DETERMINATION OF TOTAL SELENIUM AND SELENO-AMINO ACIDS IN YEAST AND AQUATIC ORGANISMS BY LIQUID CHROMATOGRAPHY AND INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY

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A Dissertation
Presented to
the Faculty of the Graduate School
University of Missouri-Columbia

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In Partial Fulfillment
of the Requirements for the Degree

Doctor of Philosophy

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by

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DECEMBER 2007

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ACKNOWLEDGMENTS

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ABSTRACT

Selenium can be toxic to aquatic life at very low concentrations, even though it is an essential element to many organisms. Selenium-laden agricultural drainwater in the Imperial Valley of California may be affecting the survival of endangered desert pupfish, which inhabit the waters of that region. It is becoming increasingly important to ensure an appropriate balance between selenium deficiency and toxicity when developing new water quality criteria for the protection of aquatic life. Because the bioavailability and toxicity of selenium are dependant on its chemical forms, the primary goal of this dissertation research was to develop methods for routine speciation analysis of selenium in tissues, which has been identified as a critical data need in the derivation and interpretation of selenium residues in fish and waterfowl.

Initial experiments were conducted to optimize the selenium determination for dynamic reaction cell (DRC) ICP-MS using methane as the reaction gas. In the DRC mode, the Ar-Ar background signals could be reduced greatly but at the price of a large loss of net selenium signal intensities. Overall, the results were less satisfactory than expected and the added complexity for the DRC mode seemed unwarranted. Therefore, a method for total selenium using on-line stable isotope dilution analysis with conventional

ICP-MS (SIDA-ICP-MS) was developed. Masses at 77, 78, 79, 81, and 82 were monitored and quantitation of Se was determined based on both ^{78}Se and ^{82}Se. SIDA-ICP-MS was successfully applied to the determination of total selenium in biological materials, such as selenized yeasts, certified reference materials, lab-cultured oligochaetes and desert pupfish.

Although cation-exchange chromatography separation proved to be feasible, separation and quantitation of seleno-amino acids was best accomplished by ion-pairing reversed-phase liquid chromatography (RPLC). For either separation method, ICP-MS detection at mass 82 using the standard mode produced good results. The efficiencies of different extraction methods were evaluated and the chromatographic conditions were investigated using selenomethionine–dosed samples and a reference yeast certified for selenomethionine content. It was found that methanesulfonic acidic hydrolysis demonstrated a higher extraction efficiency of selenomethionine than enzymatic digestion. Selenomethionine (SeMet) was the only significant Se-containing species detected in the biological samples that were examined.

Chapter 1: Selenium

1.1 Introduction to selenium

Selenium was first discovered as an element in the early 19th century. An outstanding and experienced Swedish chemist Jöns Jakob Berzelius identified selenium in 1817[1]. A Swedish manufacturer of sulfuric acid asked Berzelius to examine a reddish deposit, which remained in the lead chambers, after burning copper pyrites. As the investigation went on, Berzelius found this unknown substance with properties very much like those of tellurium. He named selenium after *selene*, which signifies the Greek Goddess of the moon, while *tellus* is the name of our own planet[2]. While Berzelius was the first to isolate and characterize selenium, its toxicity was observed much earlier. In the 13th century, an Italian scholar, Marco Polo, found problems with the hooves of horses after grazing on certain plants in regions of China. The plant mentioned by Polo must have been a selenium "accumulator", which is able to take up selenium from the soil and concentrate it to toxic levels[3]. Damaged hooves are typical symptoms of selenium poisoning in animals.

1.1.1 Background of this research

Selenium can be toxic to aquatic life at relatively low concentrations. The U.S. Environmental Protection Agency (EPA) suggests that if selenium is as toxic to saltwater fish in the field as it is to freshwater fish in the field, then the status of the fish community should be monitored whenever the concentration of selenium exceeds 5.0 μg/L in salt water[4]. In the U.S., scientists, natural resource managers and water quality

regulators are focusing considerable attention on the issue of selenium contamination in aquatic ecosystems.

Selenium as an environmental contaminant can be released from natural weathering, leaching of selenium containing rocks and soils, irrigation, and mobilization from mining and coal combustion. It has been reported to be problematic in several major reservoirs, resulting in some adverse ecological effects including reproductive and developmental impairment of aquatic birds and fish and, in some cases, even the elimination of entire communities of fish[5–10]. Hamilton[11] proposed that the historical declines of now endangered fish species may be related to selenium contamination. On the other hand, selenium is also an essential element to many organisms[12–14]. Therefore, it is important to ensure appropriately-balanced consideration of selenium sufficiency and toxicity when developing new water quality criteria for the protection of aquatic life from selenium.

The development of scientific methods for the determination of selenium in aquatic organisms is part of a larger study conducted by the Columbia Environmental Research Center (CERC), U.S. Geological Survey (USGS). The study focuses on the effects of selenium on a federally-listed endangered species, the desert pupfish (*Cyprinodon macularius*). Desert pupfish inhabit agricultural drains of the Imperial Valley of southern California and shoreline pools adjacent to the Salton Sea. Irrigation in the Imperial Valley began in 1901 and the Salton Sea took its present shape after an accidental diversion of the Colorado River in 1905. At Kesterson National Wildlife Refuge in California, studies led by Ohlendorf found that elevated selenium levels in irrigation sub-surface drain water caused adult mortality, reproductive failure, embryonic

mortality and developmental abnormalities in many aquatic bird species nesting in the refuge[15–18]. Selenium concentration in some prey items could reach as high as 175 µg/g dry weight. In 1985, the Department of Interior (DOI) launched the National Irrigation Water Quality Program, to study the irrigation-induced water quality problems in western states. Recent evidence suggests a relationship between the specific forms of Se in fish and ovarian lesions and embryo deformities.

The U.S. EPA is currently evaluating the existing data regarding the ecological effects of selenium on aquatic life as part of the process of establishing new water quality levels for the element, which may include site specific criteria that depend on water quality characteristics. Studies about the Salton Sea area began in 1986. Selenium-laden agricultural drainwater in the Imperial Valley of California may be affecting the survival of endangered desert pupfish, which inhabit the waters of that region[19, 20]. Selenium in the drainwater exists primarily as selenite and selenate ions. USGS measured total dissolved Se concentrations that ranged from 0.89 to 21.5 µg/L in filtered irrigation drain water from the Salton Sea (T. May, personal communication, March, 2007). The waterborne inorganic selenium is rapidly converted to organo-selenium forms (such as selenomethionine) via bacteria, microphytes, plankton and aquatic plants. Se can bioaccumulate into the top predators including predatory fish and waterfowl. The biomagnifications and further chemical transformation can occur as selenium is passed up the food chain. Tests performed in the 1990's indicated that the seleno-amino acid, selenomethionine was far more toxic to aquatic organisms than are inorganic selenate and/or selenite forms[21]. Fan, et al.[20] found that the embryos of deformed stilt (*Himantopus mexicanus*) were more abundant in proteinaceous selenomethionine than

were normal embryos. These findings are consistent with the hypothesis that the proteinaceous selenomethionine underlies selenium ecotoxicity and/or toxicity, which might be a useful indicator of selenium risk in aquatic ecosystems. However, virtually all of the quotable data in tissue is total selenium and the current protective criteria are based upon total Se concentrations. Consequently, a method for routine speciation analysis of selenium in tissues has been identified as a critical data need in the derivation and interpretation of selenium residues in fish and waterfowl[22].

1.2 Chemistry and biochemistry of selenium

1.2.1 Chemistry of selenium

Selenium has an atomic weight of 78.96 and its atomic number is 34. It belongs to Group 16/VIA with oxygen, sulfur, tellurium and polonium, and its neighbors are arsenic and bromine. More than 30 years ago, Frost predicted that selenium will challenge the advances in biochemistry and nutrition[23]. Today, the increasing interest in selenium research has confirmed his comments.

Selenium exists in four oxidation states: 0 (elemental selenium), 2- (e.g., Na_2Se, sodium selenide; ($NH_2CH(COOH)CH_2CH_2SeCH_3$) selenomethionine, 4+ (e.g., Na_2SeO_3, sodium selenite; H_2SeO_3, selenious acid) and 6+ (e.g., Na_2SeO_4, sodium selenate; H_2SeO_4, selenic acid). Elemental Se is very stable and highly insoluble. In soils, selenates are favored under alkaline conditions, which is of great importance because of its availability to plants. Selenium in the 4+ oxidation state (selenite) binds tightly to clays and iron hydroxides, which accounts for its insolubility in some types of soils and its low concentrations in natural waters[24].

1.2.2 Biochemistry of selenium

In contrast to the inorganic forms of selenium, the organo-selenium compounds are of considerable interest and several of them play essential roles in cell biochemistry and nutritional science. In feed ingredients, such as forage, grains, and oilseed meals, selenium is incorporated into the amino acids and proteins as selenomethionine and selenocysteine. Compared with their sulfur analogues, organo-selenium compounds are more easily oxidized, less stable when exposed to light, air and heat, and they also have unpleasant odors. These properties can be attributed to the increase in the atomic number and the resulting decrease in bond stability. Many organo-selenium compounds have been isolated from various materials. Some organo-selenium compounds have been synthesized and have found their way into industry and the medicinal sciences[25].

1.3 Selenium in the environment

The illustration of where selenium comes from and how it passes up into the human food supply will help us better understand the multidisciplinary nature and the importance of selenium research. The primary source of selenium we consume is through the food and drink we take up from the environment. Therefore, the concentrations and forms of selenium are controlled at the very beginning of the food chain process. Whether our dietary selenium intake is inadequate or just enough for our health benefit, or in excess, depends fundamentally on different geological conditions. Selenium is present in soils where plants and crops grow. Animals eat these plants and therefore, humans consuming the crops or animal-derived foods are exposed to selenium.

Additional exposure to selenium can occur when we drink water. The concentration of selenium in water and soils varies markedly around the world.

1.3.1 Selenium in waters

In general, selenium concentrations in potable fresh water are less than 1 μg/L. Currently, the World Health Organization (WHO), set the maximum admissible limit of 10 μg/L for selenium in drinking water[26]. The levels of selenium in seawater are often lower. Surveys showed that less than 0.5% of the public water supply in the USA exceeded the WHO standard; which is the same as the US Public Health Services limit[27]. An average Se concentration of 0.06 μg/L was reported for tap water in Stockholm, Sweden[28]. In Germany, a range of 1.6 to 5.3 μg/L for both tap and spring waters was found[29]. According to the WHO and the US National Academy of Sciences, drinking water contributes little to overall selenium intake and waters are rarely a significant source of selenium[30,31]. Usually, the Se level in drinking water remains well below the limit set by WHO. There were occasional/isolated instances where considerably higher selenium concentrations were found in drinking water. In an area of South Dakota, a value of 1.6 mg/L was found in spring water[32]. In one district in China, up to 12.27 μg/L of selenium was found in domestic supplies. In another place where human selenium deficiency syndrome (Keshan disease) occurred, the selenium level in the local drinking water supply was 1.72 μg/L, and even lower in well water, at 0.37 μg/L[33].

Generally, groundwater contains higher selenium concentrations than surface water mainly due to the longer times for rock-water interactions[34]. The total selenium in groundwater ranges from 0.1 to 400 μg/L, depending on geochemical factors, it may even

rise to 6 mg/L[35]. It has been reported that groundwater contains 1,000 µg/L Se in Montana and in the Enshi District, Hubei province, China, Se levels are as high as 275 µg/L[36,37].

In natural water, the predominant species of selenium are the oxyanions, selenite (Se^{4+}) and selenate (Se^{6+}). However, evidence has shown that dissolved organo-selenium forms are present in some aquatic ecosystems[38]. Dissolved selenium oxyanions are primarily absorbed by bacteria and microphytes and biotransformed into organo-selenium forms and elemental selenium. Furthermore, selenium can be transferred through different aquatic consumers, such as insect larvae, zooplankton and larval fish, into the top predators including predatory fish and waterfowl. Selenium biomagnifications and further chemical transformation can occur as selenium is passed up through the food chain. Selenium from industrial and agricultural activities may be discharged into the aquatic environment and possibly increase Se concentrations. Sewage sludge, gold mining, petroleum refineries and agricultural drain water are the main sources of selenium contamination to the water system[39].

1.3.2 Selenium in soils

Selenium concentration in soils is closely related to its geological parent materials. Selenium is originally produced from volcanic activity and is found naturally in igneous (volcanic) rocks, granite, sandstone, limestone, coal and some crude oil deposits[40]. Through the weathering of Se-rich rocks, water and wind erosion, and sedimentation processes selenium particles are deposited into top soil. Selenite and selenate are produced by fungi and bacteria from less soluble forms of selenium[41].

Selenium bioavailability, however, is determined by many factors including soil pH, the redox potential, soil texture, organic-matter contents, the presence of competitive ions, artificial fertilization, and the rate of rainfall[42]. The mobility and uptake of selenium into plants and land animals relies more on its form than on its total concentration.

In the acidic soils, selenium can form insoluble complexes with iron hydroxide and become less available. Selenate is the predominant form in alkaline soils, which makes selenium more available to plants. For instance, at pH 6, only 47% selenium was transferred from soil to ryegrass leaves. Increasing the pH to 7 improved selenium assimilation up to 70%[43]. Fertilizers containing sulfate can compete with selenate and lead to decreased selenium uptake by plants[44]. Rainy regions tend to have lower selenium content because selenium can be leached/flushed from the top soil.

The selenium content in soils ranges from 0.01 to 2 ppm while the mean value is 0.4 ppm worldwide. Soils rich in selenium are referred to as being "seleniferous". In China, soils developed under tropic and sub-tropic conditions (yellow oil and red oil) can accumulate more than 0.3 ppm selenium. In contrast, soils like brown earth and dark brown soil developed under humid/sub-humid conditions, have lower selenium concentrations present[45]. In the western states of the United States, especially the Great Plains, some soils can have high selenium levels of up to 1,000 ppm. Very high concentrations ($\geq$ 1,200 ppm) have been reported in some seleniferous areas[36, 46, 47].

1.3.3 Selenium in plants

Some plants, such as algae, appear to require selenium as an essential nutrient for their metabolism. However, selenium has not been scientifically demonstrated to be essential for the growth of many higher order plants, through which humans obtain their nutritional needs[48]. Plants differ greatly in their ability to accumulate selenium from soils and therefore could be divided into three major groups: (1) selenium accumulators, plants that are able to accumulate extraordinary high levels of selenium in stems and leaves when grown in selenium-rich soil[44]; (2) secondary selenium accumulators, they can accumulate high selenium contents from soil that contains medium or low of the element[44]; (3) non-accumulators, this category includes most forage, cereal grains, and oilmeal crop plants. These crop plants normally contain between 0.01 and 1.0 mg/kg dry weight of selenium[49].

The selenium content in plants varies greatly with Se availability in soils. In China, foods with lower selenium content are found in some low-Se areas, such as Sichuan, northern Shanxi, and Heilongjiang Provinces. However, in a few seleniferous regions of the Enshi district in Hubei Province, maize flour contained 7.5 μg/g, rice 2.5 μg/g, and leafy vegetables up to 7.6 μg/g of selenium[33]. In North and South Dakota, Nebraska, Kansas and Colorado, wheat, barley, corn, oats, rye and cereals from selenium-rich soil accumulated up to 30 μg/g selenium compared to less than 0.1μg/g in other parts of the country[32, 50].

After absorption, the distribution of selenium in various tissues mainly depends on the species, the stage of growth, the chemical form of selenium absorbed, and physiological conditions. In Se-accumulating plants, the element is not incorporated into

proteins, while for non-Se-accumulating plants, protein-bound selenium is the dominant form[51]. Both selenates (Se^{+6}) and selenites (Se^{+4}) are the major chemical forms that plants absorb from the soil and are converted to selenoamino acids (Figure 1.1) such as selenomethionine (SeMet), selenocysteine (SeCys), and Se-methyl-selenomethionine (MeSeMet)[52]. For example, in wheat, corn, soybeans and rice, SeMet makes up 50.4-81.4%, 45.5-82.0%, 62.9-71.8% and 54.9-86.5% of the total selenium, respectively[53]. When selenium concentrations in plant tissues are between 2 μg/g in non-Se-accumulating plants, such as rice[54], and several thousand μg/g in Se-accumulating plants like *Astragalus bisulcatus*[55], they begin to have toxicity symptoms, such as stunting, chlorosis, and withering of leaves. The mechanism of selenium toxicity is mainly attributed to the substitution of selenoamino acids, such as selenomethionine and selenocysteine for methionine and cysteine. These alterations/replacements can lead to lower catalytic activity for some proteins because of the differences between the sulfur and selenium in size and ionization properties[51]. In addition, interference with chlorophyll synthesis[56] and nitrate assimilation[57] can also induce selenium toxicity in plants. Interestingly and fortunately, plants can protect themselves against selenium toxicity by converting selenium into volatile compounds such as dimethylselenide and dimethyldiselenide that are released into the air. This protective mechanism shows great significance in phytoremedation of contaminated soil[58].

Selenomethionine (SeMet)

Selenocysteine (SeCys)

Se-methyl-selenomethionine (MeSeMet)
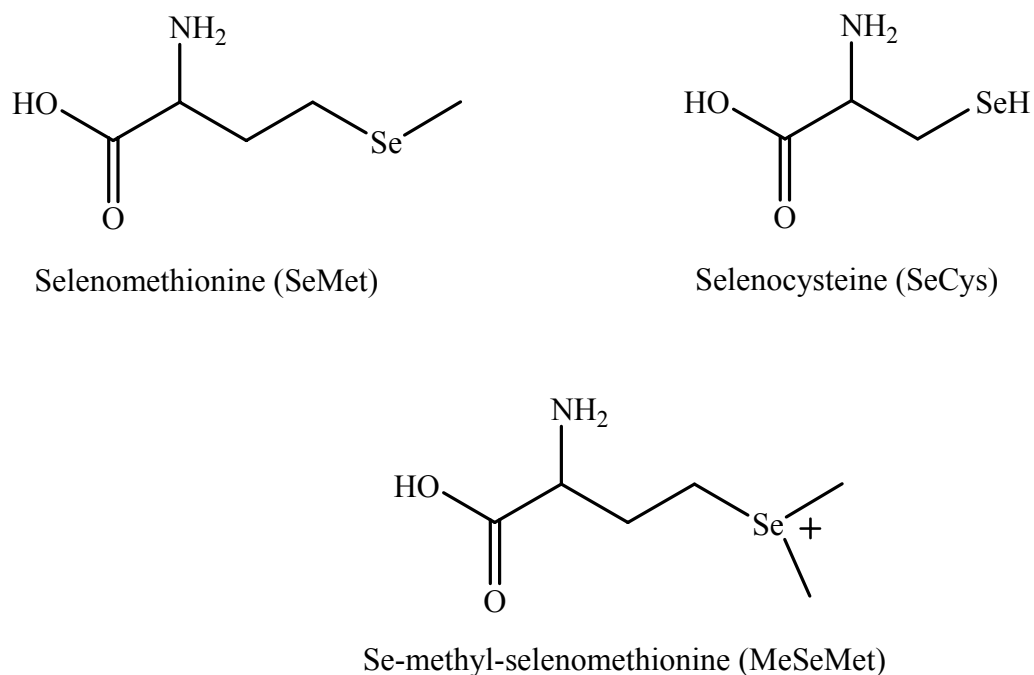
Figure 1.1: Structural formulae for SeMet, SeCys, and MeSeMet

1.3.4 Selenium in animals

Food animals raised on low-selenium feedstuffs accumulate relatively low levels of the element in their tissues and in edible products, such as milk and eggs. Animals fed on relatively high-selenium containing feed yield food products with much higher selenium contents. Apparently, the selenium concentration in animal tissues gives a good indication of the foods they consume. The condition of chronic selenium toxicity is known as selenosis, which is problematic for agriculture. As mentioned earlier, the first

account of selenium toxicity or selenosis in animals was observed by Marco Polo in China in the 13th century. There are early reports of similar selenosis occurrences in farm animals in Spain, Ireland, Germany, Mexico, Canada, Venezuela, and other countries, where seleniferous soils are found[59,60]. The animals exhibited symptoms such as damaged hooves, blindness, vomiting, spasms, paralysis, reluctance to move, suffered abdominal pain, labored respiration and death[61]. These symptoms were referred to as alkali diseases because the herds often grazed on plants growing in alkaline-rich soils. It was not until the 1930's that the role of selenium began to be understood and finally connected to these unfortunate accidents[62]. Grazing on selenium-enriched plants or contaminated hay is believed to be the cause of selenosis. Even today, the most effective way to protect the livestock from selenosis is to transfer them from the "bad lands" to lands with lower Se concentrations in the soil.

For many years selenium was known as a toxin, which could poison livestock and cause serious economic threats to farmers. The benefits of selenium as an essential trace element were not recognized until 1957. Schwarz et al. discovered selenium as a key component in preventing liver necrosis in rats[63]. Se plays a necessary role in growth and fertility. Some diseases related to selenium-deficiency are (1) exudative diathesis, a typical disease of selenium-deficient chicks[64]; (2) white muscle disease, a degenerative disease of the striated muscles, which lightens the color of muscle[61]; (3) pancreatic degeneration, a condition that occurs in severely selenium-depleted poultry[61,65]; (4) impaired reproduction, a decreased hatchability and increased embryonic mortality[61,66]; (5) impaired immune response, less ability to protect against attacks by pathogens and malignancy[67,68,69]. In order to prevent selenium deficiency syndromes and, accordingly,

the considerable losses to beef, dairy and sheep producers, either sodium selenite or sodium selenate can be added as a feed supplement in commercial animal agriculture in many parts of the world. In 1997, Food and Drug Administration (FDA) ultimately approved the maximum selenium supplement levels to be 0.3 ppm for cattle, chickens, turkeys, swine, ducks, and sheep[70].

The concentration of selenium, based on fresh weight, in a number of food sources followed the trend: sea foods > commercial beef > pork > ground beef > chicken. The selenium concentration in all foods increased significantly after cooking, air or freeze-drying[71]. In general, meat cuts containing muscle from most species contain Se concentrations of 0.3-0.4 μg/g (fresh weight basis), if raised with 0.1-0.3 μg/g selenium supplementation in livestock diets[72]. Organ meats normally take up more selenium; the livers contain about four times as much selenium as skeletal muscle. The kidneys of swine and lambs can accumulate 10-16 times the amount of Se found in muscles[72]. Selenomethionine is principle Se-containing species in plants, while selenocysteine is the dominant Se-containing species found in animal tissues[73].

1.4 Selenium in human health

1.4.1 Selenium retention and metabolism

The importance of selenium in animals has been recognized, it makes people wonder whether it is also of great significance to humans. It is well-known that selenomethionine can not be synthesized in higher animals and humans. It can only be obtained from feed sources[74,75]. After absorption, the distribution of selenium in organs and tissues depends greatly on its chemical form, namely as inorganic and organic

selenium. Once selenomethionine enters the body, it is either metabolized directly, or is incorporated into proteins in place of methionine[76]. Most inorganic selenium is not retained in tissues[77]. Generally, about 30% of tissue selenium is in the liver, 15% in the kidney, 30% in muscles, and 10% in plasma[78]. At lower intakes, selenium concentrations in the liver and muscle tissues are significantly lower, while the relative distribution percentages in the kidney are maintained[79]. Selenium is excreted from the body mainly through urine, feces, and in exhaled breath. Urinary excretion seems to be the primary route of body selenium regulation. For healthy North American adults, approximately 80% of selenium was excreted via urine and about 20% was through feces[80]. When an excessive amount of selenium is consumed, dimethylselenide (DMSe), a volatile form of selenium, is exhaled with a garlic-like odor[81].

The biochemical significance of about 25 essential selenoproteins has been discovered in animals, microbes, and humans. Some Se-containing enzymes can affect cellular differentiation, growth, and development[82]. In combination with Vitamin E and fatty acids, selenium plays a significant role in the enzyme, glutathione peroxidase (GPX), which helps to prevent oxidative damage to tissues from hydrogen peroxide, lipid and phospholipid hydroperoxides. Selenium is also of great importance in thyroid hormone metabolism[24,31,83]. Selenium is linked to the functioning of the immune system, due to the significant amounts of selenium found in immune related tissues such as the liver, spleen, and lymph nodes[84].

