the presence of H$_2$PO$_4^-$ all using conditions compatible with ICP-MS and possibly with ESI-
MS. Future method development must consider the limiting factors in the separation which are: 1) keeping organic concentrations as to make the method compatible with ICP-MS while also 2) keeping AA concentrations low enough to provide adequate ionization should the method be used with ESI-MS. Future work is continuing with this separation and its applications.

![Figure 5.5: Separation 2 for separation of A) $\text{H}_2\text{PO}_4^-$ (130 $\mu$g mL$^{-1}$) and MPA (10 $\mu$g mL$^{-1}$), B) CWADPs 2,4, 6-9 using gradient program from Table 5.1, and C) IBHMP, DIPMP, and CMPA.](image)

5.3.1.3 Optimization of Separation 3

The PRP-X100 column has been explored for this application using FPD for detection.[22,37] In these publications, the authors discussed conditions under which MPA could be separated from $\text{H}_2\text{PO}_4^-$, as resolution is needed as FPD will detect all phosphorus analytes. Hooijschuur and coworkers concluded that MPA and $\text{H}_2\text{PO}_4^-$ are separated using 0.5% FA in DDW, but these conditions cannot elute alkyl methylphosphonic acids with a carbon number greater than two from the PRP-X100 column.[37] Therefore a gradient separation is needed, in which Hooijschuur used 70% MeOH, which is incompatible with ICP-MS. Adjustments were then made to make this method compatible with ICP-MS.
A 4.6 x 100mm with 5µm particle size PRP-X100 column was used; however, MPA and H$_2$PO$_4^-$ could not be resolved with only 0.5% FA in DDW. Upon the addition of 5% MeOH to the mobile phase, separation was achieved. Various concentrations of FA were explored and optimized at 0.5% FA as higher concentrations yielded coelution while lower levels increased retention time, but gave better resolution. To then elute other CWADPs, both the NH$_4$HCO$_2$ and MeOH concentrations were increased. As NH$_4$HCO$_2$ increases, retention due to ionic interactions decreases and the hydrophobic interactions seem to be primary.[22] By independently increasing NH$_4$HCO$_2$ and MeOH, retention times were decreased, therefore optimum conditions utilized the increase of both additives throughout the gradient. This causes an increased background level for $^{31}$P. Using a shorter column allowed for the decreased retention time of hydrophobic CWADPs with a maximum 80% B (equating to 0.24 M NH$_4$HCO$_2$ and ~18% MeOH) during the gradient. Further increasing MeOH leads to plasma instability and eventual extinguishing of the plasma. After optimizing of the gradient, 10 CWADPs and H$_2$PO$_4^-$ could be resolved in less than 25 minutes. Figures of merit for Separation 3 are displayed in Table 5.3, and address reproducibility and detection limits (calculated as recommended by IUPAC, 3σ of integrated peak area of 7 blanks divided by the slope of the calibration curve).[38] The chromatogram is shown in Figure 5.6. The more hydrophobic CWADPs: CMPA, DIPMP, and pinacolyl methylphosphonic acid (PMPA), were too hydrophobic to be eluted from the PRP-X100 column using this method.

One concern with Separation 3 is that the relatively high NH$_4$HCO$_2$ and MeOH levels may lead to dirtying of the instrument components and reduce stability/sensitivity. Perhaps since the gradient was only briefly held at 80% B, there were no significant
effects as the analysis was run over 16 hours without any deleterious effects. Long term stability is addressed in the figures of merit section. Longer columns were used with increased resolution, but late eluting compounds (IPHEP, IBMHP) could not be eluted in a reasonable amount of time.

Figure 5.6: LC-ICP-MS chromatogram of CWADPs mixture including H$_2$PO$_4$ in DDW at a concentration of 10 µg mL$^{-1}$ for each analyte using parameters for Separation 3. Dashed line represents the gradient program.

