MULTIELEMENT SPECIATION USING HPLC-ICPMS

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ABSTRACT

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Elements of arsenic and selenium have been the centres of attention for researchers regarding their roles in metabolism. Selenium can be both essential and toxic depending on its concentration level, while arsenic is a toxic element. Both elements are also in focus of interest concerning their speciation analysis. In literature, there are studies on their antagonistic and/or synergic interactions. In this study, an analytical method based on enzymatic extraction followed by determination using HPLC-ICP-MS was developed for the simultaneous extraction and speciation of arsenic and selenium. Developed methodology was applied to green onion plants that were grown in Hoagland nutrient solution spiked with arsenic and/or selenium in order to investigate possible arsenic-selenium interactions.

In the samples fed by As(III) or As(V) spiked solution, most of the arsenic was absorbed by the plant and deposited in the root as As(III) whereas transport to bulb and leaves was limited. On the other hand, selenium absorption was lower compared to arsenic; when plants were supplemented with Se(IV), most of the selenium was found in root and leaves in the form of selenomethionine and selenomethyl selenocysteine. Selenium was preserved as Se(VI) and only very small percentage was converted to organoselenium compounds in plants supplemented with Se(VI). Analysis of feed solutions revealed that conversion between arsenic and selenium

species in feed solution was insignificant. This result is a significant novel contribution to the present research findings.

Synergic and antagonistic relations were shown in a detailed manner in case of simultaneous supplementation of arsenic and selenium species.

It is known from the literature that different interaction mechanisms were present for different plants. Results given in this study are valid only for green onion plants grown under specific conditions and should not be generalized for other plants.

Keywords: Arsenic, selenium, speciation, HPLC-ICPMS, green onion.

ÖZ

HPLC-ICPMS İLE ÇOK ELEMENTLİ TÜRLENDİRME

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Arsenik ve selenyum metabolizmadaki rolleri nedeniyle araştırmacıların sürekli ilgisini çeken elementler olmuşlardır. Selenyum derişim düzeyine göre hem gerekli hem de toksik, arsenik ise toksik bir elementtir. Her iki element de türlendirme analizi açısından da ilgi odağıdır. Literatürde, bu iki elementin canlılarda antagonistik veya sinerjik etkileri üzerine çalışmalar bulunmaktadır. Proje kapsamında arsenik ve selenyumun eşzamanlı türlendirmesi için enzimatik özütleme ve HPLC-ICP-MS sistemi kullanarak analitik bir yöntem geliştirilmiş ve arsenik ve/veya selenyum katılmış, Hoagland çözeltisinde yetiştirilmiş yeşil soğan bitkilerinde arsenik-selenyum etkileşimi incelenmiştir.

As(III) veya As(V) ile beslemede, bu elementin çözelti ortamından büyük oranda alındığı ve kök bölümünde çoğunlukla As(III) formunda depolandığı, gövde ve yapraklara geçişinin ise sınırlı olduğu gözlemlenmiştir. Selenyum alınımının ise arseniğe oranla daha az olduğu, bitkinin Se(IV) ile beslenmesi durumunda selenyumun kök ve yaprak bölümlerinde selenometiyonin ve selenometil selenosistin formlarında depolandığı gözlemlenmiştir. Se(VI) ile besleme durumunda ise selenyumun çoğunlukla Se(VI) formunda korunduğu, düşük bir yüzdesinin organik selenyum formlarına dönüştüğü tespit edilmiştir. Besleme çözeltisinde As(III)-As(V) ve Se(IV)-Se(VI) çiftleri için bir türden diğerine geçiş olmadığının gösterilmiş olması, bu tezdeki sonuçların özgünlüğüne önemli bir katkıdır.

Selenyum ile arseniğin birlikte verildiği durumlar incelendiğinde sinerjik ve antagonist sonuçlar alınmış ve bunlar ayrıntılı biçimde saptanmıştır.

Literatürde farklı bitkiler için farklı etkileşim mekanizmalarının olduğu bilinmektedir. Bu proje kapsamında verilen sonuçlar sadece belli koşullarda yetiştirilmiş yeşil soğan bitkisi için geçerli olup diğer bitkiler için genel bir yargı yapmak için yeterli değildir.