1.4.2 Selenium toxicity and deficiency

As an essential trace element, selenium has the narrowest range between dietary deficiency (<40 μg/day) and toxic levels (>400 μg/day). Overall selenium toxicity in humans has rarely been reported. Chronic selenosis was reported in Enshi District, Hubei Province of China during the 1960's. The reports were due to the consumption of locally produced crops grown on seleniferous soils with Se concentrations of up to 6,000 μg/g selenium. In the seleniferous areas, there were 477 cases of human selenosis reported between 1923 and 1988[85,86]. Hair, skin, nails, teeth, and the nervous system were most affected by selenosis in the reported cases. In addition to the high levels of selenium in soils, burning of locally mined coal was also blamed for the selenosis incidents. High levels of selenium and other toxic elements in the smoke were reported[87].

Selenium deficiency is more widespread than selenosis. Keshan disease (KD) is an endemic cardiomyopathy (heart disease), which mainly occurs in children and women of child-bearing age. It was named after Keshan County, Heilongjiang Province of China, where it was first observed. The occurrence of Keshan disease happened in the same areas as white muscle disease in animals. Further investigation showed that soil and crops in this region were low in selenium. The measured selenium content in hair samples was less than 0.12 μg/g[85,88]. Typical features of this disease are fatigue after mild exercise, coughing, swollen body, cardiac arrhythmias, loss of appetite, cardiac enlargement, and congestive heart failure[89]. Selenium supplementation proved to be effective in the prevention of Keshan disease. In recent years, improvements in selenium dietary intake have led to an observed corresponding drop in disease prevalence; the disease is no longer a public health problem in China. Kashin-Beck disease (KBD) is

another selenium-responsive condition. It is a condition causing chronic degeneration and necrosis of the joints. It was reported in Russia, Siberia, Japan, China and Korea[90]. It was first discovered in 1849 by Russian physician I. M. Urenskii in Eastern Siberia. But more detailed studies were made by Kashin and Beck, after whom the disease is named. KBD occurs in areas where the soil selenium availability is low. A study completed by Moreno-Reyes et al. also suggested that KBD was associated with iodine deficiency[91].

1.5 Selenium in food

1.5.1 Introduction

People receive the majority of their dietary selenium from food. The amount of selenium concentrated in plant-based food is determined by the content in soils, which typically vary from 0.01 to 2 ppm across the world. Correspondingly, the dietary intake of selenium for humans can differ markedly in different regions. Besides soil contents, other factors such as agricultural practices, types of diet and domestic harvests, national agricultural and trade policies, and even the financial conditions of customers can be related.

It has been reported that the average selenium dietary intake ranges from 20 to 300 μg/day[92]. People living in North and South Dakota, and Nebraska generally have the highest selenium intakes in the United States because of local selenium-rich soils[32, 50]. Since most people in the United States have access to food produced in many different areas, it may actually help in obtaining an adequate selenium dietary intake. It was reported that beef, chicken, white bread, pork and eggs accounted for 50% of total

selenium in a typical American diet[93]. For the U.S. population, Se intake ranges between 71 and 152 μg /day per person. The Recommended Dietary Allowance (RDA) for adult in USA is 55 μg per day[94]. Selenomethionine (SeMet) is the major selenium-containing protein in cereal grains, corn, rice, and soybeans, which comprises between 45.5% and 86.5% of total selenium[53]. In wheat harvested from seleniferous soils (up to 31 ppm Se), half was found in the form of selenomethionine[95]. SeMet was also identified in most extracts of Indian mustard, sunflower, and white lupine[96]. Phytoplankton was the only plant found that showed significant amount of selenite[73]. In selenium-enriched plants such as broccoli, onions, sprouts and wild leeks, Se-methylselenocysteine was identified as the major organo-selenium compound[73]. According to the World Health Organization (WHO)[31], selenium-rich sources in food include: organ meats and seafood (0.4 to 1.5 μg/g); muscle meats (0.1 to 0.4 μg/g), cereals and grains (less than 0.1 to greater than 0.8 μg/g), dairy products (less than 0.1 to 0.3 μg/g), and fruits and vegetables (less than 0.1 μg/g). It appears that meat and seafood are more reliable sources of selenium than plants. Consumption of food rich in vitamin E, like nuts and seeds can increase the effectiveness of selenium.

1.5.2 Selenium in bread and cereals

Bread is an important selenium source for many people, especially in western societies. Different types of bread contain varying amounts of selenium. A study showed that selenium contents in US and Canadian brown bread contained 0.41 to 0.68 μg/g and 0.06 to 0.71 μg/g of selenium[97], respectively. In another study, eighteen healthy women were given bread rich in selenium at 100, 200 and 300 μg/day for 6

weeks. The selenium concentration in serum rose by 20, 37 and 53 μg/L[98]. Cereals are another important dietary selenium source. In North America, grains usually contain high selenium contents. While in Finland, the selenium content of cereal grains averages 0.01 μg/g or less[99]. With the importation of grains, the dietary selenium intake was doubled[100]. In many countries, such as China, Thailand, and Japan, rice is the most common source of dietary selenium. The range of mean selenium levels in rice has been reported as 0.02 μg/g in China[101], 0.05 μg/g in Thailand[102], 0.10 μg/g in the UK[103] and 0.32 μg/g in the USA[104].

1.5.3 Selenium in Brazil nuts

Brazil nuts are produced by *Bertholletia excelsa*, a giant tree of the tropical rainforest of Amazon basin, located mainly in Brazil. Brazil nut-rich forests can also be found in Amazonian regions of Peru and Bolivia. Brazil nut trees can grow up to 165 feet and live 500 years or more. The flowers can only be pollinated by the large female, metallic-green colored orchid bees because of their long tongues. After the flowers are pollinated, they may develop into grapefruit-sized fruits, which can weigh up to five pounds. The fruit has a hard woody shell and can contain some 20 seeds/nuts.

Brazil nuts (Figure 1.2) have been reported as the richest natural source of selenium, which could contain up to 500 μg/g. It is about 2,500 times more than any other nuts[105,106]. For every 100 grams of Brazil nuts, they can contain 67 g of fat, 14 g of protein and almost 3 mg of selenium. The difference in the soil selenium content accounts for the wide variation in the amount of selenium contained in the nuts. According to Barclay et al, the selenium contents of cashew nuts, coconut and

macademia nuts are 0.17-0.39 μg/g, 0.05-0.08 μg/g and 0.034-0.087 μg/g, respectively[103]. While Brazil nuts are considered as a great source of dietary selenium, it is important to recognize its potential health effects[107]. Based on the data from National Institutes of Health, one ounce of dried, unblanched Brazil nuts can have 544 μg of selenium, which is about 1,000% of the recommended intake[108]. The major selenium species identified in Brazil nuts were selenomethionine and selenocystine[109].

Figure 1.2: Pictures of woody seed capsule with top half removed (left), Brazil nut seeds (middle) and shelled Brazil nuts.
(Courtesy of waynesword.palomar.edu/images/brznut3.jpg and www.naturalrecipes.co.uk)

1.5.4 Meat, eggs, milk and fish

Selenium can be found in many other food sources at variable levels. Pork produced in USA, Australia, Sweden, and UK contains selenium with concentrations of 14.4-45.0, 9.4-20.5, 11.3 and 14.0 μg/100 g, respectively[110,111]. Selenium accumulation levels in milk within the US can range from 10 to 260 μg/kg[112]. A higher intake of selenium has been reported for coastal populations because they tend to include more fish in their diet. A study found that selenium intake from fish was 60-220 μg/day per person[113]. Higher levels of selenium were found in tinned sea food than the fresh

products, canned tuna can have 0.70 (range 0.637 to 0.789) μg/g and 0.39 (range 0.347 to

0.437) μg/g in canned crab[97].

1.5.5 Selenium in selenium supplements

Many people believe that an increase in selenium dietary intake will provide

beneficial effects on their health. The increased research in human cancer prevention

shows great interest in the feasibility of using selenium supplementation to lower cancer

risks. The study's results supported the hypothesis that supplemental selenium intake can

reduce total cancer mortality and total cancer incidence. Selenium supplementation will

be beneficial for people with lower dietary selenium intake and for people with iodine

deficiency[114,115].

Over the last ten years, various nutritional supplements based on selenium

appeared on market shelves. They are mainly in the forms of sodium selenate and

selenite, selenomethionine, selenocysteine and some other organic forms. Since

selenomethionine cannot be synthesized in humans, and most selenium is in the form of

L-selenomethionine in enriched wheat, corn and grains[53], this organo-selenium

compound is considered the most appropriate form for selenium supplementation. In the

search for an economical source of organo-selenium as a supplement, yeast became the

primary candidate. It is mainly attributed to the ease of management, changeable

growing conditions, and can ensure the quantity produced in a better way. Generally,

sodium selenite is used as a culture medium and its addition can be done at different

stages of enrichment such as during the growth phase and/or at the non-growth phase at

different selenium levels.   If supplemented with 30 μg/mL sodium selenite during the

exponential growth phase, the selenium-accumulation ranged between 1,200 and 1,400 μg/g in dried baker's yeast (*Saccharomyces cerevisiae*)[116]. The pH and dissolved oxygen level in the culture medium are the most important factors that influence incorporated forms of selenium[116]. Typically, the commercial supplemental products contain 1,000 to 2,000 μg/g of selenium[117]. According to the study of Rayman et al., the selenomethionine concentration ranged from 60% to 84% of selenium in ten selenium-enriched yeast samples[118]. Another study reported that selenomethionine consisted of 85% of total selenium in enriched yeasts[119].

Unfortunately, there is a lack of good manufacturing practices (GMPs) in the production of yeast supplements[120]. Not all the manufacturers check product quality, purity of the yeast strain, the percentage of SeMet, moisture content, the level of toxic impurities (As, Pb, Hg, Cd), and microbiological contaminants before putting their products in the market. Some of them just provide the information of total selenium and no selenium species are specified. For the products with both selenite/selenate and ascorbic acid (vitamin C) present, elemental selenium may be formed over time because of red-ox reactions. While this reaction does not occur with organic selenium, it could be used to check the inorganic selenium levels in yeast products[121]. A review by the EPA concluded that the NOAEL (No Observed Adverse Effect Level) of selenium for an adult is 853 μg selenium/day[122]. The Recommended Dietary Allowance (RDA) for adult in USA is 55 μg per day[94]. The Reference Dose (RfD) for a 70 kg adult is 350 μg selenium/day[117]. The RfD is an estimated value of daily exposure to the population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effect during a lifetime. This is to say, for an American adult, a supplemental

dose of 200 μg/day along with the typical dietary selenium intake will not pose adverse

effects and is safe to take.

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Chapter 2: Analytical Techniques for Selenium

2.1 Introduction to analytical techniques for selenium

The range of analytical techniques in the field of selenium is very broad. Viable instrumental determinations for selenium analysis comprise two parts: separation and detection. Separation methods include gas chromatography (GC), high-performance liquid chromatography (HPLC), and capillary electrophoresis (CE). The detection part mainly consists of atomic spectroscopy, neutron activation analysis (NAA) and mass spectrometry (MS). This chapter discusses the main separation and detection methods used for selenium analysis. Different coupled techniques will be briefly evaluated.

2.2 Separation techniques

2.2.1 Introduction

In speciation analysis, there are several separation techniques that have been employed including HPLC, GC, and CE. Nowadays, multidimensional separation schemes are gaining increasing popularity for the more complex samples. Among all the separation methods, HPLC is by far the most popular technique employed for selenium (Se) analysis.

2.2.2 High-Performance Liquid Chromatography (HPLC)

Reversed phase LC, ion pairing LC, and strong cation-exchange chromatography are the main separation methods used in this work. Size-exclusion chromatography and anion-exchange chromatography will be briefly discussed in this section.

2.2.2.1 Reversed-Phase Liquid Chromatography

Reversed-phase high-performance liquid chromatography (RP-HPLC) is a widely used, well-established separation method. The reason for its central role is resolution. In RP-HPLC, the stationary phase is non-polar and the mobile phase is polar. The majority of packing materials used for RP columns are silica-based and contain covalently bonded alkyl chains of different lengths. The hydrocarbon group forming the hydrophobic phase is usually a linear aliphatic hydrocarbon of eighteen (C_{18}), eight (C_8) or four (C_4) carbons. Due to the large number of silanol groups, steric hindrance limits the initial bonding reactions, and leads to incomplete derivatization of the silica hydroxyl groups. In order to prevent other mechanisms occurring during separation, the free residual silanol groups are blocked or capped by a second reaction with a small reagent, such as trimethylchlorosilane[1,2]. Polymer-based resins, such as polystyrene-divinylbenzene, are also used as support materials and offer a broader operating pH range[3]. Separation in RP-HPLC is based on hydrophobicity. Generally, the more polar a compound, the less it is retained on the column. In some cases, there may be partitioning and adsorption involved. The mobile phase typically is made of a mixture of water, ion-pairing reagent, and an organic modifier, such as methanol or acetonitrile. The organic modifier is added to help promote the elution of solutes. The stronger the retention of a given solute, the more organic modifier is needed to elute the solute from the column. The ion-pairing reagent, such a trifluoroacetic acid (TFA), serves to maintain the pH, thereby minimizing ionic interactions between the analyte and the stationary phase[4,5].

2.2.2.2 Ion-pairing liquid chromatography

Ion-pairing liquid chromatography (IPLC) is a method for improving the separation of polar and ionic analytes. When the separation of compounds is not complete, an ion pairing agent can be used to enhance peak shape and alter retention times. It relies upon the addition of ionic compounds to the mobile phase to promote the formation of ion pairs with charged analytes. The overall separation principle is the same as that for RP-HPLC, except that the mobile phase contains a specific ion-pairing reagent. The IPLC reagents are comprised of an alkyl chain with an ionizable terminus, which contains a charge opposite to the analyte of interest, as well as a hydrophobic region to interact with the hydrophobic stationary phase. The counter-ion combines with the ions of the eluent, forming ion pairs in the stationary phase. This interaction results in a different retention time, thus facilitating separation of analytes. It is also suggested that there is a dynamic equilibrium between the ion pairing agent in the stationary and mobile phases. The ionic solute interacts with the counter-ion covered stationary phase. When an analyte with the opposite charge is introduced, it is retained onto the stationary phase. It is believed that the retention time of analytes using ion-pairing liquid chromatography is a combination of both mechanisms[6,7].

2.2.2.3 Ion-exchange chromatography

Ion-exchange chromatography affects the separation of analytes due to their pH–dependent charges. Its separation mechanism depends primarily on reversible, ionic interactions between charged solutes and a charged ion-exchange group on the stationary phase. Ion-exchange chromatography occurs as a multi-step process, including the

movement of the solutes from the mobile phase into the stationary phase, ionic binding to the solid support, and finally, selective displacement and elution of the solute. Charged analytes displaying relatively weak interactions with the ion-exchange stationary phase will be retained less strongly on the column and elute earlier than charged analytes which react more strongly with the stationary phase.

2.2.2.3.1 Cation-exchange chromatography

Cation-exchange chromatography involves the interaction of positively charged analytes with negatively charged sites on the stationary phase. The selenium species comprise weak acids, amino acids and selenonium compounds. The charges of these groups depend on their dissociation constants and the pH of the solution, except for the selenonium groups, which are positively charged independent of pH. Cation-exchange columns usually contain a carboxylate, sulfonate, and phosphate groups.

2.2.2.3.2 Anion-exchange chromatography

In anion-exchange chromatography, negatively charged analytes interact with the positive groups coupled on the column matrix, the competing ions are the negatively charged mobile phase ions. Anion-exchange columns typically have polyamino, diethylaminoethyl, or quaternary ammonium groups as the active functional groups for the solid support.

2.2.2.4 Size-exclusion chromatography

In size-exclusion chromatography (SEC), the column packing material consists of particles containing pores of well-defined size. As the mobile phase diffuses through these particles, it carries the solutes along, which depending on their size, flow into and out of the pores. The smaller molecules are able to penetrate the pores more easily; therefore, they tend to have a longer retention time. The flow of larger molecules is hindered and not able to go through the pores and is eluted from the column with shorter retention times. Unlike other HPLC techniques, SEC does not depend on any selective interaction with the stationary phase. Since the method does not use harsh elution conditions, SEC does not deactivate enzymes and is routinely used as an important step in their purification.

2.2.3 Gas chromatography

Gas chromatography (GC)[8] is used to separate volatile and thermally stable organic compounds. A gas chromatograph is comprised of a flowing mobile phase, an injection port, a separation column containing the stationary phase, a detector, and a data recording system. The organic compounds are separated due to differences in their partitioning behavior between the mobile gas phase and the stationary phase in the column. Mobile phases are generally inert gases and are not adsorbed onto the column, such as He, Ar and N_2. Since the partitioning behavior is dependant on temperature, the separation column is normally kept in a thermostatically-controlled oven. There are capillary and packed GC columns. A capillary column is a thin fused-silica (purified silicate glass) capillary that has the stationary phase coated on the inner surface. This

type of column provides much higher separation efficiency compared to packed columns, which are typically glass or stainless steel coils filled with the stationary phase, or a packing coated with the stationary phase. The most common stationary phases in gas-chromatography columns are polysiloxanes[9], which contain various substituent groups to change the polarity of the phase. After the polymer coats the column wall or packing material, it is often cross-linked to increase the thermal stability of the stationary phase and prevents it from gradually bleeding out of the column.

2.3 Mass spectrometry

2.3.1 Introduction

Mass spectrometry (MS) involves the transfer of analytes from a liquid/solid phase to the gas phase, ion generation, followed by the separation of ions according to their mass-to-charge ratio (*m/z*). The spirit of MS is the conversion of neutral analyte molecules into ions. Since Thomson's pioneering work in 1912[10], mass spectrometry has become a powerful, versatile, and sensitive tool to determine the structure of compounds.

In a mass spectrometer, all sample molecules undergo the following process: the sample molecules (analytes) are introduced into the instrument through a sample inlet, analytes are converted into ions in the ionization source, ions are then separated according to *m/z* within the mass analyzer, the ion energy is transformed into electrical signals in detector, recorded, stored and then transmitted as a mass spectrum[11]. Generally, there are four basic components in all mass spectrometers (Figure 2.1): a sample inlet, an ionization source, a mass analyzer, and an ion detector. The mass

analyzer and the ion detector must operate at low pressures of $10^{-3} \sim 10^{-9}$ torr to prevent possible collisions between ions and background molecules.
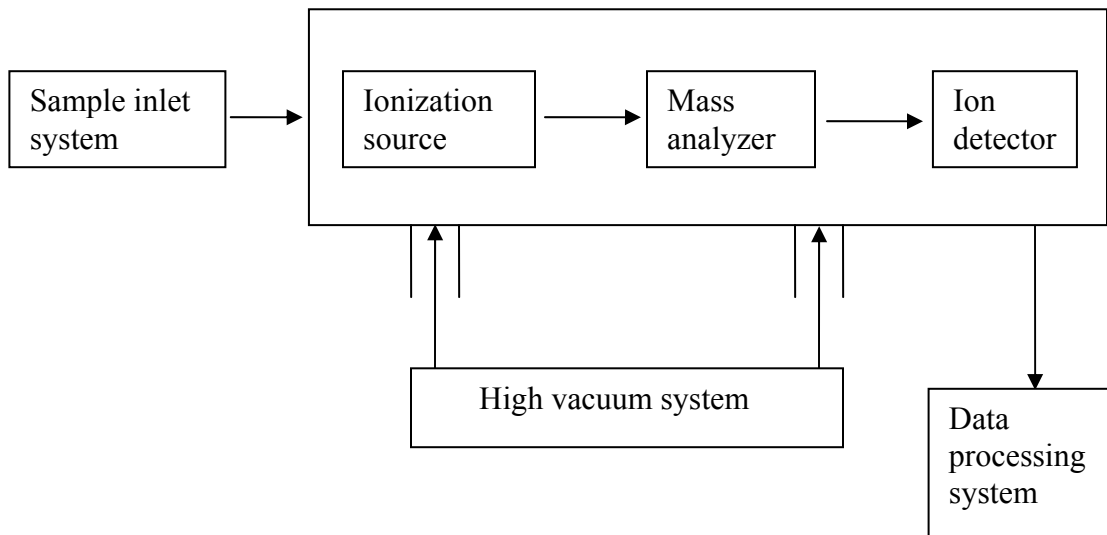
Figure 2.1: Schematic diagram of a mass spectrometer

Over the past decade, mass spectrometry has undergone tremendous technological improvements allowing for its application to the analysis of proteins, peptides, carbonhydrates, drugs, agricultural and industrial products, and many other biologically relevant molecules. A number of ionization methods were recently developed including electrospray ionization (ESI), inductively-coupled plasma (ICP), and matrix-assisted laser desorption ionization (MALDI). This section provides an overview of mass spectrometry, focusing on ionization sources and their significance in the development of mass spectrometry for selenium analysis.

A mass spectrometer measures the mass-to-charge ratio (*m/z*) of ions. Ions are generated by inducing either the loss or gain of charge from a neutral species. Once

formed, ions are electrostatically directed into a mass analyzer where they are separated according to their *m/z* and finally detected. Basically, in MS the ionized analyte or the fragment ions resulting from degradation of starting analyte species are measured to give the molecular mass and structural information of the analytes.

2.3.2 Ionization sources

2.3.2.1 Inductively coupled plasma-mass spectrometry (ICP-MS)

Inductively coupled plasma-mass spectrometry (ICP-MS) is used for elemental determination[12]. An ICP-MS instrument consists of an ICP source and a mass spectrometer. The analyte is broken down to atoms within the high temperature of the plasma. The elemental species are ionized and detected in the mass spectrometer. Typically, a quadrupole mass spectrometer is used because of its robustness, ease-of-use, and speed.

Within the ICP source, an argon plasma is maintained by the interaction of an RF field with ionized argon gas[13,14]. The ICP torch is surrounded by the RF load coil, which is connected to a radio-frequency (RF) generator. As power is supplied to the load coil, oscillating electric and magnetic fields are established at the end of the torch. When a spark is applied to the argon flowing through the ICP torch, seed electrons are stripped off of the argon atoms, forming argon ions. These ions are held within the oscillating fields and collide with other argon atoms, forming an argon discharge or plasma. When the sample aerosol is introduced into the ICP torch, it is desolvated, the molecules are atomized, and the atoms are ionized by the high temperature and Ar^+ in the plasma. Once the elements are converted into ions, they are guided toward the mass spectrometer

through interface cones. The purpose of this interface region is to transfer the ions produced at atmospheric pressure (1-2 torr) into the low pressure region of the mass spectrometer (< 10^{-5} Torr). Typically, the interface consists of two inverted funnel-like devices called cones. The purpose of these cones is to sample the center portion of the ions from the ICP torch. The sampling cone is located next to the plasma and the skimmer cone is located several millimeters behind the first cone. Each cone has an opening of approximately one-millimeter in diameter at the apex that permits the ions to pass through. The region between the two cones is evacuated to a pressure of a few Torr by a mechanical roughing pump. The holes in the cones or orifices must be large enough to prevent clogging from unvaporized materials and small enough to maintain a consistent vacuum on the other side of the interface. For best instrument performance and stability, ICP-MS has some limitations concerning the amount of total dissolved solids in the samples.

2.3.2.2 Electrospray ionization-mass spectrometry (ESI-MS)

Initial pioneering studies on the electrospray ionization of large molecules were performed more than thirty years ago by Malcolm Dole[15,16]. An ESI source of sufficiently advanced design for routine use in mass spectrometry was developed by the Fenn group[17,18] in the mid-1980s. Electrospray ionization (ESI) is an ionization method that allows the transfer of ions from solution to the gas phase, from which the ions can be subjected to mass spectrometric analysis. The ESI source has undergone continued development since the earliest examples, but the general arrangement has remained basically the same.

In an ESI inlet system, a dilute sample solution is pumped through a capillary, which is held at a potential of 3-4 kV with respect to the counter electrode. The applied electric field at the capillary tip disperses the emerging liquid into a fine spray of charged droplets. The droplets are repelled from the tip towards the source sampling cone on the counter electrode. As the droplets travels towards the cone, they desolvate and reduce in size to such a point that surface-coulombic forces overcome surface-tension forces and the droplets break up into smaller droplets[19,20]. This process continues until either an ion desorbs from a droplet[21,22] or the solvent is completely removed[15]. After going though a series of regions with decreasing pressures, the ions, produced at atmospheric pressure, are separated from neutral species, ions and cluster of ions with neutrals, and are ready to enter the mass analyzer. A very good review article covering the applications of ESI-MS to biological sciences and biochemistry was recently published by the Biochemical Society[23].