Table 5.3: Figures of merit for Separation 3 using ICP-MS for detection, based on 250 ng mL$^{-1}$ CWADPs mix

<table>
<thead>
<tr>
<th>CWADP</th>
<th>RSD (%)</th>
<th>Retention Time</th>
<th>RSD (%)</th>
<th>Peak Area</th>
<th>Detection Limit ng of standard mL$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>EHDA</td>
<td>0.6</td>
<td>0.6</td>
<td>2.5</td>
<td>21.7</td>
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</tr>
<tr>
<td>MPA</td>
<td>0.3</td>
<td>0.3</td>
<td>4.1</td>
<td>18.3</td>
<td></td>
</tr>
<tr>
<td>EDA</td>
<td>0.4</td>
<td>0.4</td>
<td>8.1</td>
<td>19.9</td>
<td></td>
</tr>
<tr>
<td>IA</td>
<td>0.3</td>
<td>0.3</td>
<td>3.3</td>
<td>23.3</td>
<td></td>
</tr>
<tr>
<td>DMHP</td>
<td>0.3</td>
<td>0.3</td>
<td>2.5</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>PPA</td>
<td>0.4</td>
<td>0.4</td>
<td>7.1</td>
<td>22.9</td>
<td></td>
</tr>
<tr>
<td>EPA</td>
<td>0.6</td>
<td>0.6</td>
<td>3.3</td>
<td>20.4</td>
<td></td>
</tr>
<tr>
<td>IMPA</td>
<td>0.1</td>
<td>0.1</td>
<td>6.6</td>
<td>19.5</td>
<td></td>
</tr>
<tr>
<td>DEHP</td>
<td>0.1</td>
<td>0.1</td>
<td>11.5</td>
<td>26.6</td>
<td></td>
</tr>
<tr>
<td>IPHEP</td>
<td>0.1</td>
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<td>0.3</td>
<td>0.3</td>
<td>16.1</td>
<td>81.1</td>
<td></td>
</tr>
</tbody>
</table>

Detection limits calculated using IUPAC method
5.3.1.4 Optimization of Separation 4

This method was adopted from previous arsenic speciation methods which have been used to separate arsenic compounds such as As\(^V\) and MMA\(_4\)[39] which are similar in pK\(_A\) and structure to H\(_2\)PO\(_4\)\(^-\) and MPA, respectively. An ammonium carbonate buffer system was used, while attempting to keep buffer concentration low for compatibility with ESI-MS. At the high pH of a carbonate buffer system (pH = 8.5), all the analytes were negatively charged. The separation mechanism is not exactly known as the retention order of several of the analytes is not based solely on anionic or hydrophobic interactions. At a buffer level of 10% B (~12mM (NH\(_4\))\(_2\)CO\(_3\)) optimum resolution between MPA and H\(_2\)PO\(_4\)\(^-\) was achieved. The gradient was changed to increase (NH\(_4\))\(_2\)CO\(_3\) levels and elute the remaining CWADPs. Addition of MeOH up to 20% of the mobile phase had no significant impact; possibly due to the more prevalent anionic interaction. Using 100% B resulted in the elution of DEHP, but almost 25 minutes later than IMPA, so it was not shown in this method. Additionally, there are several possible CWADPs that will not elute off the column under the given conditions (IPHEP, IBHPA, DIMP, CMPA, & PMPA). Once optimized the method was able to separate 7 CWADPs and H\(_2\)PO\(_4\)\(^-\) in under 25 minutes. Figures of merit for Separation 4 are displayed in Table 5.4, and address reproducibility and detection limits (calculated as recommended by IUPAC, 3\(\sigma\) of integrated peak area of 7 blanks divided by the slope of the calibration curve).[38] The optimized Separation 4 of 6 CWADPs in the presence of H\(_2\)PO\(_4\)\(^-\) with ICP-MS for detection is shown in Figure 5.7. EPA elutes with H\(_2\)PO\(_4\)\(^-\) under these conditions, and cannot be completely resolved from MPA.
Figure 5.7: Separation 4 of CWADPs 1A, 2, 4-7 with the gradient program shown above as the dashed line. EPA (CWADP #3) was not injected in this example.

Table 5.3: Figures of merit for Separation 4 using ICP-MS for detection, based on 250 ng mL\(^{-1}\) CWADPs mix

<table>
<thead>
<tr>
<th>CWADP</th>
<th>RSD (%) Retention Time</th>
<th>RSD (%) Peak Area</th>
<th>Detection Limit ng of standard mL(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMHP</td>
<td>0.3</td>
<td>4.6</td>
<td>11.3</td>
</tr>
<tr>
<td>EMPA</td>
<td>0.4</td>
<td>8.5</td>
<td>17.9</td>
</tr>
<tr>
<td>MPA</td>
<td>0.4</td>
<td>12.4</td>
<td>12.4</td>
</tr>
<tr>
<td>EPA</td>
<td>0.4</td>
<td>2.7</td>
<td>12.4</td>
</tr>
<tr>
<td>1A</td>
<td>0.3</td>
<td>1.8</td>
<td>27.2</td>
</tr>
<tr>
<td>IMPA</td>
<td>0.3</td>
<td>14.3</td>
<td>44.6</td>
</tr>
<tr>
<td>EHDAP</td>
<td>0.6</td>
<td>2.6</td>
<td>88.1</td>
</tr>
</tbody>
</table>

Detection limits calculated using IUPAC method

Due to the low (NH\(_4\))\(_2\)CO\(_3\) levels, this method was easily adapted to ESI-MS. This is the main motivation behind the difference between column dimensions; for Separation 3, the 100mm x 4.6 mm column was used with a 1.0 mL min\(^{-1}\) flow rate and for Separation 4, the smaller inner diameter (ID) column, utilized lower flow rates (0.5 mL min\(^{-1}\)). The lower flow is advantageous to ESI-MS as it is concentration dependent.
Additionally, the 2.1 mm ID column provided better resolution than the 4.6 mm ID column, but high levels of H$_2$PO$_4^-$ were detrimental to the separation (discussed further below) when using the 2.1 mm ID column, similar H$_2$PO$_4^-$ levels could be tolerated using the 4.6 mm ID column. Using the above conditions, the same separation could be accomplished using LC-ICP-MS and LC-ESI-MS (Figure 5.8). The addition of ESI-MS provides further confirmation of identity based on both retention time and molecular mass information. In the case of MPA and EPA, which are not baseline resolved, ESI-MS can resolve the two using molecular information, as shown in Figure 5.8C. Retention times of LC-ICP-MS and LC-ESI-MS are not identical as two separate HPLC systems were used, and there is most likely a difference in the pump performance in regard to gradient formation (Figure 5.8A,B). The majority of these CWADPs are well characterized by ESI-MS so this data is not discussed.

![Figure 5.8: A) LC-ICP-MS chromatogram and B) reconstructed ion chromatogram of LC-ESI-MS for Separation 4 or CWADPs 1A and 2-7 in DDW at 1 µg mL$^{-1}$. CWADP 1A was injected in both cases, but not detected in ESI-MS chromatogram. C) represents the mass spectrum as Dashed line represents the gradient program.](image)
5.3.2 Analysis of EHDAP

During the analysis, it was noticed that the analysis of EHDAP as a fresh solution, its corresponding peak eluted at ~5 min in Separation 3 and at 28 min in Separation 4. During the course of the research, the standard seemed to degrade to a secondary form (most likely via hydrolysis as it was stored in DDW). Further analysis revealed the movement of the EHDAP peak to ~11 min in Separation 3 and ~15 min in Separation 4. Attempts are currently being made to identify the possible degradant form of EHDAP. The unknown is designated CWADP #1A. Even though its structure has not yet been confirmed, 1A was injected and analyzed using both Separation 3 & 4 as it remained stable for over 2 months in its secondary form. If 1A is in fact a reproducible degradation product and then its is reason is could be found in real samples as a CWADPs, then 1A becomes the 11th CWADP to be analyzed in this study, thus bringing the total number of standards separated in Separation 3 to 11 and Separation 4 can separate 8 CWADPs.