2.3.2.3 Matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS)

In MALDI experiments[24,25], a droplet of a solution, containing a molar matrix-to-analyte ratio between 5,000:1 and 500:1, is deposited on a sample plate. After solvent evaporation is completed, the plate is transferred to the vacuum system of the mass spectrometer. Short laser pulses (usually 337 nm N_2 laser is used) are applied to the sample spot and the matrix absorbs the laser energy[26,27]. The absorbed laser energy causes rapid evaporation of the matrix molecules into the gas phase carrying some of the analyte molecules[28], which are consequently protonated to $[M+H]^+$ or $[M+2H]^{2+}$. The protonated species are then detected by the mass spectrometer. Typical matrices for

MALDI analysis are 2,5-dihydroxybenzoic acid[27], 3-hydroxypicolinic acid[29], α-cyano-4-hydroxy-cinnamic acid[30] and 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid)[31]. MALDI is usually combined with a time-of-flight (TOF) mass spectrometer[32].

MALDI is a very sensitive and the typical analyte concentration can be as low as a few picomoles. Its practical mass range is up to 300,000 Da. It is reasonably tolerant to samples containing salts, buffers and other additives and is suitable for the analysis of complex mixtures.

2.3.2.4 Electron Impact (EI)

The electron impact source consists of a heated metal filament that produces electrons which are accelerated to another electrode called the ion trap[33,34]. These resulting electrons are used to ionize the sample molecules. Normally, the energy of the electrons (usually 70 eV) is higher than the ionization energy of the molecule. When these electrons pass near neutral molecules, they may impart enough energy to remove outer shell electrons of the molecule, resulting in additional free electrons and positive ions. The excess energy breaks bonds within the newly formed ion, resulting in the formation of fragments (both neutrals and ions). Low energy electrons produce molecular ions and larger fragments, whereas high energy electrons produce many smaller fragments and possibly no molecular ions. After the ions are produced, they are driven by a potential applied to an ion-repeller electrode, away from the ion source into the accelerating region of the mass spectrometer, where mass analysis takes place.

2.3.2.5 Chemical ionization (CI)

Chemical ionization (CI) sources exhibit close similarities to EI sources. But a reagent gas is introduced into the beam of electrons, and they are fragmented by the electrons. There are four general pathways to form ions from a neutral analyte: proton transfer, electrophilic addition, anion abstraction, and charge exchange. Proton transfer normally yields protonated analyte molecules, $[M+H]^+$, but acidic analytes may produce $[M-H]^+$ ions by protonating some other neutrals. Electophilic addition chiefly occurs by attachment of complete reagent ions to the analyte molecule (e.g., $[M+NH_4]^+$ in the case of ammonia as a reagent gas). Hydride abstractions are abundant representatives of anion abstraction (e.g., aliphatic alcohols form $[M-H]^+$ ions)[35,36]. Charge exchange yields radical ions of low internal energy, which are similar to the molecular ions formed in low-energy electron impact ionization. Often, CI allows for the detection of the molecular ions.

2.3.3 Mass spectrometers

Once ions are produced, they need to be separated according to their mass-to-charge ratios. There are many different types of mass spectrometers to effect this determination: quadrupole analyzer[37], time-of-flight (TOF)[38], magnetic and electrostatic field mass analyzers[39], ion trap[40] and Fourier Transform ion cyclotron resonance (FTICR)[41]. The most widely used mass spectrometer is the quadrupole mass analyzer. The mass spectrometers used in this study are all equipped with this type of mass analyzer. The major advantages of a quadrupole mass analyzer are its relative low cost,

high scan speeds, and the ability to work at relatively high pressure. The major drawback for this type of analyzer is its limited mass resolution[37].

A quadrupole mass analyzer[37] consists of four cylindrically shaped rods positioned at the corners of a square. The pairs of opposite rods are connected together electrically to radiofrequency (RF) and direct current (DC) voltage sources. The pairs share the same but opposite voltage. The ions from the source are extracted and accelerated along the z-axis towards the detector. The quadrupole actually serves as a "mass filter" according to the RF and DC voltages. The ions entering the quadrupole can either traverse a stable trajectory, in which they finally reach the detector, or they could experience an unstable trajectory, in which they are removed prior to the detector. In this way, a quadrupole only allows the passage of ions with a certain m/z ratio. A complete mass scan can be performed by carrying the RF and DC voltage in such a way that their ratio remains constant.

2.3.4 Tandem mass spectrometry

In tandem mass spectrometry, two or more stages of mass analysis are combined in one experiment[42]. Each stage provides an added dimension in terms of isolation, selectivity, or structural information to the analysis. It generally involves multiple steps of mass selection or analysis, separated by some form of fragmentation. One of the most commonly available tandem mass spectrometers is the triple quadrupole (QqQ) instrument. Since the triple quadrupole system is used for the studies decribed here, this type of tandem mass spectrometer will be discussed in detail.

In the QqQ configuration[23], the first (Q1) and third (Q3) quadrupoles act as mass filters, and the middle (q2) quadrupole is used as a collision cell. The collision cell is an RF only quadrupole (non-mass filtering) using an inert gas, like He to induce collisional dissociation of selected parent ion(s) from Q1. Subsequent fragments are passed through to Q3 where they may be filtered or scanned fully. This process allows for the study of fragments (daughter ions), which are crucial pieces of information needed for structural elucidation. For example, Q1 may be set to "filter" for ions with a certain mass, which are fragmented in q2. The third quadrupole (Q3) can then be set to scan the entire m/z range, giving information about the sizes of fragments made. Thus, the structure of the original ion can be deduced.

In a triple quadrupole mass spectrometer, several types of experiments can be performed. To obtain information on the composition of the sample, a full scan can be done. For structural information of an unknown compound, a product ion scan will be of help. A neutral loss or precursor ion scan is informative when a group of molecules are examined. Selected reaction monitoring (SRM)[43] and multiple reaction monitoring (MRM)[44] are particularly useful for the detection of specific compounds in a mixture.

2.4 Coupled techniques

2.4.1 Introduction

It is possible to combine chromatographic methods (GC and LC) with mass spectrometry and thereby take advantage of both techniques. LC is a powerful separation technique. MS excels in sensitivity and identification. MS can provide very specific information about the chemical composition of the analytes, which is more informative

than a UV-Vis detector. The addition of MS to chromatographic separation truly provides a second dimension to the chemical analysis. This fully automated analytical method, not only can reduce the analysis time, but also decreases the chance of cross-contamination and sample losses due to fraction collection. However, it is necessary to evaluate the compatibility of the mobile phase with respect to the LC separation and MS detection to ensure feasibility of the LC-MS coupled method. Sometimes, an interface is needed. There are a variety of coupled LC-MS techniques, such as LC-ICP-MS, LC-ESI-MS, GC-MS, and LC-MALDI-MS. The first two will be discussed in detail due to the methods used in the work, the others will be briefly mentioned.

2.4.2 Liquid chromatography-inductively coupled plasma mass spectrometry (LC-ICP-MS)

ICP-MS, as a multi-element detector for LC, offers the advantages of its excellent detection limits and its capability for measuring isotope ratios of analyte peaks. The latter ability is of great use in measuring concentration, as well as, in speciation studies. Isotope dilution saves the time of acquiring multiple chromatograms, from which to generate calibration curves and compensate for matrix effects. Furthermore, HPLC offers potential advantages for ICP-MS by removing problematic matrix interferences on-line.

In the coupled LC-ICP-MS system, the outlet of LC column is connected to the inlet of the nebulizer of the ICP portion of the instrument. Short and small tubing is preferred to minimize the dead volume and to keep the good chromatographic resolution. Thompson and Houk were the first to investigate the feasibility of LC-ICP-MS in 1986[45].

In their work, ion-pair reversed-phase liquid chromatography was used and the sample was introduced by ultrasonic nebulization with aerosol desolvation. The separation and selective detection of various As and Se species were studied, giving detection limits near 0.1 ng (as the element). It was concluded that HPLC-ICP-MS could make speciation studies more promising with stable tracer isotopes. Vanesa and Laura carried out the determination of total Se, as well as, Se speciation analysis in yeast and wheat flour with an anion-exchange column coupled on-line to ICP-MS[46]. The use of mobile phases with a high percentage of organic solvents can be a bigger problem due to possible carbon deposition on the sampler and skimmer cones, which results in the decreased signals and elevated noise. These difficulties can be overcome by introducing oxygen into the nebulizer gas or increasing the RF power[47, 48].

2.4.3 Liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS)

ICP-MS has great sensitivity in elemental analysis; however, it does not provide any molecular information. It identifies the compounds solely by comparing the chromatographic retention times with known standards. In biological systems, the determination of selenium could be very complicated due to the complexity of matrices. Molecular characterization is in great need to provide the structural identification. Fortunately, this task can be fulfilled by electrospray ionization (ESI). The distinguishing Se isotopic profile is particularly helpful to recognize Se-containing species. It can help interpreting ESI-MS/MS spectra, since MS/MS spectra of the single isotopomeric species can be generated. ESI is a soft ionization technique that yields protonated (or

deprotonated) molecular ions. The molecular mass of a compound can be calculated from the $[M+nH^+]^{n+}$ family of ions, which is performed by automatic computational (deconvolution) methods. Collision-induced-dissociation (CID) MS-MS analysis is normally used to characterize the molecular structure in triple quadrupole (QqQ) MS instrument[49]. When interfacing ESI-MS to LC separations, additional issues must be considered in solvent selection. A more volatile solvent with low concentrations of salts is preferred. It is proven that lower flow rates can enhance solvent desolvation, ions formation, and transfer[50]. Typical LC runs at flow rate of 1 mL/min, while ESI favors a lower flow rate of about 5-10 μL/min[51]. To improve the sensitivity, a post-column flow splitter or the introduction of a nebulizer gas may be beneficial[52].

2.4.4 Gas chromatography-mass spectrometry (GC-MS)

Gas chromatography-mass spectrometry (GC-MS) is a powerful technique for characterizing volatile organic molecules. Volatile selenium species are normally in the reduced and methylated forms, such as dimethyldiselenide (DMDSe), dimethylselenide (DMSe), hydrogen selenide (H_2Se), and methaneselenol (MeSe). These compounds are thermally labile enough to be separated by packed[53,54] or capillary[55] columns. Capillary GC provides higher resolution and better efficiency than packed GC, but overloading the column is the biggest concern. GC has also been used to investigate the transformation of inorganic Se species into methylated forms with animals[56]. Recently, Wolf et al.[57] derivatized a more volatile compound, methylselenocyanide (CH_3SeCN), from selenomethionine (SeMet) with cyanogen bromide (CNBr). A method for the determination of methionine (Met) and SeMet in yeast with isotope dilution GC-MS was

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reported by Yang et al.[58] and represented a step forward in understanding the metabolism of these compounds. The use of GC-based methods for non-volatile Se species is restricted by the fact that proper derivatizations are a time-consuming process.

2.5 Hydride generation atomic absorption spectrophotometry (HG-AAS)

Atomic absorption spectrophotometry (AAS) is one of the most common instrumental methods for analyzing metals. Each metal absorbs a characteristic wavelength of light. In AAS, the sample is atomized into the vapor phase. A beam of electromagnetic radiation passes through the vaporized sample. Some of the radiation is absorbed by the metal of interest. The instrument measures the change in intensity, which is then converted into an absorbance reading. After calibration, the amount of absorption can be related to the concentrations of various metals through the use of Beer-Lambert's law. However, AAS suffers from interferences, poor reproducibility, and poor detection limits. An alternative method, hydride generation (HG), can be used for some elements. In HG-AAS, the hydride generation system mixes liquid samples with sodium borohydride ($NaBH_4$) and hydrochloric acid (HCl), creates a volatile hydride of the analyte, and sends the volatile hydride to the optical cell to be atomized and detected. The atomization efficiency is therefore greatly increased after hydride generation process.

For selenium, HG-AAS requires the Se (IV) oxidation state (selenite), since Se (VI) is not reproducible[59]. Therefore, all selenium must be in the Se (IV) oxidation state for analysis. This is normally accomplished by oxidizing all Se in samples to Se (VI) with nitric acid or hydrogen peroxide and the resulting Se (VI) is reduced to Se (IV) with boiling HCl[60]. The course of the reduction of Se (VI) to Se (IV) can be summarized as:

$$HSeO_4^- + 3H^+ + 2Cl^- \rightarrow H_2SeO_3 + Cl_2 \text{ (aq) } +H_2O \qquad (2.1)$$
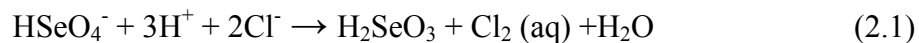
Hydride generation can be performed efficiently by reacting the acidified sample with sodium borohydride (NaBH$_4$). The general reaction is:

$$3BH_4^- + 3\,H^+ + 4\,H_2SeO_3 \rightarrow 4SeH_2 + 3\,H_2O + 3H_3BO_3 \qquad (2.2)$$
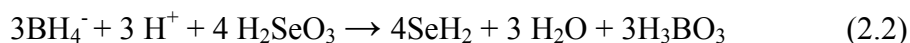
HG-AAS was used to determine concentrations of Se compounds in plant extracts by Zhang and Frankenberger[61]. They were able to separate Se into non-amino acid organic Se, Se-amino acids, selenite (Se [IV]) and selenate (Se [VI]) by an anion-exchange method.

2.6 Hydride generation atomic fluorescence spectrometry (HG-AFS)

Atomic fluorescence spectrometry (AFS) involves the optical emission of photons from gas-phase atoms that have been excited by photon absorption. The major advantage of fluorescence detection is the great sensitivity because of the low background.

Marc and Roser[62] proposed the determination of selenite, selenate, selenocystine (SeCys) and selenomethionine (SeMet) by hydride generation-atomic fluorescence spectrometry (HG-AFS). The separation was performed on an anion-exchange column. Polona and Vekoslava[63] also studied the selenium species in selenium-enriched pumpkin seeds by HG-AFS on anion- and cation-exchange columns.

2.7 Neutron activation analysis (NAA)

Neutron activation analysis (NAA) is the most common form of activation analysis[64]. It was discovered by Georg Hevesy and his student Hilde Levi in the 1930's when they found a different reaction for rare-earth elements upon neutron irradiation. In 1936, they reported this new qualitative and quantitative method based on the discrimination by half-life of the emitted radiation. In NAA, the analyte is irradiated with a flux of neutrons. It induces a nuclear reaction and results in the excited radioactive intermediate of the analyte elements. The intermediates then de-excite/decay by emitting beta particles (β^-) and delayed gamma rays (γ) with a unique half-life. The delayed gamma radiation is detected using a high-resolution gamma-ray spectrometer in order to obtain both qualitative and quantitative analytical information.

There are two Se isotopes that are often used for selenium determination by NAA, ^{75}Se and ^{77m}Se[65]. ^{75}Se has a half-life of 118.45 day which is much longer than that of ^{77m}Se (17.38 sec). Due to the advantage of shorter half-life time, many measurements can be taken via ^{77m}Se. In general, the samples are analyzed with 5 sec irradiation at a thermal neutron flux of $8 \cdot 10^{13} n \cdot cm^{-2} \cdot sec^{-1}$, 15 sec decay and counted for 25 sec. The nuclear reactions are involved as the following:

$$^{76}\text{Se} + {}^{1}\text{n} \rightarrow {}^{77m}\text{Se} \tag{2.3}$$

$$^{77m}\text{Se} \rightarrow {}^{77}\text{Se} + \gamma \tag{2.4}$$

Typically the counting is accomplished using a high-resolution gamma-ray spectrometer. The 161.9 keV gamma-ray net peak area from the decay of ^{77m}Se ($T_{1/2}=$

17.38 Sec) is used to determine the selenium concentration by standard comparison. For the purpose of quality control, NIST standard reference material 1577 Bovine Liver is analyzed under the same experimental conditions.

Selenium concentrations in nails, blood, urine and hair can serve as an indicator of selenium intakes[66]. Se levels in plasma and sera reflect the day-week changes in dietary Se intake[66], whole blood responds to week-month changes and nails normally have higher Se values than hair[65]. In addition, nails have a few advantages as the matrix for the study of Se. They are easily obtained, transported, stored and reflect long-term Se status. An increase in selenium concentrations in both fingernails and toenails was observed after taking selenium supplements containing 200 μg Se/day[67]. Less variability in the toenail Se concentration over time was monitored than that of fingernails. This shows that the toenails are a better dietary monitor for long-term Se status, which is mainly due to the fact that toenails are normally sheltered from the environment and less responsive to short-term differences[67].

Typically, NAA offers superior sensitivities on the order of parts per billion or better and hence is recognized as the "referee method", especially when developing a new procedure or solving the discrepancy between methods.

2.8 References

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Chapter 3: Selenium Speciation

3.1 Introduction to selenium speciation

Selenium, as a trace element, is known to be essential and nutritional for life and is toxic at levels above those required for health. Daily dietary consumption of less than 40 µg will result in selenium deficiency, whereas dietary values above 400 µg will be toxic[1]. The dual behavior of selenium depends not only on the doses of the element, but more importantly upon its chemical form and oxidation states. For example, with chromium, Cr^{6+} is highly toxic and Cr^{3+} is beneficial as a micro nutrient. The specific chemical form, such as isotopic composition, oxidation state, and/or complex or molecular structure is referred to as chemical species[2]. Elemental speciation analysis is defined as the analytical activities of identifying and/or measuring the quantities of one or more individual chemical species of an element in a system[2–5]. Therefore, selenium speciation analysis encompasses the identification, quantification and structural characterization of selenium species. Selenium speciation is of great importance to understand its biological significance and metabolism in toxicology, nutrition and clinical chemistry. This chapter will describe selenium speciation in biological samples, such as yeasts, nuts, wheat flour, green onions, body fluids and tissues.

3.2 Selenium speciation in yeasts.

Increased research in human cancer prevention shows great interest in the feasibility of using selenium supplementation to lower cancer risks. The results supported the hypothesis that supplemental selenium intake can reduce total cancer

mortality and total cancer incidence. Beneficial effects observed in the Se-supplemented group implied a protective effect of selenium against prostate cancer[6]. The reduction of tumor yield in an animal model of breast cancer was associated with selenium yeast[7]. Animal studies have also shown that selenium-enriched broccoli, onions, garlic, and Brazil nuts are very effective in the inhibition of tumors[8]. The cancer preventative effects of selenium have led to the emergence of selenium dietary supplements sold in various chemical forms. In order to better understand the mechanism by which selenium-supplementation shows beneficial effects, the study of the absorption, distribution, bioavailability of Se species is desirable.

Many hyphenated techniques have been applied to the speciation analysis of selenium in high-Se yeast. Cyanogen bromide is a common reagent to cleave peptide bonds on the C-side of selenomethionine (SeMet) and methionine residues. The reaction product is volatile methylselenocyanide (CH_3SeCN)[9]. Yang et al. reported the derivatization of yeast samples with cyanogen bromide[10] and methyl chloroformate[11, 12] with detection by GC-MS after final chloroform extraction. The low extraction efficiency, possibly caused by the incomplete degradation of proteins with cyanogen bromide, was also observed in a recent study[10]. In addition, the GC-based derivatization for non-volatile selenium species is fairly tedious and time-consuming. Therefore, HPLC-ICP-MS is preferable.

Anion-exchange chromatography was evaluated by Huerta et al. for the study of enzymatic hydrolysates with protease and lipase and the detection was completed by isotope dilution analysis ICP-MS equipped with an octapole reaction system[13]. Their study showed the primary Se species found in yeast was selenomethionine, which

accounted for 68% of total selenium. The findings were consistent with the analysis results accomplished by Uden et al., who discovered selenomethionine as the major Se compound in yeast enzymatic extracts[14].

The use of two-dimensional chromatography, by size-exclusion and reversed-phase HPLC coupled with ICP-MS, was developed for the separation of Se species in water extracts of nutritional yeast supplements[15]. In this study, electrospray ionization (ESI) MS-MS was adopted to identify the eluted Se species based on the selenium isotopic pattern. Casiot et al[16] compared eight solid-liquid extraction procedures for the recovery of selenium species from yeast samples and the Se speciation was accomplished by size-exclusion, anion-exchange and revered-phase LC with ICP-MS detection. Their results showed that a leaching procedure with water and methanol recovered 10-20% of selenium, leaching with pectinolytic enzymes released an additional 20% of selenomethionine, the addition of sodium dodecyl sulfate (SDS) increased the yield of Se through the solubilization of selenoproteins. The major Se species was selenomethionine from the proteolytic enzymatic digestion. This study was the first comparison of different sample preparation procedures. Two dimensional size-exclusion-reversed-phase HPLC-ICP-MS was reported to detect Se compounds in the tryptic digestates from selenized yeast, which was characterized by MALDI MS, orthogonal MALDI MS/MS and nano LC-electrospray QTOF MS/MS[17]. It illustrated that the possible loss of selenium from selenomethionine could be due to the degradation to vinylglycine and the formation of new Se peptides by methylation.

The first identification of selenodiglutathione (GS-Se-SG) and a selenotrisulfide of glutathione and cysteinylglycine (GS-Se-SCG) in biological matrices was

accomplished by Lindemann et al[18]. In their study, size-exclusion chromatography and a porous graphitic carbon LC column, combined in parallel with ICP-MS and nano-electrospray MS/MS was used to separate and detect Se compounds in an aqueous yeast extract.

Anion-exchange chromatography coupled with hydride generation atomic fluorescence spectrometry (AEC-HG-AFS) was developed for the determination of Se-methyl-selenocysteine (MeSeCys) selenomethylcysteine (SeMeCys), selenomethionine (SeMet), selenocystine (SeCys$_2$) and selenite in selenious yeast tablet and human urine[19]. GC-atomic emission detection (AED) was proven effective for the detection of selenomethionine-Se-oxide and SeMeCys in the enzymatic hydrolysate with protease XIV from a reference yeast (SelenoExcell)[20].

3.3 Selenium speciation in foods and other biological samples

Selenomethionine can not be synthesized in humans and most of our dietary selenium is from bread, cereal, meat, and milk. The Recommended Dietary Allowance (RDA) for an adult in the USA is 55 μg per day. Selenium content in foods is closely dependent on the regions where the food crops are grown and therefore quite variable all across the world. To date, there is little information available about the chemical forms of selenium in foodstuff. The main reason for the lack of information is the difficulty and complexity of the food-sample matrix. With more availability of many hyphenated techniques, such as HPLC coupling with ICP-MS, ESI-MS and MALDI-MS, selenium speciation is facing a promising future. The studies in this field will help us to better understand the beneficial effects of selenium rich/enriched food.

Brazil nuts (*Bertholletia excelsa*) have been recognized as one of the very few foodstuffs naturally enriched with selenium. The first investigation of selenium species in Brazil nuts was accomplished by Vonderheide et al[21]. Ion-pairing chromatography ICP-MS with hexanesulfonic acid as the ion-pairing agent was selected for the analysis of the enzymatic hydrolysis products with proteinase K. Selenomethionine was found as the most abundant seleno-amino acid. Kannamkumarath recently studied the association of selenium to proteins in Brazil nuts with five different extraction methods[22]. Size-exclusion chromatography was applied to the separation of Se-containing proteins with UV detection in parallel with ICP-MS detection. Further characterization of the enzymatic hydrolysate of defatted nuts was achieved by capillary electrophoresis (CE)-ICP-MS and selenomethionine was the dominant selenium compound in the nut extract. The preparation of a potential laboratory reference material from Brazil nuts for selenium speciation was evaluated by HPLC-UV-HG-AFS for species stability and homogeneity[23]. Their tests showed that the candidate reference materials passed the relevant tests recommended by the community bureau of reference.