5.3.3 Sample Applications

Once each method was optimized, applications were performed on real-world samples. The majority of the literature focuses on tap water, ground water, and soil extracts.[16,18,24,26] This is the first work to examine more complicated samples. Several different beverages were analyzed including tap water, bottled water, grape juice, apple juice, soda, and sports drink. Tap water and bottled water samples were directly spiked with a CWADPs mixture and the resulting chromatograms for Separation 3 and Separation 4 were very similar to Figure 5.6 and Figure 5.7, respectively, with the
difference being that a small amount of $\text{H}_2\text{PO}_4^-$ was in the tap water sample, but not enough to mask EPA. Samples of more complicated matrices and increasing $\text{H}_2\text{PO}_4^-$ levels provide a case for a 1:10 or 1:20 dilution with DDW (to keep other materials from dirtying the instrument) and then samples were fortified with mixtures of CWADPs. All samples were filtered through a 0.22 µm syringe filter prior to injection. Results were similar for grape juice, apple juice, soda, and sports drink (sports drink shown in Figure 5.9). For Separation 3, both water and beverage samples without CWADPs, exhibit a ‘peak’ at ~15 min. This peak appears in all samples including blank DDW injections and gradient blanks with no injection. Most likely this is due to a $^{31}\text{P}$ background increase from the mixing of the mobile phases and consequential formation of polyatomic interferences during the gradient elution; this is reproducible in retention time and peak area. This ‘peak’ elutes at the same time, ~17 min, and considered background, thus being subtracted out.

![Figure 5.9: LC-ICP-MS chromatogram of sports drink diluted with DDW 1:20 unspiked (A) vs. spiked (B) with CWADPs 1A and 2-10 at a concentration of 1 µg mL$^{-1}$)](image)

Figure 5.9: LC-ICP-MS chromatogram of sports drink diluted with DDW 1:20 unspiked (A) vs. spiked (B) with CWADPs 1A and 2-10 at a concentration of 1 µg mL$^{-1}$)
In Separation 3, beverages that had a high level of H$_2$PO$_4^-$ encountered difficulties as the EPA peak is overtaken by the H$_2$PO$_4^-$ peak. Analysis of solid foods was then attempted. Because the analytes are soluble in DDW, it seemed to be the easiest and most efficient mode of extraction. DDW was added to lettuce leaves, vortexed, and the supernatant was spiked with CWADPs mix. Here, the content of H$_2$PO$_4^-$ in the lettuce extract is higher than those of the beverages and the chromatography is affected as peaks 1A, 4, and 5 are coeluting, as shown in Figure 5.10. This led to the exploration of the effect of H$_2$PO$_4^-$ on the chromatography.

Increasing H$_2$PO$_4^-$ levels were added to determine the tolerance of H$_2$PO$_4^-$ before separation was affected (beyond the unavoidable EPA overlap). Figure 5.11 shows the effect of increasing H$_2$PO$_4^-$ concentrations on the chromatography. As alluded to earlier, at levels of 100 µg H$_2$PO$_4^-$ mL$^{-1}$, the peak relating to EPA is masked by the H$_2$PO$_4^-$. At high levels (10,000 µg H$_2$PO$_4^-$ mL$^{-1}$), there is a high effect and even though H$_2$PO$_4^-$ would not overlap the 1A and others, it does shift the peaks (refer to lettuce extract). This problem could be somewhat alleviated by using a slower gradient around the elution.
time of 1A and DMHP as it would provide more resolution between these peaks and 
H$_2$PO$_4^-$.

![Figure 5.11: Effect of increasing H$_2$PO$_4^-$ levels on the chromatography of Separation 3. Significant deleterious effects are shown >1,000 µg H$_2$PO$_4^-$ mL$^{-1}$](image)

Separation 4 was applied to similar samples including beverages and lettuce extracts. However, tolerance of Separation 4 for H$_2$PO$_4^-$ is less when compared to Separation 3. Levels up to 1,000 µg H$_2$PO$_4^-$ mL$^{-1}$ can be tolerated by Separation 3, but 100 µg H$_2$PO$_4^-$ mL$^{-1}$ causes problems with the Separation 4 when using ICP-MS for detection due to excessive overlap as EPA & MPA are masked. This is most likely due to the column dimensions as the loading capacity of the 2.1 mm ID column is less than the 4.6 mm ID column. When using ESI-MS for detection, this problem is partially alleviated as H$_2$PO$_4^-$ is virtually invisible, thus leading to the visibility of MPA and EPA not possible by ICP-MS; however, the signal is somewhat suppressed compared to samples of less H$_2$PO$_4^-$ (Figure 5.8B); Figure 5.12 shows sports drink analysis by HPLC-ESI-MS.
Figure 5.12: Separation 4 applied to A) LC-ICP-MS of sports drink fortified with 1A, 2-7 at 1 µg mL⁻¹ B) LC-ESI-MS reconstructed ion chromatogram of sports drink fortified with 1A, 2-7 at 1 µg mL⁻¹ C) LC-ESI-MS reconstructed ion chromatogram of sports drink unfortified. H₂PO₄⁻ interferes drastically in LC-ICP-MS masking EPA and 1A. Suppression of MPA, EPA, 1A occurs in ESI-MS fortified sports drink - most likely due to the higher matrix/H₂PO₄⁻ contained in sport drink.

After successful applications of Separations 3 & 4 to beverages and lettuce extract, more complicated samples were attempted. Samples such as rice, potatoes, and milk were analyzed (data not shown), but H₂PO₄⁻ levels were extremely high (>1,000 µg mL⁻¹) and chromatography was distorted. Analysis was attempted for a sample of infant formula. The infant formula was spiked with the CWADPs mix and then extracted with DDW. The sample was centrifuged at 5000 rpm and the supernatant taken and filtered. Then it was injected and compared to an unfortified blank. The results are shown in Figure 5.13. The infant formula has a high amount of interferences (in addition to H₂PO₄⁻) and only 6 of the standards could be identified due to the high background of the blank infant formula. This application obviously leaves room for improvement as future work focuses on sample preparation with possibilities of solid phase extraction or other pretreatments to hopefully specifically extract CWADPs while leaving the sample matrix behind.
5.4 Conclusions

Four separation methods for the determination of CWADPs using LC-ICP-MS were developed. Separation 1 was the most efficient separation in terms of resolving MPA and H$_2$PO$_4$\(^{-}\), but peak overlap renders Separation 1 as only a screening method. Separation 2 has the potential to separate 8 CWADPs in the presence of H$_2$PO$_4$\(^{-}\), including the more hydrophobic ones that never elute under conditions for Separation 3 & 4, all using conditions possibly suitable for ESI-MS. Current work is continuing with both Separation 1 & 2. Separation 3 is able to separate 10 CWADPs in the presence of H$_2$PO$_4$\(^{-}\). Separation 3 exhibited the best tolerance for H$_2$PO$_4$\(^{-}\) and is applicable to several matrices. Separation 4 is able to separate 7 CWADPs in the presence of H$_2$PO$_4$\(^{-}\) and is compatible with ESI-MS, thus helping to overcome its short comings of less H$_2$PO$_4$\(^{-}\) tolerance when compared to Separation 3. Detection limits were lower than or
comparable to the majority of currently published LC methods and easily meet the required limits of detection of 1 mg mL\(^{-1}\) for the OPCW tests.[22] Preconcentration of CWADPs has been reported with a decrease in detection limits by a factor of 100.[28] This research represents the first application to problematic samples including beverages and lettuce extracts. These methods can be used in cases of food tampering/contamination involving CWAs.