Anion-exchange chromatography-ICP-MS was employed to determine the Se species in the enzymatic extracts of Se-rich (natural American Flour) and enriched wheat flour[24]. Selenomethionine appeared to be the major Se species in both types of flours. The measured selenomethionine concentration was 256 ± 22 μg/kg and 975 ± 103 μg/kg dry weight in Se-rich flour and Se-enriched flour, respectively, which accounts for 59.6 ± 6.4 % and 74.5 ± 10.2% of the total Se in that order. Ion-pairing reversed-phase HPLC-ICP-MS was utilized for the total selenium and selenium species analysis for the aqueous and enzymatic extractions in Se-enriched garlic and onion samples and reference

materials with natural selenium contents[25]. γ-glutamyl-Se-methylselenocysteine (γ-glutamyl-SeMeCys) was identified in Se garlic samples with the same LC separation combined with ESI-MS with heptafluorobutanoic acid (HBFA) or trifluroacetic acid (TFA) as the ion-pairing agent. The analysis results explained that the selenium distribution in samples of the same type with distinct selenium levels was closely related with the total selenium contents. The Se-distribution could be different in spite of the similar total Se-concentrations in the samples. γ-glutamyl- Se-methylselenocysteine is the carrier of methylselenocysteine (MeSeCys), which is the biological precursor of methylselenol (CH_3SeH). After β-elimination of MeSeCys, CH_3SeH will generate reactive oxygen species in tumor cells to induce the apoptosis process[26]. It is the major metabolite in anticancer activity. The presence of γ-glutamyl-SeMeCys was further confirmed by McSheehy with size-exclusion LC-ESI-MS/MS[27].

Se-enriched green onions (*Allium fistulosum*) were investigated using IP-RP and SEC-HPLC techniques for selenium speciation with ICP-MS detection[28]. SEC analysis showed that about 44% of total Se was incorporated with the proteins in the plant extracts with sodium hydroxide. IP-RP separation results demonstrated that SeCys$_2$, MeSeCys, SeMet and inorganic selenium were present in the enzymatic plant extracts. The confirmation of γ-glutamyl-SeMeCys with ESI ion-trap-MS proved that green onions may be used for Se-dietary purposes when Se-enrichment was applied.

Three different HPLC methods were utilized for the selenium speciation studies in Se-enriched chives (*Allium schoenoprasum*) grown in Se^{4+}, Se^{6+} and SeMet-supplemented media[29]. In this study, size-exclusion chromatography (SEC) evaluated the Se-containing proteins in chives, seleno-amino acids were determined by ion-pairing

reversed-phase chromatography and a chiral crown ether column was employed for the separation and identification of enantiomers of seleno-amino acids. The species in Se-enriched chives with chiral speciation agreed well with the results derived from the ion-pairing reversed-phase chromatography (IP-RP). Fortification with Se^{6+} resulted in the highest total Se-concentration, up to 700 μg/g among the three different media. However, approximately 30% of the total Se was inorganic selenium, which excluded its potential use for dietary selenium source. For the samples supplemented with Se^{4+} and SeMet, the accumulated selenium showed high selenocystine ($SeCys_2$) and selenomethylcysteine (SeMeCys), especially for the SeMet enrichment. The primary enantiomer was L-SeMeCys in the perchloric acid-ethanol extracts and L-SeMet was present in the enzymatic extracts.

Anion-exchange and cation-exchange LC separation coupling with UV-HG-AFS detection was employed for the Se species in Se-enriched pumpkin (*Cucurbita pepo* L.) seeds[30]. Se-enrichment was realized via spraying the leaves with 1.5 mg/L Na_2SeO_4 aqueous solution during the flowering period. SeMet was found to be the major Se species from the enzymatic extracts, which accounted for an average 81% of total Se content. It showed the most of Se^{6+} was converted to SeMet after foliar application. One edible mushroom, shiitake (*Lentinula edodes*), is quite popular in the daily diet in eastern Asian countries, such as Japan and Korea. The primary selenium species in the aqueous extract of Se-enriched shiitake was identified as SeMet[31]. The separation was performed on a size-exclusion column and the detection was achieved by ICP-MS detection. ESI-MS was carried out to confirm the selenium compounds.

The enzymatic hydrolysate of oysters was analyzed with anion-exchange and cation-exchange chromatography-ICP-MS[32]. The identified selenium species were trimethylselenonium (TMSe$^+$) and SeMet, which consisted of 56% of total selenium measured. The enzymatic hydrolysis was proven to be an efficient method to fragment seleno-proteins/peptide into seleno-amino acids in liquid extracts.

Selenium speciation in other biological matrices, such as plants, human fluids and tissues plays a critical role in understanding the biological significance and metabolism in toxicology, nutrition and clinical chemistry. *Chlorella* algae (coccoid green algae) samples collected from a Se-laden agricultural evaporation pond of the San Joaquin Valley, CA, and the trichloroacetic acid extract was detected by cation-exchange HPLC-ICP-MS with a dynamic reaction cell (DRC)[33]. The presence of dimethylselenonium propionate (DMSeP) was confirmed with HPLC-ESI-MS. As described in Chapter 1, plants can protect themselves against selenium toxicity by converting selenium into non-toxic volatile compounds such as dimethylselenide (DMSe) and are released into the air[34, 35]. The ability of algae to volatize DMSe from DMSeP may be part of biogeochemical cycling of selenium.

Two dimensional-reversed-phase and anion-exchange chromatography were employed for the separation of Se^{4+}, Se^{6+}, SeCys$_2$ and SeMet in conjunction with HG-UV-AFS detection[36]. The goal of the study was to develop a routine method for quality control purposes. The analysis results showed Se^{4+} was the only species detected in infant formulas and principal seleno-amino acids as SeCys$_2$ and SeMet and minor inorganic Se^{4+} were present in the nutritional supplements. Whole milk powder was used as the certified reference material to check the feasibility of the method.

As can be seen, many analytical techniques have been developed in the field of selenium speciation, which leads to the discovery of some new functions of selenium. The separation, detection and quantification can be easily accomplished using different HPLC methods coupling with ICP-MS or AFS. Different mass spectrometry methods, such as ESI-MS and MALDI-MS, could provide more structural information and complete the identification of unknown species.

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Chapter 4: Investigation of DRC-ICP-MS for Selenium Detection

4.1 Introduction

This chapter describes optimization experiments conducted for the determination of selenium by dynamic reaction cell (DRC)-ICP-MS. This type of analyzer has recently been promoted as a means to eliminate interferences in the determination of selenium. The goal was to develop a low-cost, routine method for selenium detection with high sensitivity that could be applied to the determination of total selenium and organic selenium speciation in biological samples. The performance characteristics of DRC-ICP-MS were evaluated and results obtained for selenium in tissue were compared to the standard mode ICP-MS.

4.2 Detection of total selenium by DRC- and standard ICP-MS

4.2.1 Introduction to DRC-ICP-MS

Because of increasing interest in selenium in human health and in environmental issues, there is greater demand for accurate determination of selenium in biological matrices. Historically, the accurate determination of sub parts-per-million concentrations of selenium by ICP-MS has suffered severely from polyatomic interferences, especially argon dimers. Selenium has six naturally occurring (stable) isotopes, which are listed in Table 4.1 by atomic mass and natural abundances, along with the most common interferences. Polyatomic interferences are primarily formed in the plasma from the argon plasma gas as well as sample matrix components. Unfortunately, such interferences are present in virtually all ICP-MS systems and for selenium there are potential

interferences with all six isotopes. Seemingly, the best choice for selenium would be ^{80}Se, because that isotope has by far the greatest natural abundance. However, $^{40}Ar_2^+$ causes an extremely high background signal at mass 80 which severely limits the detection of ^{80}Se. In order to achieve the best detection limit for selenium, as well for the other elements, spectroscopic interferences must be addressed properly.

Table 4.1: Spectral interferences of Se isotopes by ICP-MS.

Se isotopes	Atomic mass	Abundance (%)	Interferences
74	73.92247	0.89	$^{36}Ar^{38}Ar^+$, $^{37}Cl_2^+$, $^{40}Ar^{34}S^+$, $^{38}Ar^{36}S^+$
76	75.91921	9.37	$^{40}Ar^{36}Ar^+$, $^{38}Ar_2$, $^{31}P_2^{14}N^+$, $^{40}Ar^{36}S^+$
77	76.91991	7.63	$^{36}Ar^{40}Ar^1H^+$, $^{38}Ar_2^1H^+$, $^{40}Ar^{37}Cl^+$
78	77.91731	23.77	$^{40}Ar^{38}Ar^+$, $^{31}P_2^{16}O^+$, $^{38}Ar^{40}Ca^+$
80	79.91651	49.61	$^{40}Ar_2^+$, $^{79}Br^1H^+$, $^{32}S^{16}O_3^+$
82	81.91669	8.73	$^{40}Ar_2^1H_2^+$, $^{12}C^{35}Cl_2^+$, $^{34}S^{16}O_3^+$, $^{81}Br^1H^+$

One approach to avoid spectral interferences is the use of a high-resolution mass spectrometer with a sector field[1]. The major disadvantages of this method are the significant loss of instrumental sensitivity at the required high resolving powers (R=10,000) and its high cost. Because selenium determinations in biological samples are often needed at very low concentrations, the use of a high-resolution instrument for routine selenium applications is limited. Another approach is to apply a cold-plasma

combined with a shielded ICP torch[2], but this technique is not particularly effective for selenium because of its high ionization potential.

An altogether different approach is to prevent interfering ions from entering into the mass analyzer, which in essence is the method of the ICP-MS equipped with a collision cell (CC) or dynamic reaction cell (DRC). DRC-ICP-MS offers an alternative for the elimination of spectroscopic interferences and theoretically improves ICP-MS performance for certain elements. DRC incorporates a quadrupole that is oriented between the ion lens system and the mass analyzer (a quadrupole in these experiments). The cell chamber is pressurized with a reactive gas or a mixture of gases, which then reacts with the interfering atomic and molecular ions of argon. Afterwards, the isobaric interferences are converted to non-interfering species, ejected from the DRC before entering the analyzer quadruple and reaching the detector.

Gasses such as H_2[3,4], He[5], NH_3[6], O_2[6], and CH_4[7] are usually employed in the reaction cell. The gas-phase ion-molecule chemistry takes advantage of the differences in the reactivities of analytes and interferent ions. The selection of a gas depends on its chemical reactivity with the interfering species in the gas phase. It can be predicted based on the thermochemistry of the interferences and reaction gas. Once the interferences are eliminated, the ions that could be transmitted to the analyzer and therefore be detected are the ions of interests (Figure 4.1).

For example:

$$^{40}Ar^{40}Ar^+ \ + \ CH_4 \ \longrightarrow \ CH_4^+ \ + \ ^{40}Ar \ + \ ^{40}Ar \tag{4.1}$$

70
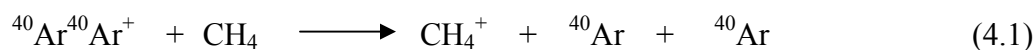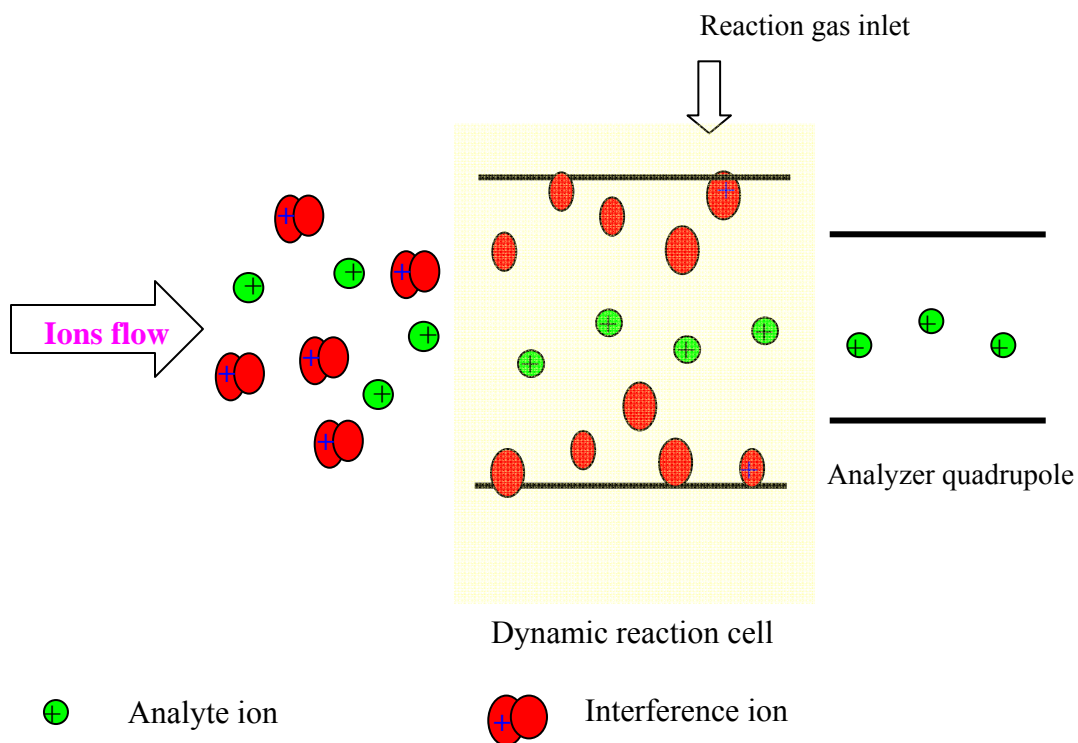
Figure 4.1: The dynamic reaction cell (DRC) eliminates polyatomic interferences by using ion-molecule reactions.
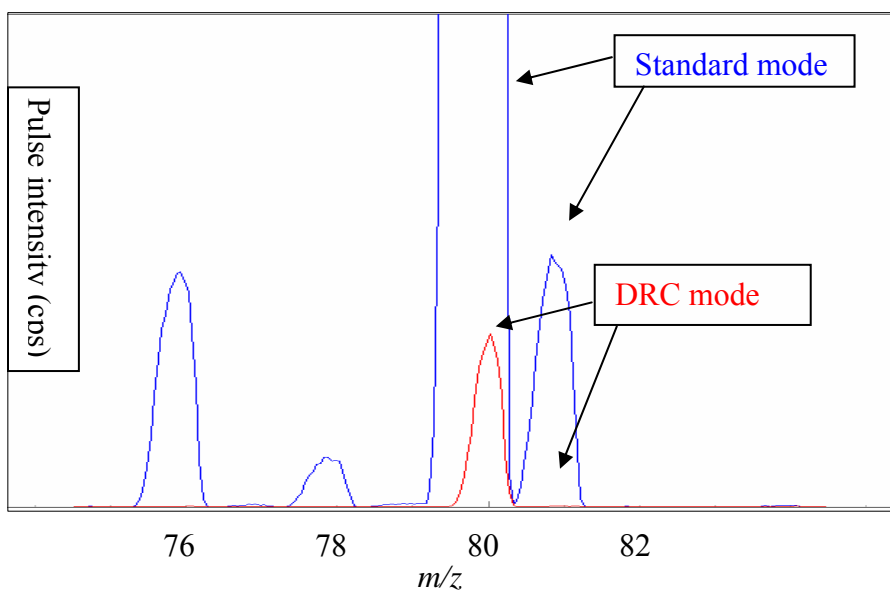
Figure 4.2: Comparison of relative background signals resulting from Ar dimers at Se analytical masses for an ELAN DRC-*e* in standard mode and in DRC mode with methane at a flow of 0.4 mL/min.

In the dynamic reaction cell, the $^{40}Ar^{40}Ar^+$ is converted to a neutral species, which is minimally transferred to the analyzer quadrupole. Therefore, $^{80}Se^+$ can be detected with much better sensitivity and lower detection limit (Figure 4.2).

In conventional/standard ICP-MS, the background at mass 80 from $^{40}Ar_2^+$ is about 6×10^5 counts per second (cps). For the ELAN DRC-*e*, the $^{40}Ar_2^+$ background was reduced to about 400 cps using CH_4 at a flow of 0.4 mL/min in the DRC, and the background for all other important Se masses was negligible (Figure 4.2). Background intensities of less than 40 cps were achievable at mass 80 using higher CH_4 flows, but not without consequences (discussed later).

4.3 Experimental

4.3.1 Instrumentation

An ELAN DRC-*e* (PerkinElmer SCIEX Inc., Ontario, Canada) instrument was used for all experiments. The instrument consists of an inductively coupled plasma (ICP) source with a plasma-shielded torch, an ion lens, dynamic reaction cell (DRC), and a quadrupole mass spectrometer with a dual mode detector. The sample introduction system consisted a cyclonic quartz spray chamber with an inserted Meinhard nebulizer. Both are made of quartz, which is resistant to contamination and is easy to clean. The integrated peristaltic pump was used to ensure a constant introduction of the liquid sample to the nebulizer, which converts the liquid sample into a finely divided aerosol. The spray chamber then isolates only the smallest droplets resulting in only about one percent of the aerosol being transmitted into the ICP torch. The DRC gas used was 99.999% pure methane (Matheson Tri-gas, Parsippany, NJ, USA).

4.3.2 Optimization of the instrumental parameters

The DRC-ICP-MS instrument was tuned for optimum signal-to-noise (S/N) ratio of ^{80}Se prior to analysis. The optimal instrumental settings are provided in Table 4.2. The primary instrumental parameter affecting the S/N ratio was the reaction cell gas flow rate, but lens voltage and cell path voltage were also somewhat important. The effects of CH_4, 5% H_2, and isobutane were tested as cell gasses. The reduction of argon dimer interferences using hydrogen and isobutane in the cell was less pronounced than that observed for CH_4. Therefore, CH_4 was selected as the reaction gas.

Table 4.2: Instrumental settings for ELAN DRC-*e* ICP-MS.

Mass Spectrometer---

ICP Rf power	1350 W
Nebulizer gas flow	0.9 L/min
Auxiliary gas flow	1.2 L/min
Plasma gas flow	15 L/min
Spray chamber	Cyclonic quartz
Interface cones	Platinum
Lens voltage	5.75 V
Autolens	on

Dynamic reaction cell parameters---

RPa	0
RPq	0.45
Quadrupole rod offset	0 V
Cell rod offset	-16 V
Cell path voltage	-18 V

Data acquisition/Analyzer parameters---

Correction for ^{78}Kr, ^{80}Kr and ^{82}Kr

Dwell time per amu	200 ms
Sweeps	20
Readings	1
Replicates	3

4.3.3 Reagents and materials

All chemicals were of analytical reagent grade. Ultra pure Milli-Q water (18 MΩ cm^{-1}) was obtained from a Millipore Milli-Q-Plus water purifier (Millipore Bedford, MA, USA). Nitric acid from J. T. Baker (Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA) was further purified by sub-boiling distillation in house. HPLC-grade methanol (Fisher Scientific) was used to prepare the standards at different concentrations. A stock standard solution of 1,000 mg/L of Se as SeO$_3^{2-}$ stabilized in 2% nitric acid was purchased from SPEX Certiprep (Metuchen, NJ, USA). Working standard solutions were prepared daily with ultra pure water.

4.3.4 Quality assurance

Analysis of method blanks, reference tissues, spiked samples and check standards were included during the analysis of most test samples. Check standards typically included 0, 4 μg/L, 10 μg/L and 20 μg/L Se solutions. For some experiments, 500 μg/L Ga or Ge was used as the internal standard to attempt to correct for signal drift and sample ionization effects.

4.4 Results and discussion

4.4.1 Elimination of polyatomic interferences

The addition of 3% MeOH was found to enhance the Se detection by a factor of 3–4. Higher concentrations of MeOH provided little improvement in response. Because one commonly-used digestion method for total selenium in tissue results in a final sample solution matrix of 4% (v/v) HNO_3, the instrumental optimization was performed with 4% HNO_3 and 3% MeOH in ultra-pure water (UP H_2O). Each analysis day, a system performance check was conducted with a 10 µg/L Mg, Cu, Rh, Cd, In, Ba, Ce, Pb, U mixture in 1% HNO_3 solution (PerkinElmer, atomic spectroscopy standard). For the selenium determinations, isotopes of 77, 78, 80, and 82 were usually monitored, depending on the experiment. The blank solution used in the instrumental optimization was 4% HNO_3 and 3% MeOH with UP H_2O. All standards and samples were also made with 4% HNO_3 and 3% MeOH in UP H_2O.

Figure 4.3 shows the effect of CH_4 cell gas flow rate on the signals of a blank solution and a standard solution of 100 µg/L selenium as SeO_3^{2-} at mass 78. It is apparent that with the increasing CH_4 flow, the signal response decreases due to the elimination of argon interfering species. Figure 4.4 demonstrates the same effect of cell gas on the selenium signal at mass 80. As can be seen from the slope of the plots, the background signal of ^{80}Se was more dramatically decreased compared with that of ^{78}Se. This is primarily because ^{80}Se has greater abundance (49.61%) vs. only 23.77% for ^{78}Se, but more importantly because the Ar-Ar^+ background is so much greater at mass 80. In the standard ICP-MS operating mode, the average background signals for ^{78}Se and ^{80}Se were about 1,000 and 600,000 cps, respectively. At 0.65 mL/min CH_4 flow rate, the

background response for ^{78}Se and ^{80}Se were 20 and 40 cps, respectively. Notably, at a gas flow rate at 0.4 mL/min, the intensity for the 100 µg/L Se at mass 80 was approximately 60,000 cps, while for the blank solution it was 400 cps. These results represented a dramatic reduction in the intensity of the Ar_2^+ interferences compared with traditional ICP-MS.
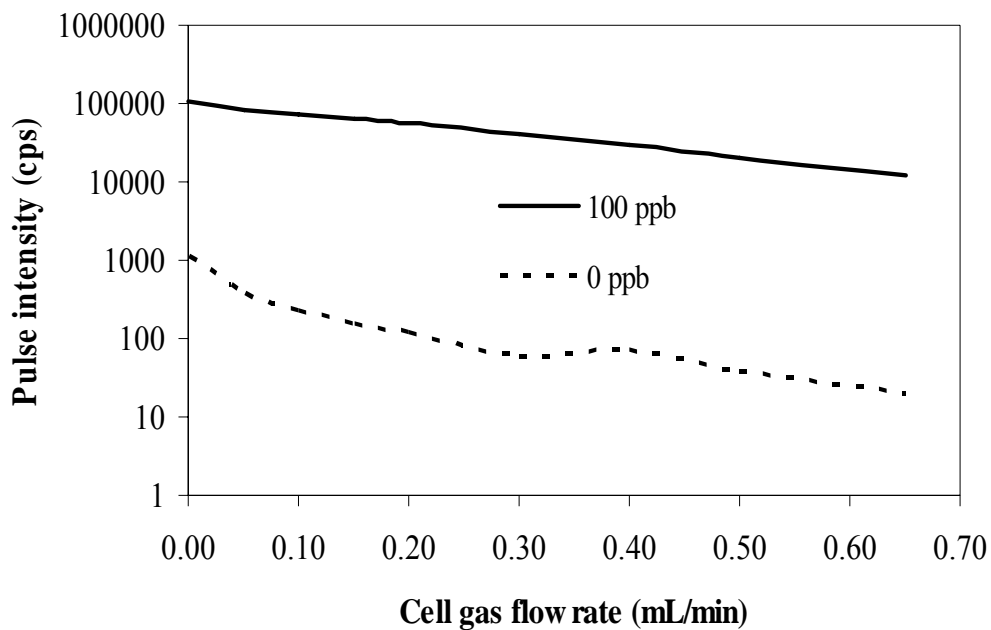
Figure 4.3: Signal intensity of ^{78}Se as a function of the CH_4 flow rate in the dynamic reaction cell.
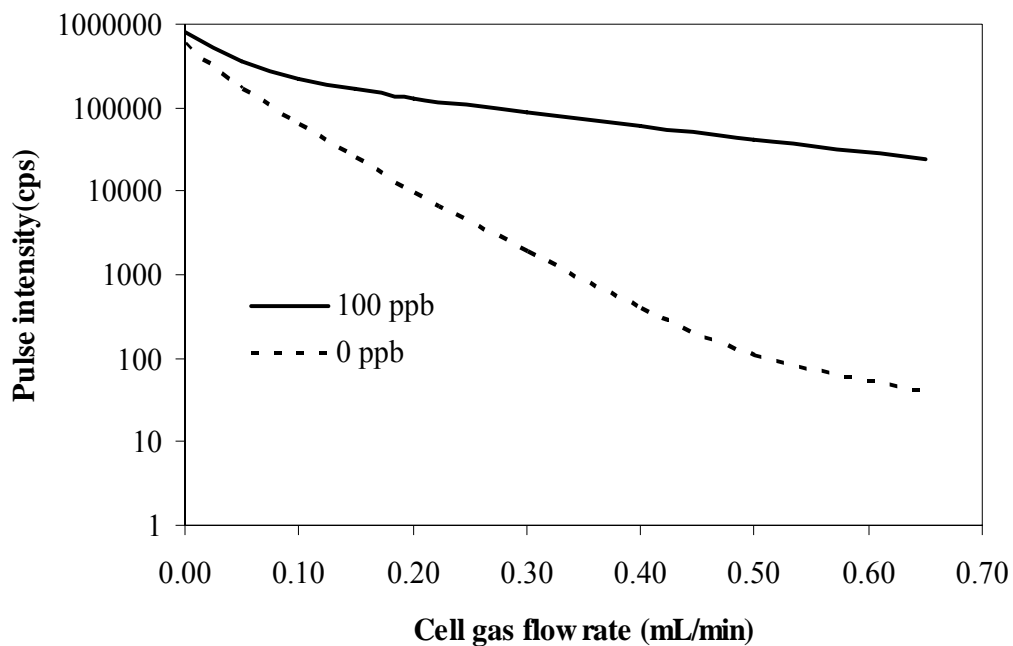
Figure 4.4:   Signal intensity of ^{80}Se as a function of the CH$_4$ flow rate in the ELAN DRC-*e* dynamic reaction cell.

4.4.2 Accuracy of isotope ratio measurements in ICP-MS

Mass discrimination is a common characteristic of mass spectrometers. It results because heavier masses are more efficiently transferred through ion focusing apparatus than lighter masses. Accordingly, when calculating isotopic ratios, a correction must be applied to account for differences in ion transfer efficiencies, which vary according to mass. For DRC-ICP-MS, pressurization of the reaction cell will affect the transmission efficiency of the ion stream because it is strongly dependent upon the mass of ions. In addition, the space charge might be altered slightly after elimination of argon interfering ions from the ion beam. Thus operation of the DRC might be expected to alter the apparent mass discrimination due to changes of ion transmission efficiencies.

Corrections for mass discrimination could be carried out by measuring the isotope ratio of standard solutions with known isotopic compositions under the same experimental conditions[8].

Table 4.3: Apparent isotopic ratios based on net intensity ratios of $^{78}Se/^{77}Se$ and $^{80}Se/^{77}Se$, and fraction of Se present as SeH^+ based on the comparison of signals at mass 82 and 83. Theoretical isotopic ratios are in parentheses.

Flow rate (mL/min)	$^{78}Se/^{77}Se$	$^{80}Se/^{77}Se$	$^{82}SeH^+/(^{82}Se+^{82}SeH^+)$
	(3.12)	**(6.50)**	**Protonation Fraction (%)**
0	3.02	22.85	0.06
0.05	2.96	13.30	2.66
0.1	2.84	9.04	6.58
0.15	2.73	7.20	9.80
0.2	2.66	6.29	12.47
0.3	2.55	5.55	16.60
0.4	2.47	5.17	19.86
0.5	2.39	4.93	22.33
0.65	2.35	4.75	25.34

Table 4.3 presents the results for the measured isotope ratios of Se vs. CH_4 flow rate for $^{78}Se/^{77}Se$ and $^{80}Se/^{77}Se$. These values were calculated from intensities corrected for any Ar background, but do not account for the possibility of SeH^+ formation. As the cell gas was increased from 0 to 0.65 mL/min, the measured isotopic ratio of $^{78}Se/^{77}Se_{measured}$ became decreasingly less than the true $^{78}Se/^{77}Se_{true}$ of 3.12. In contrast,

the $^{80}Se/^{77}Se_{measured}$ was much greater than the $^{80}Se/^{77}Se_{true}$ of 6.50 for flows up to 0.2 mL/min, but became smaller for flows greater 0.2 mL/min. Mass discrimination (discussed later) and the formation of protonated Se species (at all Se masses) largely explains the observed deviations from the true values. The higher the CH_4 flow rate, the greater the fraction of SeH^+ that was produced. This effect is demonstrated more clearly later in this chapter in Figure 4.5.

Unfortunately for selenium determination, the correction for mass discrimination is complicated greatly because of the formation of SeH^+ ions, which can be pronounced for collision or reaction cells. Selenium hydride (SeH^+) was observed by Boulyga et al. when using an ICP-MS equipped with a hexapole collision cell with hydrogen (H_2) as the reaction gas[9]. Sloth also reported the SeH^+ formation when using methane (CH_4) in the DRC-ICP-MS[6]. Selenium hydride SeH^+ may be the reaction product between selenium and hydrogen or other proton donating ions derived from the sample matrix, or from impurities of water and/or methanol in the cell gas. Consider the use of CH_4 as the reaction gas in the example below. The predominant plasma ion Ar^+ is known to react as follows:

$$Ar^+ \;+\; CH_4 \;\;\rightarrow\;\; CH_4^+ \;\;+\; Ar \tag{4.2}$$

$$\rightarrow\;\; CH_3^+ \;\;+\; Ar \;\;+\;\; H$$

$$\rightarrow\;\; CH_2^+ \;\;+\; Ar \;\;+\;\; H_2$$

$$\rightarrow\;\; ArH^+ \;\;+\; CH_3$$
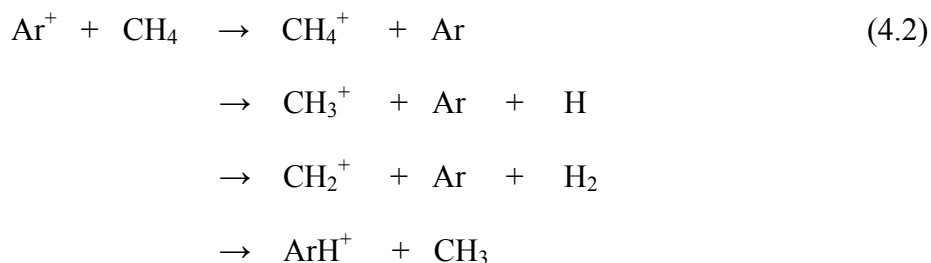
Subsequently, these intermediates can react as:

$$CH_3^+ \;\;+\; CH_4 \;\;\rightarrow\;\; C_2H_5^+ \;\;+\;\; H_2 \tag{4.3}$$

$$\rightarrow\;\; C_2H_3^+ \;\;+\;\; 2\,H_2$$
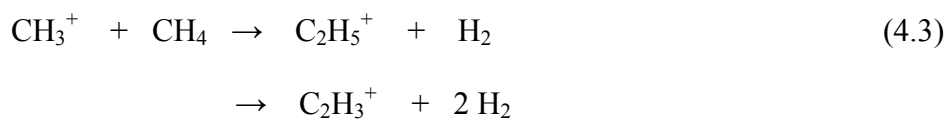
ArH^+, $C_2H_3^+$, $C_2H_5^+$, H_3O^+ and H_3^+ are good proton donors:

$$Se \; + \; ArH^+/\,C_2H_3^+/\,C_2H_5^+ \quad \rightarrow \quad SeH^+ \; + \; Ar/\,C_2H_2/\,C_2H_4 \qquad (4.4)$$

$$Se \; + \; H_3O^+ \; \rightarrow \; SeH^+ \; + \; H_2O$$

$$Se \; + \; H_3^+ \; \rightarrow \; SeH^+ \; + \; H_2$$
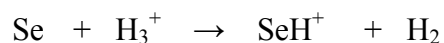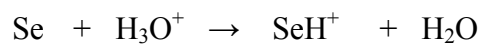
Via hydrogen atom transfer:

$$Se^+ \; + \; H_2 \; \rightarrow \; SeH^+ \; + \; H \qquad\qquad (4.5)$$
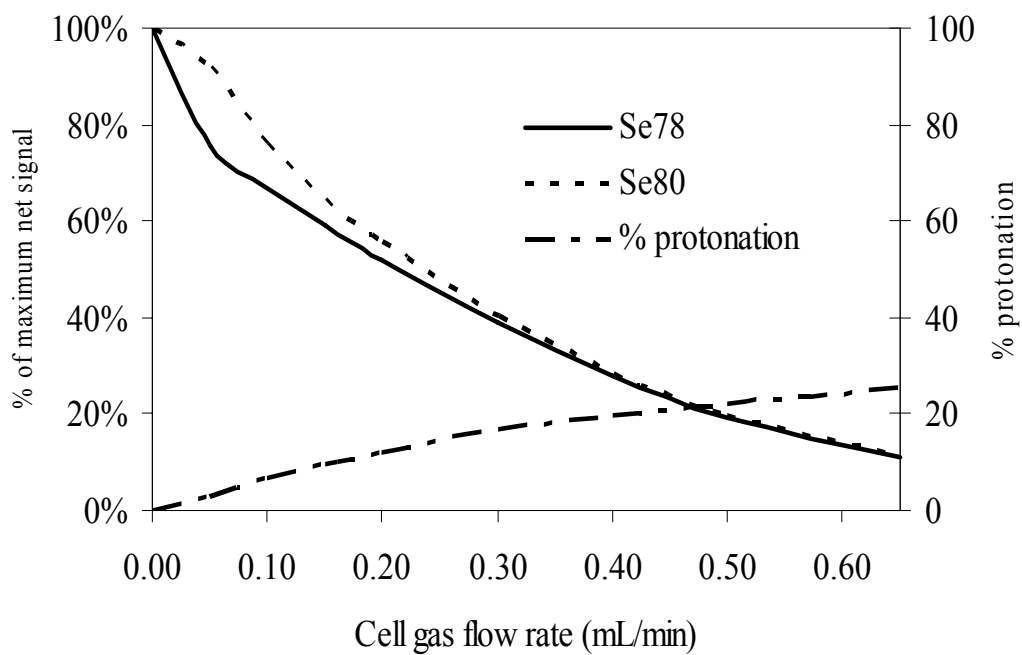
Figure 4.5:   Percentage of maximum signal intensity of ^{78}Se, ^{80}Se and the protonation percentage as a function of the CH_4 flow rate in the dynamic reaction cell.

As shown in Figure 4.5, it is obvious that the percentage of SeH$^+$ formation increased dramatically with the increase of CH$_4$ flow rate in the dynamic reaction cell, as measured by the % protonation. This relationship strongly suggests that the cell gas is the primary cause of the protonation. This contradicts the report by Sloth and Larsen, who concluded that the cell gas was not the primary cause of protonation based on experiments with deuterated methane as the reaction gas[7]. At a flow rate of 0.4 mL/min, the % protonation could reach up to 25.3%. At the same time, there was a significant decrease in signal to as low as 11.0% of the maximum net signal intensity at ^{78}Se and ^{80}Se in the absence of reaction gas. The introduction of methane changed the mass bias because CH$_4$ molecular ions may cause some ion scattering, which resulted in the change of the ion trajectory and hence ion losses. In a general sense, the serious loss of signals could be due to the formation of selenium hydrides (SeH$^+$), ion scattering, and other side reactions. The schematic figure of the protonation problem was shown in Figure 4.6.

	pf$_{As}$	pf$_{Se}$	pf$_{Se}$	pf$_{Se}$	pf$_{Br}$	pf$_{Se}$	pf$_{Br}$	pf$_{Se}$
^{75}As	^{75}AsH$^+$ ^{76}Se	^{76}SeH$^+$ ^{77}Se	^{77}SeH$^+$ ^{78}Se	^{78}SeH$^+$ ^{79}Br	^{79}BrH$^+$ ^{80}Se	^{80}SeH$^+$ ^{81}Br	^{81}BrH$^+$ ^{82}Se	^{82}SeH$^+$
Mass @ 75	Mass @ 76	Mass @ 77	Mass @ 78	Mass @ 79	Mass @ 80	Mass @ 81	Mass @ 82	Mass @ 83
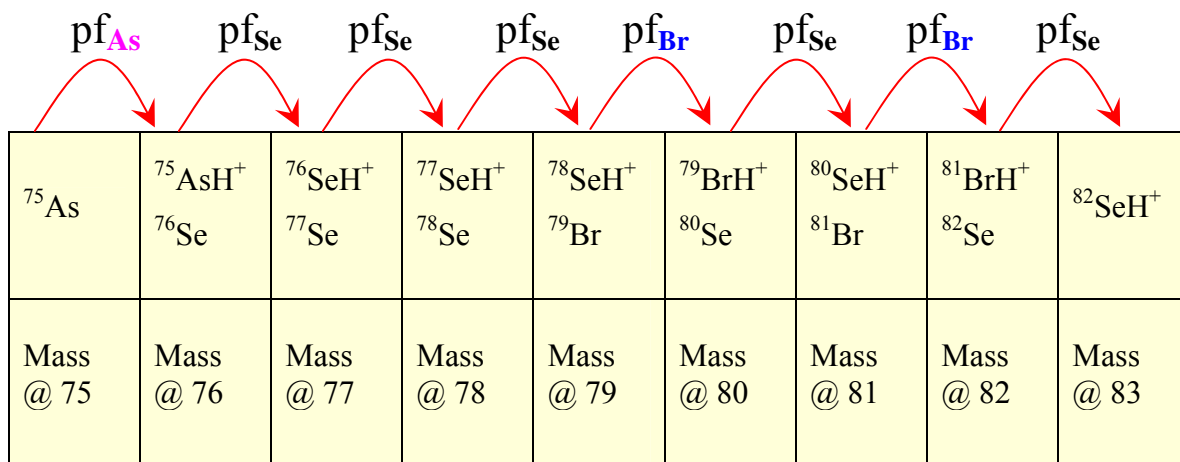
Figure 4.6:   Possible protonation reactions affecting isotopic measurements for masses between 75 and 83

The protonation fraction of SeH^+/Se was evaluated with ^{82}Se by measuring the 83/(82+83) ion ratio in the same run using a 100 μg/L Se standard in the 4% HNO_3 and 3% MeOH. The protonation fraction for As and Br was also corrected by the ion ratio of 76/(75+76) for AsH^+ with a 100 μg/L As standard and 82/(81+82) for BrH^+ with a 100 μg/L Br standard in 4% HNO_3 and 3% MeOH, respectively. Standard solutions of Se, As, and Br (all of natural isotopic composition) were analyzed daily for this purpose. The measured intensities at mass 76, 77, 78, 79, 80, 81, 82, and 83 were corrected using mathematical equations 1 to 8. Since the natural abundance of ^{74}Se is only 0.89%, the contribution to ^{75}As from $^{74}SeH^+$ or $^{74}GeH^+$ (low concentration and low protonation) was assumed to be negligible. The measured intensities for ^{76}Se were corrected by taking into account the formation of $^{75}AsH^+$ and $^{76}SeH^+$. The measured intensities at other masses were corrected the same way, considering both $(m-1)H^+$ and $(m+1)H^+$ at the same time. The intensity correction equations used were as follows:

$$^{76}Se_{corr} = [^{76}I - {}^{75}As/(1-pf_{As}) * pf_{As}] / (1-pf_{Se}) \tag{4.6.1}$$

$$^{77}Se_{corr} = [^{77}I - {}^{76}Se_{corr} * pf_{Se}] / (1-pf_{Se}) \tag{4.6.2}$$

$$^{78}Se_{corr} = [^{78}I - {}^{77}Se_{corr} * pf_{Se}] / (1-pf_{Se}) \tag{4.6.3}$$

$$^{79}Br_{corr} = [^{79}I - {}^{78}Se_{corr} * pf_{Se}] / (1-pf_{Br}) \tag{4.6.4}$$

$$^{80}Se_{corr} = [^{80}I - {}^{79}Br_{corr} * pf_{Br}] / (1-pf_{Se}) \tag{4.6.5}$$

$$^{81}Br_{corr} = [^{81}I - {}^{80}Se_{corr} * pf_{Se}] / (1-pf_{Br}) \tag{4.6.6}$$

$$^{82}Se_{corr} = [^{82}I - {}^{81}Br_{corr} * pf_{Br}] / (1-pf_{Se}) \tag{4.6.7}$$

$$^{83}I = {}^{82}Se_{corr} * pf_{Se} \tag{4.6.8}$$

Where

pf   =   protonation fraction

I   =   Measured intensity

I_{corr} = Corrected intensity

At a cell methane flow rate of 0.4 mL/min, protonation fractions for As, Se and Br were found experimentally to be about 0.002, 0.25 and 0.004, respectively.

4.4.3 Critical evaluation of DRC-ICP-MS

The analyses of many elements can be accurately performed by ICP-MS with an external standard calibration and the addition of an internal standard (usually on-line) that is similar in both mass and ionization characteristics to correct for matrix effects. However, the selection of a suitable internal standard for total Se determination is problematic because Se ionization is highly sensitive to sample matrix effects. Commonly used internal standards, including Ge and Ga, were investigated to correct for sample matrix effects, but the results were unsatisfactory, presumably because the ionization behavior of those elements did not adequately match that of Se. Matrix matching of standards to samples can also be used to correct for matrix effects, but that approach will be unreliable if sample components are unknown or inconsistent. For these reasons, stable isotope dilution analysis (SIDA) was considered for Se quantitation. The final SIDA method used is described in detail in Chapter 5. As explained, for the DRC mode it will be necessary to determine the protonation fraction and correct for both protonation and mass bias when conducting measurements that require accurate isotopic

ratios. Furthermore, the large loss of net Se signal intensities for the DRC mode could not be overcome by adjustment of operating conditions (RPq value and lens voltage etc.). The possibility was considered that the standard mode analysis of a less abundant Se isotope for which Ar-Ar background intensity is not overwhelming might be comparable to the results for DRC mode analysis using a more abundant isotope. The potential advantage of the standard mode is that protonation is minimal, which will make an analysis by SIDA less complicated than for the DRC mode. Preliminary SIDA analyses of previously acid-digested reference tissues that were individually spiked with [77]Se enriched isotope were compared using both the standard and DRC modes. The figures of merit using the optimized operating conditions for either standard or DRC mode at Se masses 76, 77, 78, 80, and 82, standard ICP-MS mode using either the [78]Se or [82]Se mass appeared to be equivalent to, or better than that for any mass/DRC combination. The average recoveries for DRC and standard ICP-MS at mass 78 were 98.8% and 96.8%, respectively. The range of recoveries was 88-109% for the DRC mode and 85-108% for the standard mode.

In summary, the performance of DRC-ICP-MS using methane as the reaction gas was investigated. The Ar-Ar background signals were reduced significantly at the price of the large loss of net selenium signal intensities in the DRC mode. The results were not satisfactory as expected. SIDA-ICP-MS in the standard mode was considered for Se determination and quantitation and details about this method will be further described in the following chapter.

4.5 References

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Chapter 5: Total Se Determination in Yeasts and Aquatic Organisms

5.1 Introduction

The current research began by dosing live oligochaetes (*Lumbriculus variegatus)* with selenized yeast enriched with selenomethionine (SeMet) to produce a food source for fish with a range of selenium concentrations. The range of concentrations varied from low to toxic concentrations for some fish species. The endangered desert pupfish was fed Se-dosed diets consisting of live, laboratory-cultured oligochaetes. Figure 5.1 shows pictures of the endangered desert pupfish and lab-cultured oligochaetes. Toxicity tests of dietary selenium to the pupfish will be used to evaluate whether selenium contamination of agricultural drains and shoreline pools adjacent to the Salton Sea pose a threat to desert pupfish. The objective of the current research is to develop an easily applied, scientifically credible procedure for determining total Se and seleno-amino acid concentrations in biological matrices, which is the focus of this dissertation. The efficiency of different digestion methods and the distribution of seleno-amino acid species in biological matrices are also evaluated (in Chapters 5 and 6).

Hydride generation-atomic absorption spectrophotometry (HG-AAS)[1,2] and instrumental neutron activation analysis[3] have been applied to determine the total selenium concentration. Chapter 5 mainly describes the total determination of selenium in yeasts and aquatic organisms by on-line stable isotope dilution analysis combined with ICP-MS. The methods for extracting selenium from small sample sizes were evaluated during the experiments.
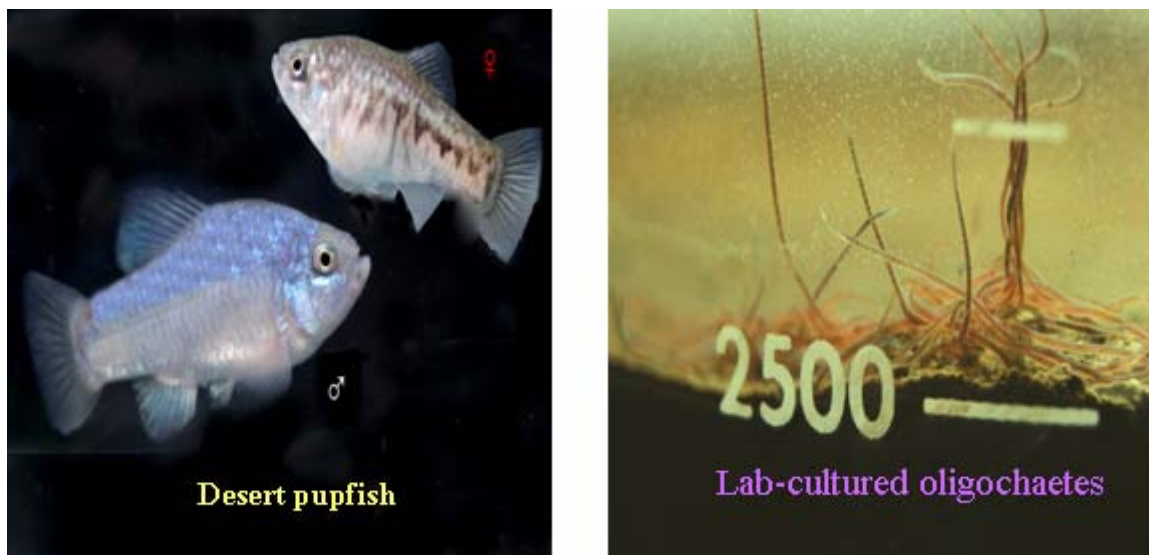
Figure 5.1: Photographs of desert pupfish and lab-cultured oligochaetes.
(Courtesy of J.M. Besser, CERC, USGS.)

5.2 Experimental

5.2.1 Instrumentation and reagents

Instrumentation and reagents were as described in Chapter 4, unless otherwise stated. The standard mode of ICP-MS was employed and masses 77, 78, 79, 81, and 82 were typically monitored. The syringe was from HENKE SASS WOLF (Tuttlingen, Germany). 0.45 μm polypropylene filters were purchased from Whatman Inc. (Florham Park, NJ, USA). All the reference materials used for quality assurance were purchased from National Institute of Standards and Technology (Gaithersburg, MD, USA) or the National Research Council of Canada (Ottawa, ON, Canada). Selenized yeast, Selenosource AF 600, was obtained from Diamond V Mills (Cedar Rapids Iowa USA), a manufacturer of animal feeds and feed supplements. The control yeast was Red Star™

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Nutritional yeast and was obtained from Lesaffre Yeast Corporation (Milwaukee Wisconsin). Ultrex II ultrapure hydrogen peroxide (H_2O_2, 30%) was obtained from J. T. Baker (Mallinckrodt Baker, Inc. Phillipsburg, NJ, USA). Se calibration standards of 0, 5, 10, 20, and 40 μg/L (ppb) were prepared from a stock solution of 10 mg/L in 4% HNO_3, 3% MeOH. Selenium enriched in [77]Se (99.96% vs. natural abundance of 7.63%) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). A 100 μg/L [77]Se solution was made in 4% HNO_3 and 3% MeOH with UP H_2O.

5.2.2 Sample preparation

Samples were lyophilized in a Virtis Genesis® 35EL freeze dryer. After lyophilization, all samples were homogenized by grinding with a glass rod against the container surface.

A digestion method suitable for comparatively small sample sizes and the number of samples was developed in anticipation of the need to determine selenium in small fish organs. For total Se analysis, 50 mg of lyophilized samples were weighed into a dry, clean borosilicate test tube (25 mL). 1 mL sub-boiled HNO_3 was added and the samples were predigested at room temperature for a minimum of 30 min. The digestion was performed in the well-heating block for 30 min at 110 ± 10°C. 1 mL high-purity H_2O_2 was added dropwise. After cooling, each sample was diluted to 25 mL with 3% v/v methanol in UP H_2O. The final analysis matrix was 4% HNO_3 and 3% MeOH. Before analysis with ICP-MS, samples were all filtered using an all polypropylene syringe and filter cartridge. The selenium determination was conducted by SIDA-ICP-MS.