5.5 Acknowledgements

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5.6 References


6.1 Conclusions and Future Directions

By implementing combinations of chromatographic techniques and ICP-MS complemented by molecular MS for speciation analysis, several important research areas were explored. By utilizing chromatographic techniques with appropriate sample preparation techniques, complicated matrices could be unraveled to enable the analysis of the given analytes. The high sensitivity and elemental specificity of ICP-MS were integral for the analysis of arsenic, selenium, and phosphorus. When ICP-MS information was insufficient to identify unknown compounds, molecular MS was utilized to ensure confident structural confirmation. The collective use of the aforementioned multidimensional analytical techniques provided an excellent approach for the elemental and molecular examination of a variety of nutritional and environmental issues. Significant new information was presented so that a more complete understanding of these systems can be achieved.

It was found that total arsenic levels must be complemented with speciation analysis to better understand the risk involved when the consumption of wheat, potatoes, yams, and rice is involved. In this particular instance the low levels of detection for the individual arsenic species could not have been reached without the use of ICP-MS. Because of the low levels of arsenic in wheat, potatoes, and yams lead to even lower levels of individual arsenic species, this work showed confident results that the arsenic is in fact inorganic in nature, and nutritionist no longer have to assume this fact as it will have been proven. In addition, this work suggests that the unhealthy implications of US
rice proposed by Williams et al. are clearer, in that inorganic arsenic levels rarely reach levels of 160 ng As g\(^{-1}\) (0.16 part per million). However the significant portion of the arsenic in high level arsenic rice samples was determined to be DMA, but toxicity studies are still in progress, and if found to be negative, these unhealthy implications could resurface.

Extraction techniques to study the possible association of selenium in proteins in selenized *Lentinula edodes* were evaluated using SEC-ICP-MS. Enzymatic digestion proved to be very effective in reducing higher molecular weight selenium fractions into more manageable as a portion of these fragments were able to be identified using HP-IPC-ICP-MS. The presence of MeSeCys, based on its chemopreventive properties, may in the future, incur health benefits of consuming selenized mushroom. The identification of the unknown selenium species resulting from protein degradation via enzymes using molecular MS could shed more light and possibly help to confirm identities of seleno proteins and/or selenium containing proteins.

Using GC with ICP-MS and molecular MS enabled studying volatiles for further understanding of the metabolic pathway of Se in genetically modified *Brassica juncea* plants as related to phytoremediation. Markers for evaluating/quantifying genetic modification were evaluated including total selenium content, MeSeCys content, and ratio of volatile DMeSe/DMeDSe, in which the latter proved to be the most sensitive to change. In addition three novel selenium volatiles were confirmed and represent the first report of these compounds in nature.

Four new methods using LC with ICP-MS to screen for the presence of chemical warfare agent degradation products in the presence of phosphate were explored. Two of
the methods (*Separation 1 & 2*) are to be further examined as they show great potential. Two additional chromatographic approaches (*Separation 3 & 4*) were able to separate > 7 CWADPs in the presence of phosphate and were applied to several food samples. *Separation 4* was compatible with ESI-MS and structural confirmation was obtained. These developed methods now can be utilized in cases involving suspected food contamination using chemical warfare agents. Further work to remove phosphate and clean up samples by removing interferences in increasingly complicated matrices (infant formula, milk, etc.) are ongoing. It may also be useful to find additional separation methods capable of resolving the more hydrophobic CWADPs unable to be analyzed by *Separation 3 & 4*.

In summary, ICP-MS in conjunction with the chromatographic techniques LC and GC offer sensitive, selective means of qualitative and quantitative speciation analysis of selenium, arsenic, and phosphorus related to variety of food related and environmental samples. The use of molecular MS techniques only further improves the methodology making the combination these analytical techniques a highly useful research approach.