5.2.3 Selenium measurement with on-line SIDA-ICP-MS

In Chapter 4, the figures of merit using the optimized operating conditions for either standard or DRC mode at Se masses 76, 77, 78, 80, and 82 were compared. The standard ICP-MS mode using either ^{78}Se or ^{82}Se appeared to be equivalent to, or better than that for any mass/DRC combination. In order to overcome the polyatomic interferences at ^{78}Se and/or ^{82}Se, on-line stable isotope dilution-ICP-MS was adopted to obtain more accurate and precise measurements.

5.3 Introduction of stable isotope dilution analysis (SIDA)

Isotope dilution analysis (IDA) is an analytical technique to apply isotopes in chemical analysis, which was first introduced by Hevesy and Hobbie in 1932[4]. The basis of IDA is that a known accurate amount of an isotope of the element of interest is added as a spike to the sample. When radioisotopes are used, the mixing by its non-radioactive counterpart results in the reduction/dilution of the specific activity defined as the activity per unit mass or volume. The underlying principle is that the specific activity of the spike and sample is conserved even upon dilution. After isolating a pure portion of the analyte-spike component from the sample, the specific activity of the isolated portion is used to back calculate the amount of analyte in the original sample. There are many variations of the basic technique. The application of non-radioisotopes --- stable isotopes was first initiated in the 1940's with C, N, O and H[5,6]. With more availability of mass spectrometers and electromagnetically separated isotopes, stable isotopes were more widely applied.

Stable isotope dilution analysis (SIDA) is an excellent and important quantification method that enables the independent yet accurate measurement of the ion current of individual stable isotopes for a given element. It shares the same principles with the ordinary IDA method. When stable isotopes are used, the isotopic ratios instead of specific activities are determined with a mass spectrometer. The extent of the isotopic dilution suffices to establish the original concentration of the analyte of interests.

SIDA offers a number of advantages that are ideally suited for studies in complex biological samples. The major merit of this method is the ability to use a non-quantitative isolation procedure in the separation of analytes, which provides considerable simplicity, speed, robustness and flexibility in the design of separations. The disadvantages of this method are that it is only suitable for elements with multiple isotopes and the cost of obtaining highly enriched isotopes can be high in some instances. In addition, for greatest accuracy, the concentration of the spiked isotope should be close to the concentration in each sample, which may require a second preparation if sample concentrations are unknown. ICP-MS is a great tool to detect the selenium isotopes with great sensitivity. Naturally, SIDA-ICP-MS can afford accurate and precise determination for selenium in biological samples because it can compensate for instrument instabilities and interferences from the sample matrix.

5.3.1 Analytical procedures for selenium determination with on-line SIDA-ICP-MS

For the standard ICP-MS mode, background intensities must be accounted for before analysis. Typically, analysis of the blank solution (e.g. 4% HNO_3 and 3% MeOH) was first performed repeatedly until a stable result was obtained, which provided the

intensities used to correct for Ar-Ar background signals at all relevant masses.

Afterwards, a 100 μg/L ^{77}Se solution in 4% HNO_3 and 3% MeOH was pumped through

the internal standard (I.S.) tubing to provide on-line addition of the stable isotope

continuously throughout the analysis. A blank solution, (4% HNO_3 and 3% MeOH), and

standards of 4 μg/L, 10 μg/L and 20 μg/L Se (natural isotopic abundance; as SeO_3^{2-}) were

analyzed to evaluate net signal intensities. More importantly, these standards were also

used to periodically check the SIDA calculation equations and to empirically determine

the actual effective concentration of the on-line ^{77}Se standard solution being delivered to

the ICP-MS. Measurement of these standards and the subsequent adjustment of the

effective on-line concentration of ^{77}Se during final calculations in essence provided a

correction for any mass bias between the signal intensities observed at $m/z = 77, 78$ and

82 for formation of small fractions of SeH^+. This procedure also corrected for the small

wearing of tubing, the constant mixing of samples and internal standard, instrumental

drift, and any sample matrix effects. A spreadsheet was formulated that incorporated the

appropriate calculations using net intensities at each mass, and which allowed for the

effective concentration of the ^{77}Se internal standard to be adjusted separately for either

^{78}Se or ^{82}Se as the analytical mass (Appendix A). Theoretically, when 100 μg/L ^{77}Se was

used for the internal standard , the effective concentration that was mixed on-line with

each sample solution was 3.7 μg/L ^{77}Se, based on the relative internal diameters of

sample and internal standard pump tubings. In practice, values of about 4.6 μg/L and 4.0

μg/L for determinations involving ^{78}Se and ^{82}Se, respectively, provided the most accurate

results for the standards. Again, these deviations from the expected value of 3.7 μg/L are

largely due to mass bias, which was empirically corrected for by the adjustment of the effective ^{77}Se concentrations after analysis.

The analyte concentrations at mass 78 and 82 were calculated using the following SIDA-MS equation[7].

$$C_{sa} = C_{sp,\,effective} * (M_{sa}/M_{sp}) * (a_{sp}^1 - (R * a_{sp}^2))/(R * a_{sa}^2) - a_{sa}^1)) \qquad (5.1)$$

C_{sa} = Concentration of element in sample (μg/mL)

$C_{sp,\,effetive}$ = Effective concentration of spike element solution (μg/mL)

M_{sa} = Molar mass of analyte element in sample (g/mole)

M_{sp} = Molar mass of spike element (g/mole)

a_{sa}^1 = Isotope abundance of isotope 1 in sample

a_{sa}^2 = Isotope abundance of isotope 2 in sample

a_{sp}^1 = Isotope abundance of isotope 1 in spike

a_{sp}^2 = Isotope abundance of isotope 2 in spike

R = Measured ratio of isotope 1/ isotope 2 in spiked sample

$$C_{78_{Se}} = 0.0046 * (78.96/76.9199) * (0.0002-(R*0.9996))/(R*0.0763)-0.2377)) \qquad (5.2)$$

$$C_{82_{Se}} = 0.0040 * (78.96/76.9199) * (0.00005-(R*0.9996))/(R*0.0763)-0.0873)) \qquad (5.3)$$

The natural isotopic ratios of ^{78}Se/^{77}Se and ^{82}Se/^{77}Se are 3.12 and 1.14, respectively. In the enriched spike they are 0.0002 and 0.00005. From the ^{78}Se/^{77}Se and

^{82}Se/^{77}Se values listed in Appendix A, both ^{78}Se/^{77}Se and ^{82}Se/^{77}Se differed distinctively from the natural isotopic ratios and the spike isotopic ratios, which suggested an optimized spiking process[7]. This is essential for the precise determination with respect to the influence of the error multiplication factor.

Signal intensities are input automatically and corrections are applied to determine the isotope ratios observed for samples. Once the effective ^{77}Se concentration was determined (empirically), the calculated concentrations for the 0, 4, 10, and 20 µg/L standards, which were measured at the beginning, middle and end of the analytical run, yielded very consistent results despite the variations in raw intensity values. For example, at mass 78, the 20 µg/L standard (ICV3 and CCV3) produced raw intensities of 14,492, 14,502 and 16,350 cps, where calculated concentrations were 19.74, 19.59 and 19.83 µg/L (Appendix A). The protonation fraction for BrH$^+$ was calculated as 0.004 from 82/(81+82) using a 100 µg/L natural bromine standard in 4% HNO$_3$ and 3% MeOH. The protonation fraction was applied to all intensities in the reference materials and pupfish samples to obtain greater accuracy for mass 82 quantitation when Br was present. The IAEA 407 whole fish tissue had the greatest Br concentrations; raw intensities at 79 and 81 were more than 100,000 cps (Appendix A). Pupfish samples also contained significant concentrations of Br, presumably because they were cultured in 5 parts per thousand saline water. With the BrH$^+$ correction, the measured concentration for IAEA 407 was 2.71 µg/g dry weight at ^{82}Se, whereas quantitation at mass 78 yielded 2.68 µg/g dry weight (Table 5.1). Both values were consistent with the certified value of 2.83 ± 0.13 µg/g dry weight. These results demonstrated that on-line SIDA with BrH$^+$ correction could be applied successfully to the Se determination in biological samples.

Both mass 78 and mass 82 should be monitored to provide assurance of accuracy. The Ar-Ar background will limit the accuracy for low concentrations at mass 78, whereas the presence of Br will limit the accuracy at mass 82.

Table 5.1: SIDA-ICP-MS Selenium Calculation Worksheet.

Sample ID	Sample description	Sample weight (g)	Dilution volume (mL)	Mass at 78	Mass at 82
39463	pupfish control day 56 rep E-1	0.0499	25.00	0.0930	0.903
39467	pupfish low day 56 rep E-1	0.0503	25.00	3.86	3.81
39471	pupfish medium low day 56 rep E-1	0.0497	25.00	4.55	4.42
39475	pupfish medium day 56 rep E-1	0.0503	25.00	7.35	7.29
39479	pupfish medium high day 56 rep E-1	0.0292	25.00	14.6	14.7
39483	pupfish high day 56 rep E-1	0.0306	50.00	22.5	22.4
37479-dup	pupfish medium high day 56 rep E-1	0.0295	25.00	14.5	14.6
QC 112	IAEA 407 whole fish tissue	0.0507	25.00	2.68	2.71
QC 117-1	NRCC SELM-1 (1+249)	0.0316	6250	1.98×10^3	2.02×10^3
QC 117-2	NRCC SELM-1 (1+249)	0.0301	6250	1.99×10^3	2.03×10^3
QC 117-3	NRCC SELM-1 (1+249)	0.0303	6250	2.03×10^3	2.04×10^3

5.3.2 Analysis of oligochaetes and yeast samples

The total selenium concentrations for lab-cultured oligochaetes (worms) and selenized yeasts are listed in Table 5.2 (original data is listed in Appendix B). These data, combined with the experimental toxicity results, indicated that dosing worms with Se-yeast resulted in predictable Se bioaccumulation, without toxicity, in a range desired to conduct dietary toxicity tests with desert pupfish. Oligochaetes fed undiluted selenized yeast (812 µg/g Se dry weight) showed reduced biomass, but oligochaetes fed yeast diets diluted with nutritional yeast (51 to 188 µg Se/g) had stable or increasing biomass and accumulated Se concentrations as high as 105 µg/g. First-order kinetic models indicated that oligochaetes fed yeast diets below toxic levels reached over 90% of equilibrium concentrations in 28-d exposures. Repeated sampling from 28- to 42-d exposures of oligochaetes to Se-enriched yeasts should provide live diets with consistent Se concentrations in the ranges desired for dietary toxicity studies.

Table 5.2: SIDA-ICP-MS results of total Se concentration for lab-cultured oligochaetes.

Dosage	Exposure (days)	Sample type	Total Se (µg/g dry weight)
Low	7 (short-term)	Oligochaetes	14.76 ± 2.64 (n=6)
Medium	7 (short-term)	Oligochaetes	50.39 ± 4.04 (n=6)
High	7 (short-term)	Oligochaetes	168.4 ± 28.1 (n=6)
Very low	28 (long-term)	Oligochaetes	7.530 (n=2)
Low	28 (long-term)	Oligochaetes	24.87 (n=2)
Medium	28 (long-term)	Oligochaetes	75.13 (n=2)
Very low	35 (long-term)	Oligochaetes	8.200 (n=2)
Low	35 (long-term)	Oligochaetes	28.71 (n=2)
Medium	35 (long-term)	Oligochaetes	85.97 (n=2)
Very low	42 (long-term)	Oligochaetes	9.800 (n=2)
Low	42 (long-term)	Oligochaetes	31.09 (n=2)
Medium	42 (long-term)	Oligochaetes	103.3 (n=2)

Table 5.3 shows the analysis results of selenium in biological certified reference materials digested along with the oligochaetes and yeast samples. Measured Se concentrations were consistently within the certified values at the 95% confidence interval (CI). It is worth noticing that SIDA-ICP-MS measurement for certified reference yeast (SELM-1) was 2028 ± 12 µg/g (n =3), which was in close agreement with the certified value of 2059 ± 64 µg/g. This reference material was used for subsequent experiments for both total Se and organic seleno-amino acid measurements.

Table 5.3: Selenium analysis of biological certified reference materials with SIDA-ICP-MS (n =3 for each).

CERC #	Reference sample	Measured Se ± 1 SD (µg/g)	Certified value ± 95% CI	% Recovery (from 95% CI)
QC 55	NIST 1566a Oyster	2.050 ± 0.020	2.210 ± 0.240	100%
QC 117	NRCC selenized yeast	2028 ± 13	2059 ± 64	100%
QC 108	NRCC Dogfish Liver	5.731 ± 0.009	6.060 ± 0.490	100%
QC-2	USGS whole striped bass	2.269 ± 0.001	2.260 ± 0.200	100%
QC-53	NIST RM50 TUNA	3.877 ± 0.013	3.600 ± 0.400	100%
QC-70	NRCC Lobster Hepatopancreas	6.365 ± 0.050	6.880 ± 0.470	99%
QC-72	NIST Bovine Liver	0.7100 ± 0.0050	0.7300 ± 0.0600	100%
QC 98	IRMM COD fillet	1.629 ± 0.025	1.630 ± 0.070	100%

Table 5.4: Spike recoveries in oligochaetes added before digestion.

Dosage	Exposure (days)	Spike Recovery (%)
Very low	42	92.50
Low	42	95.20
Medium	42	93.90

Table 5.5: Se determinations of oligochaetes duplicates (n=1).

Dosage	Exposure (days)	rep-1 Se ($\mu g/g$)	rep-2 Se ($\mu g/g$)	RPD (Relative percent difference, %)
Low	35	27.44	27.33	0.4
Medium	35	89.13	87.06	2.4
Very low	42	11.52	11.41	1.0
Low	42	31.57	31.76	0.6
Medium	42	129.0	126.3	2.1

As can be observed in Table 5.4, recoveries of selenium (SeO_3^{2-}) spiked into samples before digestion ranged from 92% – 95%, and averaged 94%. Relative percent differences for selenized yeasts ranged from 0.4% to 2.4% (Table 5.5). Selenium concentrations for digestion blanks ranged from -0.012 to 0.050 $\mu g/g$ (n = 10), with a method detection limit of 0.067 $\mu g/g$ dry weight calculated as three times the standard deviation of the measured Se blank concentrations. All digestion blanks were relatively low, but all samples were blanked corrected never-the-less.

Calibration verification solutions were always analyzed at the beginning, middle and the end of each batch analysis of samples. As shown in Table 5.6, instrumental precision for selenium determined by repeated analysis of four standards (0, 4, 10, and 20 µg/L) throughout the run was less that 2% from the actual values (n =3).

To check for possible SIDA-ICP-MS bias specific with oligochaetes and yeast tissues, splits of four yeast and three oligochaetes samples were submitted for cross-

Table 5.6: Typical instrumental blanks and calibration verification results using SIDA-ICP-MS (n=3).

Verification category	Actual concentration (µg/L)	Measured concentration (µg/L)			Mean difference from actual
		Minimum	Maximum	Mean	
Blank (IBV/CBV)	0.00	-0.01	0.00	-0.01	n/a
Calibration (ICV/CCV)	4.00	3.97	4.02	4.00	0.0%
Calibration (ICV/CCV)	10.00	10.03	10.16	10.11	1.1%
Calibration (ICV/CCV)	20.00	20.33	20.42	20.36	1.8%

check analysis by an independent method – Neutron activation analysis. Neutron activation analysis (NAA) is the most common form of activation analysis[8]. In NAA, the analyte is irradiated with a flux of neutrons. It induces a nuclear reaction and results in the excited radioactive intermediate of the analyte elements. The intermediates then de-excite/decay by emitting beta particles (β^-) and delayed gamma rays (γ) with a unique half-life. The delayed gamma radiation is detected using a high-resolution gamma-ray

spectrometer in order to obtain both qualitative and quantitative analytical information. Typically, NAA offers superior sensitivities on the order of parts per billion or better and hence is often recognized as the "referee method", especially when developing a new procedure or solving the discrepancy between methods.

Table 5.7: Comparison of selenium analysis results between SIDA-ICP-MS and NAA
for selected samples of lab-cultured oligochaetes and selenized yeasts.

CERC #	CERC Toxicology ID	Sample Type	SIDA-ICP-MS Se (µg/g dw) (n=1)	NAA Se (µg/g dw) (n=1)	Percent difference between methods (ICPMS / NAA)
36842	L-SE-1	yeast	50.89	45.00	13.1%
36844	VL-SE-1	yeast	15.42	14.45	6.7%
36846	M-SE-1	yeast	193.9	171.2	13.2%
36848	H-SE-1	yeast	785.4	789.7	-0.5%
36857	L-VL-2 day35	oligochaetes	8.140	8.150	-0.2%
36859	L-L-2 day35	oligochaetes	29.98	27.18	10.3%
36861	L-M-2 day35	oligochaetes	82.81	84.21	-1.7%
				overall RPD	5.9%

Table 5.7 compares the selenium analysis results for single determinations of selected lab-cultured oligochaetes and selenized yeasts from SIDA-ICP-MS and NAA performed by the University of Missouri Research Reactor (MURR). As can be seen,

SIDA-ICP-MS results were similar to the NAA results. The overall relative percent difference was +5.9% for SIDA-ICP-MS as compared to NAA, which was considered satisfactory for this investigation. The reason why measured concentrations for SIDA-ICP-MS tended to be greater than NAA is unclear, but sample heterogeneity may have been a possible contributing factor. In addition, the small sample size of 20-50 mg and non-cryogenically pulverized samples may cause the discrepancy between the two measurement results. Additional sample analyses using both methods would be needed to clearly determine the extent of bias for either method. A more comprehensive comparison is planned in the future.

5.3.3 Analysis of whole desert pupfish

Table 5.8: Measured total Se concentrations in desert pupfish at day 56 of study (n=4).

Sample description	Exposure (days)	Matrix	Total selenium (μg/g dry weight)
pupfish control	56	whole fish	0.8125 ± 0.0785
pupfish low	56	whole fish	3.618 ± 0.393
pupfish medium low	56	whole fish	4.098 ± 0.317
pupfish medium	56	whole fish	6.750 ± 0.472
pupfish medium high	56	whole fish	12.54 ± 1.42
pupfish high	56	whole fish	21.23 ± 1.30

Four sets of desert pupfish were analyzed after 56 days of exposure. Each sample was a composite consisting of five individual fish. The SIDA-ICP-MS measurements based on mass 82 are listed in Table 5.8 (see original data in Appendix C). The fish, according to dietary dosage, increasingly accumulated selenium from the lab-cultured oligochaetes.

The recovery of selenium (SeO_3^{2-}) spiked into the digestion blank before digestion ranged from 93% – 96% and averaged 94%. The average recovery of selenomethionine (SeMet) spiked into the pupfish samples before and after digestion was 95% and 96%, respectively. The relative standard deviation between triplicate sample determinations of whole pupfish dosed at the medium treatment was 1.89% with the mean of 6.790 µg/g dry weight. The relative standard deviation between the analysis triplicates of the same samples was 0.66% with the mean of 6.850 µg/g dry weight. The total Se measurement for the certified reference yeast sample was 2028 ± 13 µg/g dry weight, agreed well with the certified value of 2059 ± 64 µg/g dry weight.

In summary, the simple and accurate on-line stable isotope dilution with ICP-MS was successfully applied to the determination of total selenium in biological materials, such as lab-cultured oligochaetes, selenized yeasts and certified reference materials. The SIDA-ICP-MS method will be applied to all future sample analyses.

5.4 References

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Chapter 6: Determination of Seleno-amino Acids in Yeasts and Aquatic Organisms

6.1 Introduction

This chapter describes optimization experiments for the separation and quantitation of seleno-amino acids by LC-ICP-MS and the structural identification/characterization of possible unknowns by LC-ESI-MS and MS/MS. The efficiencies of different extraction methods were evaluated and chromatographic operating conditions were investigated. The goal of these studies was to develop a low-cost analytical method that is suitable for routine determination of seleno-amino acids in small (10-100 mg dry weight) samples of biological tissues.

6.2 Detection of selenium compounds by LC-ICP-MS

6.2.1 Introduction

As mentioned in Chapter 3, several different approaches have been attempted to separate selenium compounds using ion-exchange, reversed-phase, ion-pairing reversed-phase and size-exclusion chromatography. For this investigation, only two LC separation methods were evaluated: cation-exchange chromatography and ion-pairing reversed-phase chromatography.

6.2.2 Instrumentation and chemicals

Instrumentation and chemicals were as described previously, unless otherwise noted. DL-selenomethionine (SeMet), DL-selenocystine (SeCys$_2$) and Se-methyl-

selenocysteine hydrochloride (MeSeCys) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in 0.1% HCl and Milli-Q-Plus water. Their structural formulae are shown in Figure 6.1. Stock solutions of 100 mg/L calculated as selenium were stored in the dark at 4ºC and working standard solutions are prepared daily by dilution. Methanesulfonic acid (CH_3SO_3H, 99%) was purchased from Acros Organics (New Jersey, USA). Protease type XIV and pronase E (protein-cleaving enzymes), 2,3-dimercapto-propanol, and trifluoroacetic acid (TFA, 99%) were obtained from Sigma-Aldrich. An ultrasonic bath (model 2510R-DTH) obtained from Branson Ultrasonics Corporation (Danbury, CT, USA) was used for some experiments. Beckman model Avanti™ J-25 and Grant model SS40-5 (Science/Electronics, Miamisburg, Ohio, USA) centrifuges were used for enzyme digestion procedures.

Selenomethionine (SeMet)

Se-methyl-selenocysteine (MeSeCys)

Selenocystine (SeCys2)
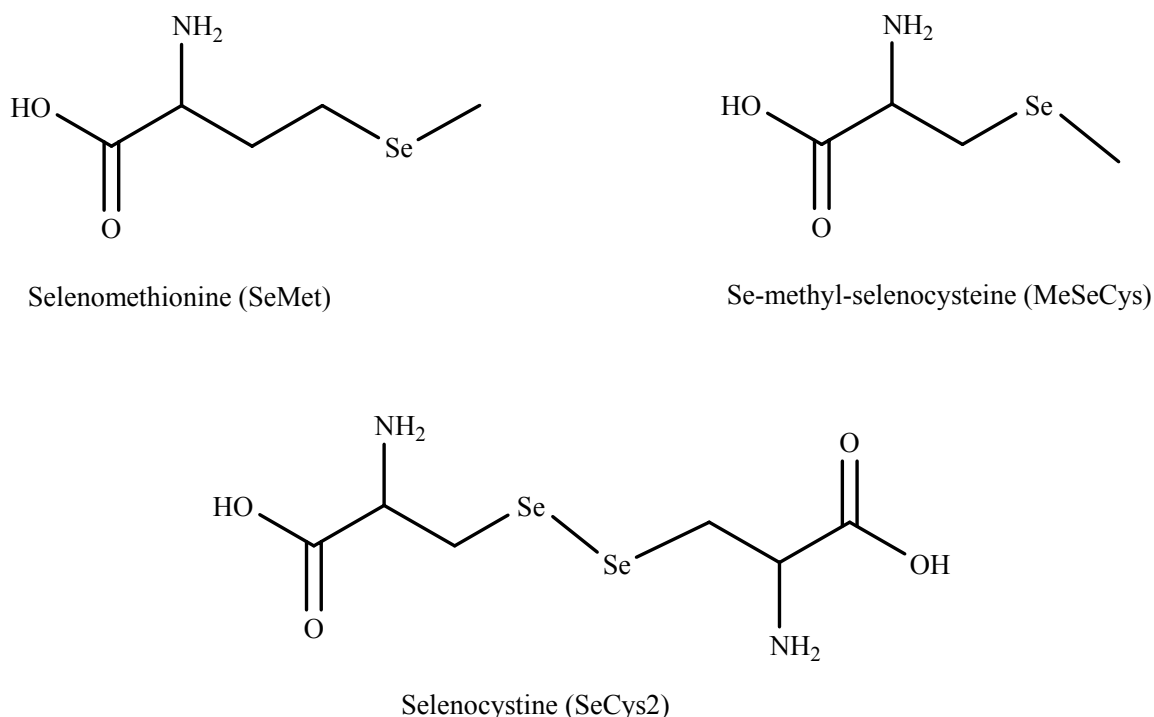
Figure 6.1: Structural formulae for SeMet, MeSeCys and SeCys$_2$.

For liquid chromatographic analysis, a PerkinElmer series 200 metal-free quaternary HPLC pump and series 200 autosampler (PerkinElmer, CT, USA) were used. The outlet of the chromatographic column was connected to the Meinhard nebulizer of the ICP-MS through polytetrafluoroethylene (PTFE) tubing. Once the batch analysis was initiated, the LC pump and the ICP-MS were triggered by software control of the autosampler, which allowed for sample introduction and chromatographic separation of selenium species. Mass 82 was monitored for quantitation because it had adequate signal response and the lowest background signal.

For the cation-exchange chromatographic separation, a polymer-based strong cation exchange OmnipacPCX-500 guard column, 4×50 mm, 8.5 µm particles (Dionex, Sunnyvale, CA, USA) was evaluated. The OmniPacPCX-500 employs a multi-phase column packing to combine ion-exchange and reversed phase mechanisms in a single column. It consists of a highly cross-linked macroporous core with a neutral hydrophobic internal surface. The polymeric colloid is functionalized to create acidic sulfonate groups, which provide the cation exchange sites. All these sites are attached to the hydrophobic core that is made of ethylvinylbenzene/divinylbenzenene polymer. For reversed-phase chromatographic analysis, a silica-based reversed phase C_{18} column VYDAC 218TP5415, 4.6×150 mm, 5 µm particles (GRACE VYDAC, Hesperia, CA, USA) was employed. The column is end-capped with n-octadecyl compounds that are chemically bonded to "TP" 300 angstrom pore-size silica through chlorosilanes. All mobile phase solutions were filtered through a 0.45 µm polyethersulfone membrane filter (PALL Gelman Laboratory, Ann Arbor, MI, USA) just before use.

6.2.3 Sample preparation

6.2.3.1 Tris-buffer aqueous extraction for water-soluble seleno-amino acids

All freeze-dried samples were ground using a mortar/pestle bathed in liquid nitrogen.

To determine the fraction of seleno-amino acids bound in proteins, a 2-step procedure was investigated. The sample was first extracted with tris-buffer to release any "free" seleno-amino acids. For each determination, a 50-mg lyophilized sample was weighed into a dry, clean centrifuge vial (15 mL). Water-soluble ("free") seleno-amino acids were extracted with 10 mL of 20 mM Tris-HCl buffer solution at pH 7.5. Incubation at 37 $^{\circ}$C in a water bath was carried out in the dark for 2 h with a shaker set at 180 rpm. The samples were then centrifuged at 38,000 g for 30 min at 4 $^{\circ}$C. Before analysis with ICP-MS, samples were all filtered using all polypropylene syringes and filter cartridges. The selenium determination was conducted by RPLC-ICP-MS.

6.2.3.2 Enzymatic digestion for protein-bound seleno-amino acids

After aqueous extraction for water-soluble seleno-amino acids, the appropriate amount of enzyme was added with 10 mL of 10 mM Tris-HCl buffer solution at pH 7.5. Afterwards, the incubation at 37 $^{\circ}$C in a water bath was carried out in the dark for 24 h with a shaker set at 180 rpm. During the enzymolysis, the sample tubes were vortexed periodically to resuspend the tissue. The samples were then centrifuged at 38,000 g for 30 min at 4 $^{\circ}$C. All samples were filtered before selenium determination by RPLC-ICP-MS.

6.2.3.3 Methanesulfonic acidic digestion for total seleno-amino acids

Lyophilized samples (50 mg) were weighed into a dry, clean borosilicate test tube (25 mL) and 5 mL of 4M CH_3SO_3H and 0.01mL 2,3-dimercapto-propanol were added. Ultrasonication was applied to the samples for 1 hour at 50ºC. Afterwards, a well block digestion was carried out with refluxing for 16 h at 125 ± 10ºC. A small fan was used to circulate air over the tops of the test tubes. After cooling, the test samples were diluted to 25 mL with ultra-pure water. All samples were filtered before selenium determination by RPLC-ICP-MS.

6.2.4 Method optimization

For the cation-exchange chromatographic separation, an aqueous solution of pyridinium formate was used as the mobile phase. The pH of the mobile phase was adjusted by dropwise addition of formic acid. The pK_a values of SeMet, $SeCys_2$ and MeSeCys ranged from 1.7 to 9, the pH of the mobile phase was adjusted to 3.2. At this pH, the -NH_2 groups of all the analyte compounds were positively charged (protonated). Consequently, these protonated groups could interact with the negatively charged sulfonate functional groups of the column and based on the extent of interaction, they could be separated. The separation efficiency and signal response was performed with 10 μg/L each of the above standards. The chromatographic separation conditions for the Se-compounds on a cation-exchange column are listed in Table 6.1.

Table 6.1: Chromatographic separation conditions for the Se compounds on a cation-exchange column.

Column	OmniPac PCX-500 guard column
Column dimensions	4×50mm (i.d. $\times$ L), 8.5 µm particles
Mobile phase	7.5 mM pyridinium formate
pH	3.2
Injection volume	100 µL
Flow rate	1 mL/min

The chromatogram is shown in Figure 6.2. The use of 7.5 mM pyridinium formate at pH 3.2 enabled the baseline separation of SeMet, $SeCys_2$ and MeSeCys. The resolution between SeMet and MeSeCys is 0.98 and 2.07 for MeSeCys and $SeCys_2$. However, the peak tailing could not be readily eliminated and the retention time for SeMet was considered to be too close to that of the unretained species. Consequently, an ion-pairing reversed-phase method was investigated.
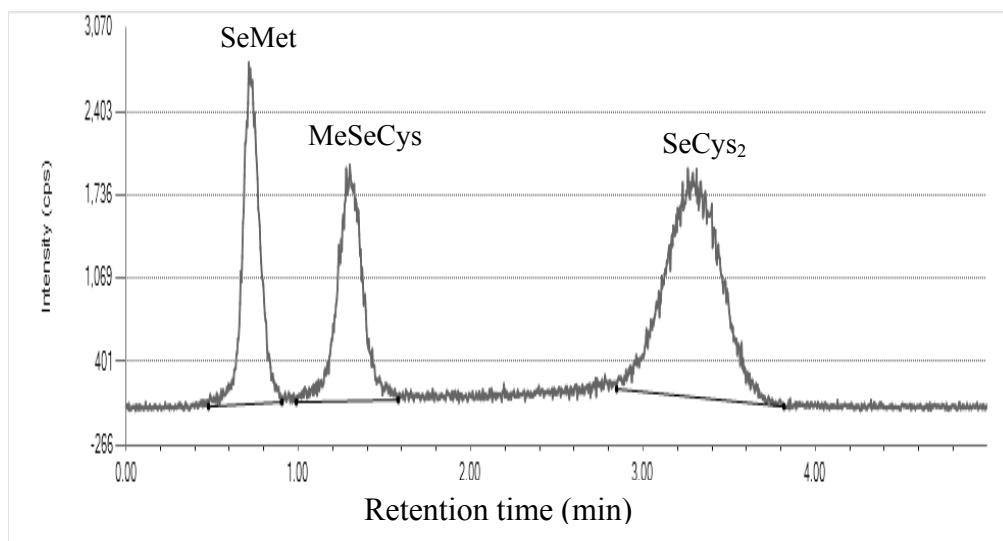
Figure 6.2: Cation-exchange chromatographic separation of SeMet, MeSeCys and $SeCys_2$ (10 µg/L each).

For ion-pairing reversed-phase chromatographic analysis, working standards of SeMet, SeCys$_2$ and MeSeCys ranging from 5-100 µg/L as Se were prepared daily before analysis from the stock solution of 100 mg/L. The effects of the different methanol concentrations on the net signal were investigated using 10 µg/L SeMet. It was found that the 2% MeOH afforded the optimal response and better resolution was achieved. Therefore, Se detection using ICP-MS, the addition of 2% MeOH is recommended.

The chromatographic separation conditions for the Se-compounds on a reversed-phase column are listed in Table 6.2.

Table 6.2: Chromatographic separation conditions for the Se compounds on a reversed-phase column.

Column	VYDAC 218TP5415
Column dimensions	4.6 × 150mm (i.d. × L), 5 µm particles
Mobile phase	0.1% TFA + 2% MeOH
Injection volume	100 µL
Flow rate	1 mL/min

The ion-pairing reversed-phase chromatogram is shown in Figure 6.3. The resolution and signal response for SeMet, SeCys$_2$ and MeSeCys were improved with the 0.1% TFA + 2% MeOH compared with cation-exchange separation results. The resolution between SeCys$_2$ and MeSeCys is 1.50 and 3.80 for MeSeCys and SeMet. Therefore, ion-pairing reversed-phase chromatographic analysis was employed for future selenium determinations.
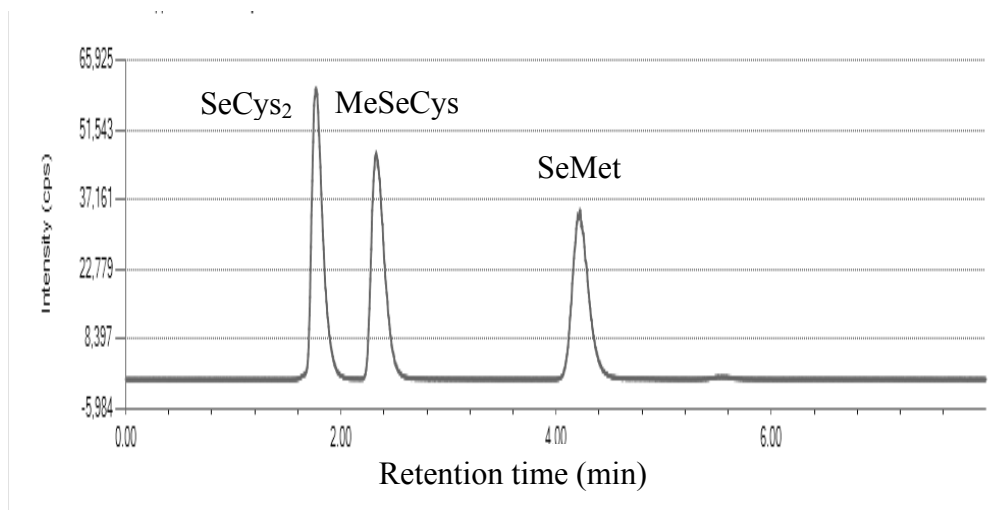
Figure 6.3: Ion-pairing reversed-phase chromatographic separation of SeCys$_2$, MeSeCys and SeMet (100 µg/L each).

6.3 Confirmation of selenium compounds by LC-ESI-MS and MS/MS

6.3.1 Introduction

Analysis of biological sample matrices is often complicated by the presence of many biogenic compounds. In order to identify any co-eluted or unknown peaks of the chromatographic separations, the use of ESI mass spectrometry can be used. In addition, ESI-MS provides greater structural information, which can be useful for the specific identification and characterization of the Se compounds. For this research, selected samples were evaluated by these methods to confirm structural species.

6.3.2 Instrumentation

The mass spectrometer employed was a Thermo-Finnigan TSQ7000 triple-quadrupole mass spectrometer with the API2 source and Performance Pack (ThermoFinnigan, San Jose, CA). Both electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) were available. The heated inlet capillary was usually kept at 250ºC and the electrospray needle voltage is 4.5kV unless otherwise noted. All voltages were optimized to maximize ion transmission and minimize unwanted fragmentation and were determined during the regular tuning and calibration of the instrument. Nitrogen sheath gas and auxiliary gas (as needed) were provided to the source from a nearby Dewar of liquid nitrogen. For direct infusion experiments, usually only the sheath gas is required and it was supplied at 40-80 psi. For LCMS experiments, the sheath gas was supplied at 80 psi and the auxiliary gas was provided at a flow rate of 40 (arbitrary units on the integrated flowmeter). For MS/MS experiments, argon was used as the collision induced dissociation (CID) gas.

The TSQ7000 mass spectrometer was connected to an integrated Thermo-Finnigan LC system consisting of a P4000 quaternary LC pump and SCM1000 vacuum degasser, an AS3000 autosampler, and a UV6000LP diode-array detector. This system was used for all LCMS and LCMS/MS experiments. For the LC separation, the same operation conditions and mobile phase were adopted from the same method as in LC-ICP-MS. All the standards of $SeCys_2$, MeSeCys and SeMet were prepared with a 1 mg/L Se with 0.1% TFA + 2% MeOH.

6.4 Results and discussion

6.4.1 Chromatographic evaluation

The C_{18} reversed-phase column was equilibrated by passing at least 75 mL of the mobile phase through the column before any injection of the selenium standards and samples. The baseline was always monitored for at least 20 minutes before the analysis to ensure that the background was low and stable. Quantification was performed in the peak area mode.

The ion-pairing reversed-phase chromatography chosen for this study demonstrated reasonable results for the separation of the selenium extracts. Effective peak efficiencies were acceptable for the standard solutions. The chromatogram was 10 min in length, and the separation of SeMet, $SeCys_2$ and MeSeCys only took less than 5 min. Since the yeasts used in the study used were enriched with SeMet, the research by the time of this dissertation was focused on the identification and quantitation of SeMet. It was also confirmed with the experimental results later.

For a 20 μg/L SeMet standard, the precision of peak area and peak height expressed as the relative standard deviation (% RSD) were 0.7% and 2.5%, respectively, based on three successive measurements. The calibration curves (in the range of 0-50 μg/L) typically gave good linearity with correlation coefficients from 0.9997 to 0.9999. The detection limit (DL) for SeMet was 0.07 μg/L, calculated as three times standard deviation of eight consecutive measurements of a 1 μg/L standard. SeMet retention time fell within 3 s for the eight standard injections, which indicated excellent reproducibility. This DL value for SeMet was considered to be low enough for direct analysis of selenium in the biological samples used in this study.

6.4.2 Confirmation of selenium compounds by LC-ESI-MS and MS/MS

The molecular ions of the three standards after LC separation were monitored in positive ion mode. SeMet, SeCys$_2$ and MeSeCys were measured at 198, 337 and 184 as [M+H]$^+$, respectively. Figure 6.4 is the mass spectrum for SeMet, SeCys$_2$ and MeSeCys from top to bottom.

The MS/MS spectra for the three Se-compounds are presented in Figure 6.5. For SeMet (*m/z* 198), the major product ions were at *m/z* 181 (loss of NH$_3$), 152 (loss of CO and H$_2$O), 135 (loss of NH$_3$, CO and H$_2$O), 109 (formation of CH$_3$SeCH$_2^+$) and 102 (formation of ion fragment NH$_2$CH(COOH)CH$_2$CH$_2^+$). For SeCys$_2$ (*m/z* 337), the major product ion was found only at *m/z* 248, corresponding to the loss of NH$_2$CH(COOH)CH$_3$. For MeSeCys (*m/z* 184), the major product ions were at 167 (loss of NH$_3$), 123 (loss of NH$_3$ and CO$_2$) and 95 (formation of ion SeCH$_3^+$).
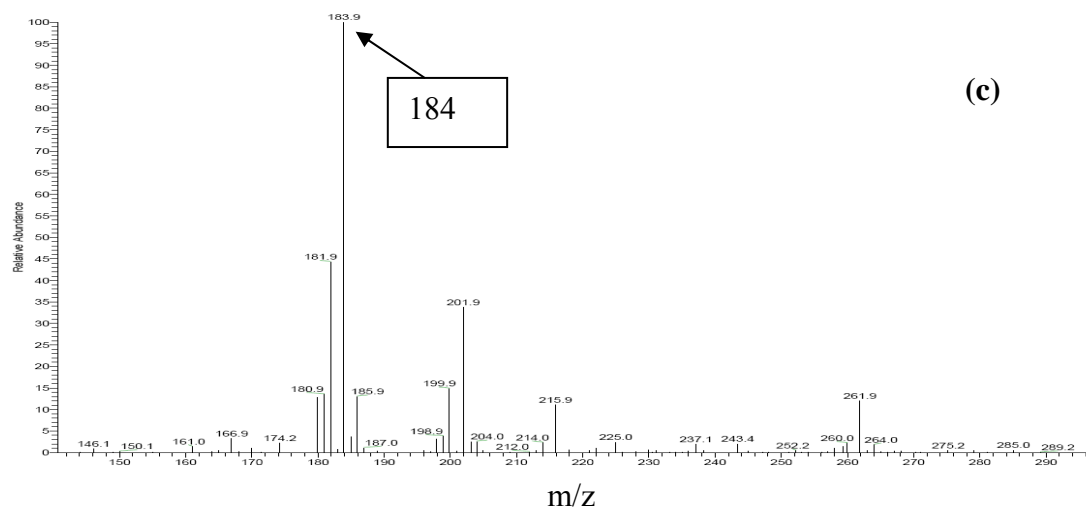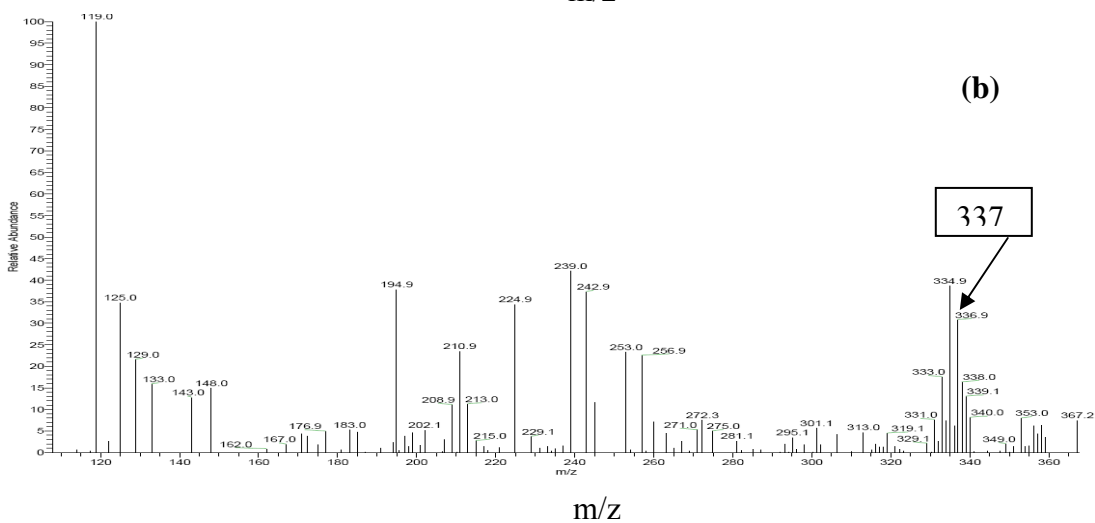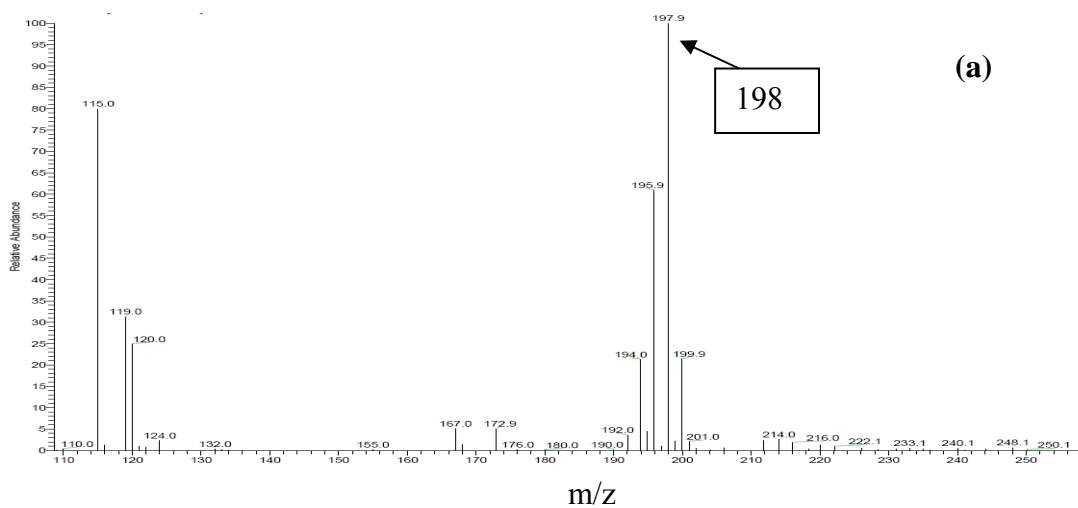
Figure 6.4: ESI-MS spectra of (a)SeMet, (b)SeCys$_2$ and (c)MeSeCys.
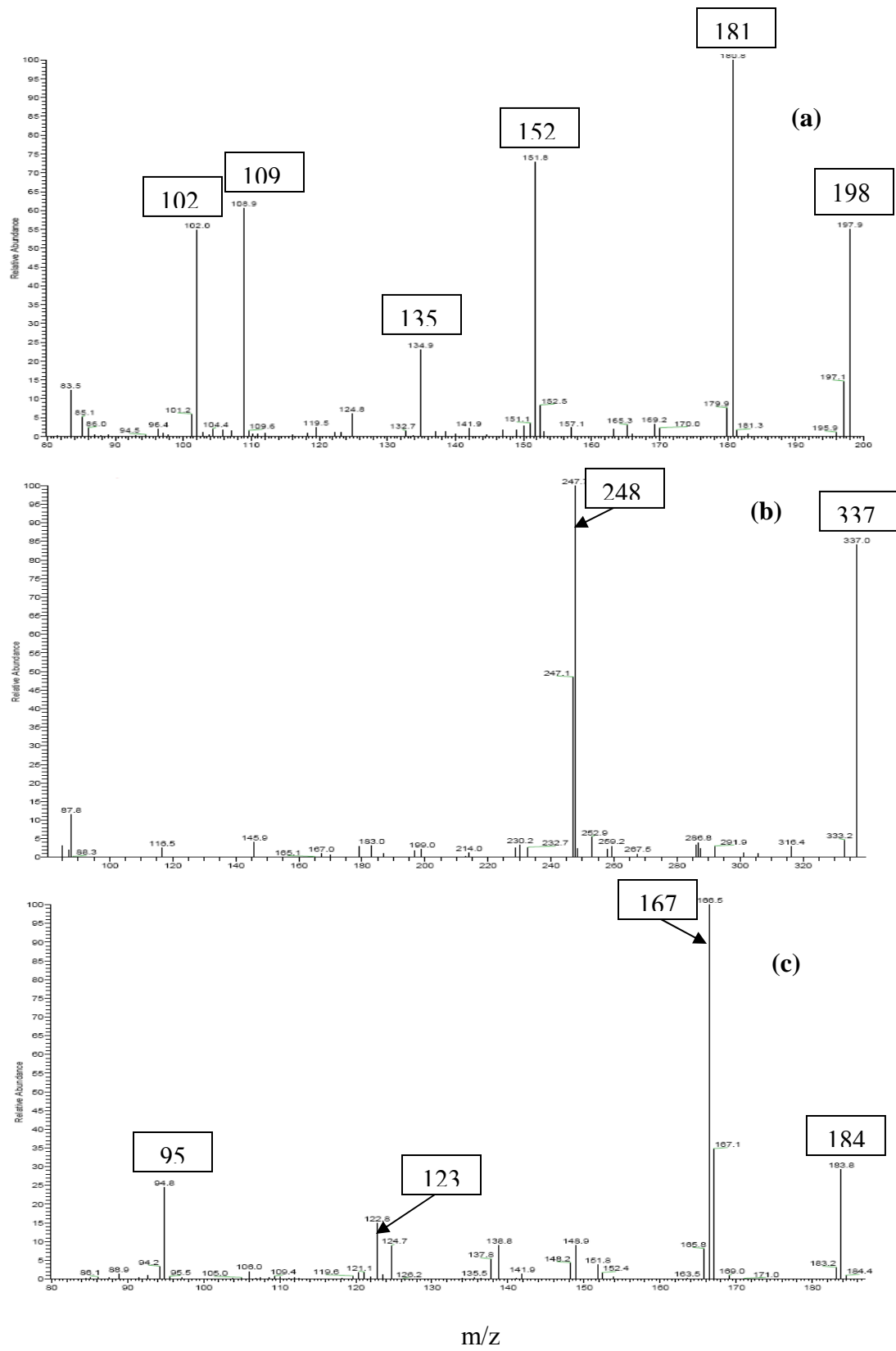
Figure 6.5: ESI-MS/MS spectra of (a) SeMet (*m/z* 198), (b) SeCys$_2$ (*m/z* 337) and (c) MeSeCys (*m/z* 184).

6.4.3 Analytical results for yeasts

Sample preparation, sample storage, and analysis procedures might all influence the recovery and quality of selenium compounds. High extraction efficiency is needed, but for some selenium species, compound stability can be problematic. Hydrolysis with constant-boiling 6M hydrochloric acid at 110 – 120 ^{o}C for 24 hours is the most conventional method for the analysis of amino acids in proteins and peptides[1]. However, under such harsh conditions, selenium-containing species are generally unstable and prone to decomposition. Incomplete cleavage of peptide bonds and the degradation of selenium compounds have been reported with this procedure[2,3]. For this reason, methanesulfonic acid (CH_3SO_3H) at 4M was used in place of hydrochloric acid for the complete amino acid hydrolysis[4]. The acid hydrolysis conditions were experimentally selected. A small amount of 2,3-dimercaptopropanol was also added to act as an anti-oxidant to retard the oxidation of SeMet[5]. It was diluted (1:9) in propanol to reduce the strong odor during handling. Without the addition of 2,3-dimercaptopropanol, the SeMet recovery was only about 78% based on in-house blank spike tests. The effect of heating temperature and the aid of ultrasonication were also evaluated. Increasing the well-block temperature from 110 ^{o}C to 125 ^{o}C resulted in the extraction efficiency of SeMet from the certified reference yeast (SELM-1) to increase from 65% to 90%. However, at least 95% recovery was desired so a brief ultrasonication treatment step was investigated before the acid hydrolysis. With this pre-treatment, the extraction efficiency improved from 90% to an average of 99% (range from 96% to 108%). The final acid hydrolysis was developed as described in 6.2.3.3.

The recovery of selenomethionine (SeMet) spiked into blank solutions before digestion was 99%. The recovery of SeMet spiked into the sample of certified reference yeast before digestion was 98%. These results indicated that the methanesulfonic acid hydrolysis procedure developed was an effective yet non-damaging digestion method for seleno-amino acids.

A secondary goal was to develop a method for protein-bound seleno-amino acids. For this, a tris-buffer aqueous extraction followed by an enzymatic digestion was briefly tested. Two types of non-specific enzymes were evaluated for the digestion of proteinaceous tissues for seleno-amino acids: pronase E and protease type XIV. Based on a limited number of experiments, results suggested there was no difference in the enzyme type and a small difference between results for two substrate/enzyme ratios. Recoveries were about 47% and 52% for substrate/enzyme ratios of 20:1 and 5:1, respectively, so the 5:1 ratio was selected for all the enzymatic digestions thereafter. The preliminary results of percent of selenium measured as water-soluble and protein-bound SeMet in one type lab-used selenized yeast and certified reference yeast are presented in Table 6.3.

Table 6.3: Preliminary results for the percent of Se measured as water-soluble and protein-bound SeMet in lab-used selenized yeast and certified reference yeast.

Sample description	Measured SeMet as of % of total Se (n =3)		
	Water-soluble SeMet $\pm$ 1SD	Protein-bound SeMet $\pm$ 1SD	Total SeMet $\pm$ 1SD
Commercial selenized yeast	3.400 ± 0.900	48.80 ± 1.50	60.70 ± 0.60
Certified reference yeast	1.500 ± 0.200	46.70 ± 1.80	66.50 ± 5.40

For this preliminary experiment, the water-soluble SeMet consisted of 2.4% and 1.5% of the total selenium for a commercial selenized yeast (that was used in toxicity tests) and certified reference yeast, respectively. The determination of protein-bound seleno-amino acids in samples of tissues from aquatic organisms was briefly attempted, but satisfactory final results could not be completed during this research. The percent of total selenium measured as protein-bound SeMet was 48.8% for the commercial selenized yeast and 46.7% for the certified yeast. The sum of water-soluble and protein-bound SeMet was much lower than the total SeMet measured using acidic hydrolysis. Some of the SeMet might be expected to be associated with non-proteinaceous material such as cell fragments. However, the disagreement may be due to the incomplete recovery with/without enzyme caused by insufficient extraction time and/or insufficient amount of enzyme. Notably, the extraction of SeMet from the certified reference material was 66.5% of total Se, which agreed very well with the reported value of 67.1%. This further confirmed that the methanesulfonic acid hydrolysis was an efficient extraction procedure from a single protein hydrolysate. Also, the analyses from enzymatic digestion and acidic hydrolysis indicated SeMet as the primary elemental species found in selenium-enriched yeast, a finding which was consistent with that of Wrobel and others[6] and Larsen and others[7].

The stability of selenium species in the extracts from the enzymatic digestion and the acidic hydrolysis were evaluated. The acidic hydrolysates were stable for at least three months at 4°C in the dark. However, there were dramatic changes in chromatograms just 24 hours after enzymatic extraction of selenomethionine-spiked blanks and oligochaete tissues, for example, the broad SeMet peak. These

transformations likely resulted from microbial decomposition/degradation of the selenium species. After analyses of these extracts, the C_{18} column apparently became contaminated with biogenic material, resulting in increasing Se background and a significantly reduced response. Apparently, the contaminated column was trapping a considerable quantity of the selenium species from each injection, only to slowly release them during subsequent analyses. Somewhat surprisingly, this problem did not seem to affect peak retention times or increase peak tailing. Performance of the column was restored by eluting with 90% acetonitrile and 10% water until the background was back to normal. The use of a guard column might have reduced this problem, but to what extent is uncertain. A guard column is generally recommended, but additional experiments would be needed to confirm satisfactory chromatographic performance. Lowering the storage temperature from 4 °C to -20 °C was attempted as a means to inhibit microbial activity, but this was an ineffective means for retarding degradation. In a follow-up experiment, the enzymatic extracts were acidified with 0.8M methanesulfonic acid immediately after centrifugation, which proved to be highly effective for stabilizing the seleno-amino acid species. No degradation was observed for at least (1 week) using this treatment.

6.4.4 Analytical results for oligochaetes and whole desert pupfish

Using RPLC-ICP-MS, the SeMet concentrations in oligochaetes and desert pupfish were determined using a standard calibration curve constructed from six standards 0, 5, 10, 20, 30, 40, 50 µg/L (ppb), along with the sample analysis. Due to the number of standards, biological samples and reference materials, all the samples can only

be analyzed once on the same day under the same experimental conditions. However, the calibration standard 20 μg/L was analyzed at the beginning, middle and the end of each batch analysis as a quality control sample. The instrumental precision will be described later in more details.

Table 6.4: Percent measured selenomethionine of total selenium in oligochaetes at week 2-7 (n=1).

Sample description	Exposure (week)	Matrix	Total SeMet (μg/g dry weight)	Total Se (μg/g dry weight)	Fraction of total Se as SeMet (%)
oligochaetes control	2-7	Oligochaetes	< 0.04 *	1.330 ± 0.040	< 3
oligochaetes low	2-7	Oligochaetes	1.550 ± 0.270	5.670 ± 0.040	27
oligochaetes medium low	2-7	Oligochaetes	2.990 ± 0.280	9.550 ± 0.050	31
oligochaetes medium	2-7	Oligochaetes	5.440 ± 0.280	15.54 ± 0.09	35
oligochaetes medium high	2-7	Oligochaetes	9.440 ± 0.270	25.89 ± 0.21	36
oligochaetes high	2-7	Oligochaetes	19.84 ± 0.27	56.72 ± 0.43	35

* Estimated detection limit is 0.04 μg/g dry weight, which is calculated as three times the standard deviation of eight consecutive measurements of 1 μg/l standard and assumed 0.05 g dry sample diluted to 25 mL.

The methanesulfonic acid hydrolysis was performed on the oligochaetes. Each sample was a composite consisting of oligochaetes from week 2 to week 7. Table 6.4 lists the analytical results for these composite samples. The error associated with the measurement was calculated with the calibration curve. The RPLC-ICP-MS method was proven to work well with this biological sample matrix. It was observed that after 2-7

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week of selenium exposure, oligochaetes dosed at high treatment could accumulate as high as 19.84 µg/g dry weight SeMet, which accounted for about 35% of the total Se at 56.72 µg/g dry weight.

The average recovery of selenomethionine (SeMet) spiked into the digestion blank before digestion was 102%. The average recovery of selenomethionine (SeMet) spiked into the oligochaete samples before and after digestion was 97% and 96%, respectively. The relative standard deviation (RSD) between triplicate sample determinations of oligochaetes dosed at the medium treatment was 1.74% with a mean of 5.55 µg/g dry weight. The relative standard deviation (RSD) between the analysis triplicates of the same samples was 0.94% with a mean of 5.50 µg/g dry weight. Total SeMet measurement for the certified reference yeast sample analyzed with the oligochaete samples was 1357 ± 29 µg/g dry weight (n =3), which agreed well with the certified value of 1381 ± 63 µg/g dry weight. The instrumental response to the standards from day to day ranged from 3700 to 4000 peak area per ppb with correlation coefficients from 0.99967-0.99997. The calibration standard 20 µg/L (ppb) was always analyzed at the beginning, middle and the end of each batch analysis of samples. The instrumental precision for SeMet determined by RPLC-ICP-MS is shown in Table 6.5.

Table 6.5: Typical instrumental calibration verification results using RPLC-ICP-MS (n=3).

Verification category	Actual concentration (µg/L)	Measured concentration (µg/L)			RSD (percent)
		Minimum	Maximum	Mean	
Calibration (ICV/CCV)	20.00	20.15	21.48	20.81	3.20

One final extraction and analysis was performed on a subset of whole pupfish samples after 56 days of exposure to selenized diets. Table 6.6 lists the analytical results for these samples. Each sample was a composite consisting of five individual fish. The error associated with the measurement was calculated with the calibration curve. It was observed that after 56 days of selenium exposure, pupfish in the highest exposure (56 µg/g dry weight Se diet) accumulated whole-body Se concentrations as high as 22 µg/g dry weight.

Table 6.6: Percent measured selenomethionine of total selenium in desert pupfish at day 56 of study (n=1).

Sample description	Exposure (days)	Matrix	Total SeMet (µg/g dry weight)	Total Se (µg/g dry weight)	Fraction of total Se as SeMet (%)
pupfish control	56	whole fish	0.4700 ± 0.3400	0.9000 ± 0.0100	52
pupfish low	56	whole fish	2.620 ± 0.340	3.810 ± 0.010	69
pupfish medium low	56	whole fish	2.750 ± 0.330	4.420 ± 0.010	62
pupfish medium	56	whole fish	5.160 ± 0.330	7.290 ± 0.010	71
pupfish medium high	56	whole fish	10.10 ± 0.55	14.66 ± 0.02	69
pupfish high	56	whole fish	16.32 ± 0.55	22.43 ± 0.04	73

The recovery of selenomethionine (SeMet) spiked into the digestion blank before digestion was 104%. The recovery of SeMet spiked into a sample of pupfish control after digestion was 94%. The relative percent difference between duplicate sample determinations of whole pupfish dosed at the highest treatment was 2.5% (mean; 16.12 µg/g dry weight). Total SeMet measurement for the certified reference yeast sample

analyzed with the whole pupfish samples was 1165 ± 116 µg/g dry weight (n =3), which was slightly below the certified value of 1381 ± 63 µg/g dry weight. Unfortunately, the column was not yet performing optimally after regeneration, which might explain the lower recovery from the certified yeast.

In summary, a methanesulfonic acidic hydrolysis was successfully developed for evaluation of seleno-amino acids in yeasts and aquatic organisms (oligochaetes and desert pupfish). Three readily available seleno-amino acids were successfully separated, but selenomethionine was the only significant species found in these samples. The acid hydrolysis method demonstrated higher extraction efficiency of selenomethionine than the enzymatic digestion. Ion-pairing reversed-phase chromatography interfaced with ICP-MS proved to be most useful for the determination of selenomethionine in biological materials, such as certified reference yeast, oligochaetes and desert pupfish.

6.5 References:

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Chapter 7: Summary and Conclusion

In order to achieve the accurate determination of selenium in biological samples, the performance of a dynamic reaction cell (DRC) using methane as the reaction gas was investigated with ICP-MS analysis. In the DRC mode, the Ar-Ar background signals could be reduced greatly but at the price of the large loss of net selenium signal intensities. The results were less satisfactory than expected and attempts to improve performance by adjusting operating conditions were unsuccessful. Therefore, on-line stable isotope dilution with conventional ICP-MS was successfully developed and applied to the determination of total selenium in biological materials, such as lab-cultured oligochaetes, selenized yeasts and certified reference materials. Masses at 77, 78, 79, 81, and 82 were monitored and quantitation of Se was determined based on both ^{78}Se and ^{82}Se.

For the determination of seleno-amino acids in yeasts and aquatic organisms (desert pupfish), a methanesulfonic acidic hydrolysis was successfully developed. Three readily available seleno-amino acids were successfully separated on a C_{18} reversed-phase column, but selenomethionine was the only significant species found in these samples. The acid hydrolysis method demonstrated higher extraction efficiency of selenomethionine than the enzymatic digestion. Ion-pairing reversed-phase chromatography interfaced with ICP-MS proved to be most useful for the determination of selenomethionine in biological materials, such as certified reference yeast and desert pupfish.

Future experiments might include the evaluation of selenomethionine transfer in food chain organisms and target organisms. These data will help to characterize transfer of selenium and selenomethionine from yeasts, oligochaetes, and fish, including maternal transfer to offspring.

Appendix A

SIDA-ICP-MS Selenium Calculation Worksheet.

SIDA Se Calculation Worksheet---										mass 78			mass 82		
Project	pupfish												BrH+ fraction for run:	0.004	
Batch No.	1361								Effective Se-77 SIDA conc.	0.0046	1		effective Se-77 IDA conc:	0.004	1
BID	39218		Divisor 1												0.013
									MLOD (calculated):		0.065				
									blank corrections:	-0.000108				-0.000005	
										SIDA calculated conc.			SIDA calculated conc.		
										(78)	(78)			(82)	(82)
Sample ID	Sample Description	Sample wt.	Dil. Vol.	Blk Corr?	77	78	79	81	(Br corr) 82	Se ug/mL (78)	Se ug/g (78)	78/77 ratio	82/77 ratio	Se ug/mL (82)	Se ug/g (82)
					net signal intensity (cps) for each mass										
IBV	0 ppb Se4 4%o-3%	0.05	25	n	18539	16	0	29	0	0.00001	--	0.00087209	-0.0000089	-0.00001	--
ICV1	4 ppb Se4 4%o-3%	0.05	25	n	18053	3409	59	-316	1439	0.00399	--	0.18884973	0.0797336	0.00402	--
ICV2	10 ppb Se4 4%o-3%	0.05	25	n	18001	7774	124	-685	3274	0.00995	--	0.43186841	0.1818722	0.01016	--
ICV3	20 ppb Se4 4%o-3%	0.05	25	n	19224	14492	221	-858	6037	0.01974	--	0.75388569	0.3140339	0.02034	--
diluent blank	diluent blank	0.05	25	n	15108	-160	-6	-987	1	-0.00021	-0.107	-0.0105635	0.0000511	-0.00001	-0.0035
blank-1	digestion blank-1	0.05	25	n	16391	-51	199	-287	5	-0.00007	-0.0331	-0.0031372	0.0002781	0.00000	0.001835
blank-2	digestion blank-2	0.05	25	n	16171	-88	164	-373	0	-0.00011	-0.05608	-0.0054578	-0.0000240	-0.00001	-0.00527
blank-3	digestion blank-3	0.05	25	n	15759	-112	230	-372	0	-0.00015	-0.0726	-0.0071287	-0.0000040	-0.00001	-0.0048
blank-spk	digestion blk spk	0.05	25	n	17984	7353	205	-489	3068	0.00934	4.670921	0.40889794	0.1706030	0.00942	4.70774
39463	pupfish control day 56 rep E-1	0.0499	25	y	20087	1723	17921	18544	746	0.00175	0.929602	0.0857856	0.0371621	0.00180	0.902609
39467	pupfish low day 56 rep E-1	0.0503	25	y	25406	8721	21493	22983	3632	0.00766	3.858749	0.34326429	0.1429393	0.00767	3.81437
39479	pupfish med high day 56 rep E-1	0.0292	25	y	33232	22302	17390	19534	9184	0.01698	14.63056	0.67111367	0.2763715	0.01712	14.66181
39483	pupfish high day 56 rep E-1 (1+1)	0.0306	50	y	30774	17336	8192	9745	7161	0.01365	22.48138	0.56333146	0.2326925	0.01372	22.42933
39575	pupfish medium day 56 rep E-1	0.0503	25	y	32564	19455	29728	32320	7981	0.01467	7.346388	0.59741996	0.2450942	0.01465	7.285307
39671	pupfish med low day 56 rep E-1	0.0497	25	y	28425	11178	40909	43535	4566	0.00893	4.547312	0.39323777	0.1606517	0.00878	4.417206
CBV	0 ppb Se4 4%o-3%	0.05	25	n	17639	-64	2522	2079	1	-0.00008	--	-0.0036192	0.0000813	-0.00001	--
CCV1	4 ppb Se4 4%o-3%	0.05	25	n	18437	3490	1515	972	1467	0.00400	--	0.18929324	0.0795440	0.00401	--
CCV2	10 ppb Se4 4%o-3%	0.05	25	n	18374	7917	1037	119	3342	0.00993	--	0.43090118	0.1818823	0.01016	--
CCV3	20 ppb Se4 4%o-3%	0.05	25	n	19354	14502	855	-386	6077	0.01959	--	0.74929617	0.3140129	0.02033	--
39463-anal spk	39463-10 ppb Se4+	0.05	25	y	27404	13652	24391	26020	5664	0.01177	5.939197	0.49816649	0.2066930	0.01185	5.927161
39479-dup	pupfish med high day 56 rep E-1	0.0295	25	y	34409	23055	19334	21942	9554	0.01695	14.45199	0.67002345	0.2776492	0.01722	14.60131
QC 112	IAEA 407 whole fish tissue	0.0507	25	y	22147	5474	104230	107327	2349	0.00533	2.679767	0.24716554	0.1060428	0.00548	2.707121
QC 117-1	NRCC SELM-1 (1+249)	0.0316	6250	y	18813	8073	204	-683	3430	0.00988	1975.007	0.42911666	0.1823257	0.01019	2015.692
QC 117-2	NRCC SELM-1 (1+249)	0.0301	6250	y	18688	7736	170	-730	3290	0.00948	1989.99	0.41398535	0.1760269	0.00977	2029.71
QC 117-3	NRCC SELM-1 (1+249)	0.0303	6250	y	18557	7869	138	-800	3299	0.00974	2031.796	0.42403383	0.1777589	0.00988	2039.81
CBV	0 ppb Se4 4%o-3%	0.05	25	n	22127	160	974	1607	4	0.00014	--	0.00723161	0.0001714	0.00000	--
CCV1	4 ppb Se4 4%o-3%	0.05	25	n	22569	4403	653	1045	1779	0.00413	--	0.19507674	0.0788090	0.00397	--
CCV2	10 ppb Se4 4%o-3%	0.05	25	n	21681	9515	523	267	3902	0.01014	--	0.43885321	0.1799932	0.01003	--
CCV3	20 ppb Se4 4%o-3%	0.05	25	n	21616	16350	516	-312	6809	0.01983	--	0.7563723	0.3149988	0.02042	--

Appendix B

SIDA-ICP-MS results of total selenium concentration for lab-cultured oligochaetes and selenized yeasts.

Dosage	Exposure (days)	Sample type	Total selenium (µg/g dry weight)
Low-1	7 (short-term)	Oligochaetes	17.65
Low-2	7 (short-term)	Oligochaetes	17.68
Medium-1	7 (short-term)	Oligochaetes	50.15
Medium-2	7 (short-term)	Oligochaetes	56.40
High-1	7 (short-term)	Oligochaetes	207.0
High-2	7 (short-term)	Oligochaetes	178.7
Low-1	7 (short-term)	Oligochaetes	14.92
Low-2	7 (short-term)	Oligochaetes	12.76
Medium-1	7 (short-term)	Oligochaetes	53.62
Medium-2	7 (short-term)	Oligochaetes	46.82
High-1	7 (short-term)	Oligochaetes	130.3
High-2	7 (short-term)	Oligochaetes	146.7
Low-1	7 (short-term)	Oligochaetes	14.49
Low-2	7 (short-term)	Oligochaetes	11.03
Medium-1	7 (short-term)	Oligochaetes	45.74
Medium-2	7 (short-term)	Oligochaetes	49.58
High-1	7 (short-term)	Oligochaetes	187.2
High-2	7 (short-term)	Oligochaetes	160.3
Very low-1	28 (long-term)	Oligochaetes	7.75
Very low-2	28 (long-term)	Oligochaetes	7.30
Low-1	28 (long-term)	Oligochaetes	24.65
Low-2	28 (long-term)	Oligochaetes	25.09
Medium-1	28 (long-term)	Oligochaetes	73.02
Medium-2	28 (long-term)	Oligochaetes	77.24
Very low-1	35 (long-term)	Oligochaetes	8.260
Very low-2	35 (long-term)	Oligochaetes	8.140
Low-1	35 (long-term)	Oligochaetes	27.44
Low-2	35 (long-term)	Oligochaetes	29.98
Medium-1	35 (long-term)	Oligochaetes	89.13
Medium-2	35 (long-term)	Oligochaetes	82.81
Very low-1	42 (long-term)	Oligochaetes	9.990
Very low-2	42 (long-term)	Oligochaetes	9.610
Low-1	42 (long-term)	Oligochaetes	30.81
Low-2	42 (long-term)	Oligochaetes	31.36
Medium-1	42 (long-term)	Oligochaetes	104.6
Medium-2	42 (long-term)	Oligochaetes	102.0
Low-2	N/A	Yeast	51.61
Medium-1	N/A	Yeast	193.9
Medium-2	N/A	Yeast	182.7
High-1	N/A	Yeast	785.4
High-2	N/A	Yeast	806.0

Appendix C

SIDA-ICP-MS results of total selenium concentration for lab-cultured pupfish

Sample description	Exposure (days)	Matrix	Total selenium (µg/g dry weight)
pupfish control rep. E-1	56	whole fish	0.9000
pupfish control rep. F-1	56	whole fish	0.8100
pupfish control rep. G-1	56	whole fish	0.8300
pupfish control rep. H-1	56	whole fish	0.7100
pupfish low rep. E-1	56	whole fish	3.810
pupfish low rep. F-1	56	whole fish	3.030
pupfish low rep. G-1	56	whole fish	3.780
pupfish low rep. H-1	56	whole fish	3.850
pupfish medium low rep. E-1	56	whole fish	4.420
pupfish medium low rep. F-1	56	whole fish	4.150
pupfish medium low rep. G-1	56	whole fish	3.660
pupfish medium low rep. H-1	56	whole fish	4.160
pupfish medium rep. E-1	56	whole fish	7.290
pupfish medium rep. F-1	56	whole fish	6.890
pupfish medium rep. G-1	56	whole fish	6.660
pupfish medium rep. H-1	56	whole fish	6.160
pupfish medium high rep. E-1	56	whole fish	14.66
pupfish medium high rep. F-1	56	whole fish	11.90
pupfish medium high rep. G-1	56	whole fish	11.69
pupfish medium high rep. H-1	56	whole fish	11.93
pupfish high rep. E-1	56	whole fish	22.43
pupfish high rep. F-1	56	whole fish	21.25
pupfish high rep. G-1	56	whole fish	21.82
pupfish high rep. H-1	56	whole fish	19.42

VITA

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Her research has focused on the determination of total selenium and seleno-amino acids in yeasts and aquatic organisms by LC-ICP-MS. The developed method will be used to study the endangered desert pupfish and other aquatic organisms. She received a Ph.D. in Chemistry from University of Missouri-Columbia in 2007.