Anahtar kelimeler: Arsenik, selenyum, türlendirme, HPLC-ICPMS, yeşil soğan,

To My Wife and our princess İpek

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AAS	Atomic absorption spectrometry
AE	Anion exchange
AFS	Atomic fluorescence spectrometry
APDC	Pyrrolidine dithiocarbamate
CAMA	Calcium acid methanearsonate
CCA	Chromated copper arsenate
CPE	Cloud point extraction
CRM	Certified reference materials
DMA	Dimethyl arsenate
DMA	Dimethyl arsenic
DMSeO	Dimethyl selenium oxide
DSMA	Disodium methanearsonate
EDTA	Ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency
GC	Gas Chromotagraphy
GSH	Glutathione
HFBA	Heptafluoro butyric acid
HPLC	High Performance Liquid Chromatography
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma optical emission spectrometry

IUPAC	The International Union of Pure and Applied Chemistry
LD_{50}	Lethal dose 50%
LOD	Limit of Detection
LOQ	Limit of Quantification
MeSeCys	Selenometyl selenocysteine
MIPS	Microwave induced plasma spectrometry
MMA	Monomethyl arsenate
MMA	Monomethyl arsenic
MSMA	monosodium methanearsonate
N	Number of samples
n.d.	Not detected
NIPs	Nodulin-26-like intrinsic proteins
NIST	National Institute of Standards and Technology
NRC	National Reseach Center of Canada
OsPT2	Inorganic phosphate transporter
pK_a	Acid dissociation constant
PSDVB	Polystyrene-divinylbenzene
RSD	Relative standard deviation
s	Standard deviation
SAX	Strong anion exchanger
SCX	Strong cation exchanger
$SeCys_2$	Selenocystine
SeMet	Selenomethionine

-SH	Thiol groups
TFA	Trifluoro acetic acid
TMAO	Trimethyl arsenic oxide
U	Expanded uncertainty
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION

1.1 Arsenic

1.1.1 Occurrence, production and use

Arsenic is a silver coloured shiny metalloid that occurs in the environment as different oxidation states, namely –III, 0, III and V. It is the 53rd most abundant element in earth crust, about 0.00015% [1–3]. In nature, it is found in soil, sediment, sea and fresh waters and ambient air in varying organic and inorganic forms. In soil, it rarely occurs as elemental form but found in conjunction with metal and sulfide minerals. Most common forms are arsenopyrite (FeAsS), orpiment (As_2S_3), realgar (As_4S_4) and loellingite ($FeAs_2$) [4]. Arsenic concentration in seawater is typically 1.5-2.0 µg/L [5–7].

Arsenic level in freshwater and groundwater shows much more variation because of different arsenic release mechanisms. Typical freshwater concentration is less than 5 µg/L but high concentrations are reported especially for ground water samples. Samples containing arsenic as high as 5000 µg/L were reported in the literature [8,9]. Arsenic rich groundwater samples are found in geologically active regions, areas of excessive mining, industrial and agriculturally active areas, and natural arsenic releasing regions such as Bengal Basin Delta. Arsenic is mostly found in water bodies as inorganic As(III) and As(V). Dominating species is strongly dependent on the pH, mineral content and source of the arsenic [10–12].

Arsenic may also be present in air mostly in suspended particles. Environmental Protection Agency (EPA) identified arsenic as one of the 33 toxic agents in urban air [13]. Arsenic in air could be originating from natural sources such as volcanoes and

soil erosion or from anthropogenic sources like fossil fuel burning, industrial wastes and pesticides [14–16]. Amount of arsenic released to air by anthropogenic sources is three times higher than natural sources.

Arsenic is produced by roasting of arsenopyrite or produced by the processing of metal ores, such as copper, silver and gold, as a by-product. It is produced and distributed mainly in the form of arsenic trioxide and is converted to other compounds in related industrial processes. China is the leading arsenic producer in the world producing more than 50% of total arsenic [17].

Arsenic is used in various fields in the industry. One of the main uses of arsenic comes from its toxic nature, as wood preservative. Chromated copper arsenate (also known as CCA or Tanalith) is used to keep fungus, insects and other microorganisms away from wood, helps to decrease rotting and increase the lifetime of wood based products [18]. CCA is applied to timber under pressure to ensure deep penetration of the compound to wood. However, its use is restricted in certain countries such as USA, Canada and European Union due to its toxic nature [19]. Additionally, arsenic is used in pesticides and herbicides for agricultural purposes. Less toxic arsenic forms are used for that aim. Among them, most popular ones are monosodium methanearsonate (MSMA), disodium methanearsonate (DSMA), calcium acid methanearsonate (CAMA), and cacodylic acid and its sodium salt [20].

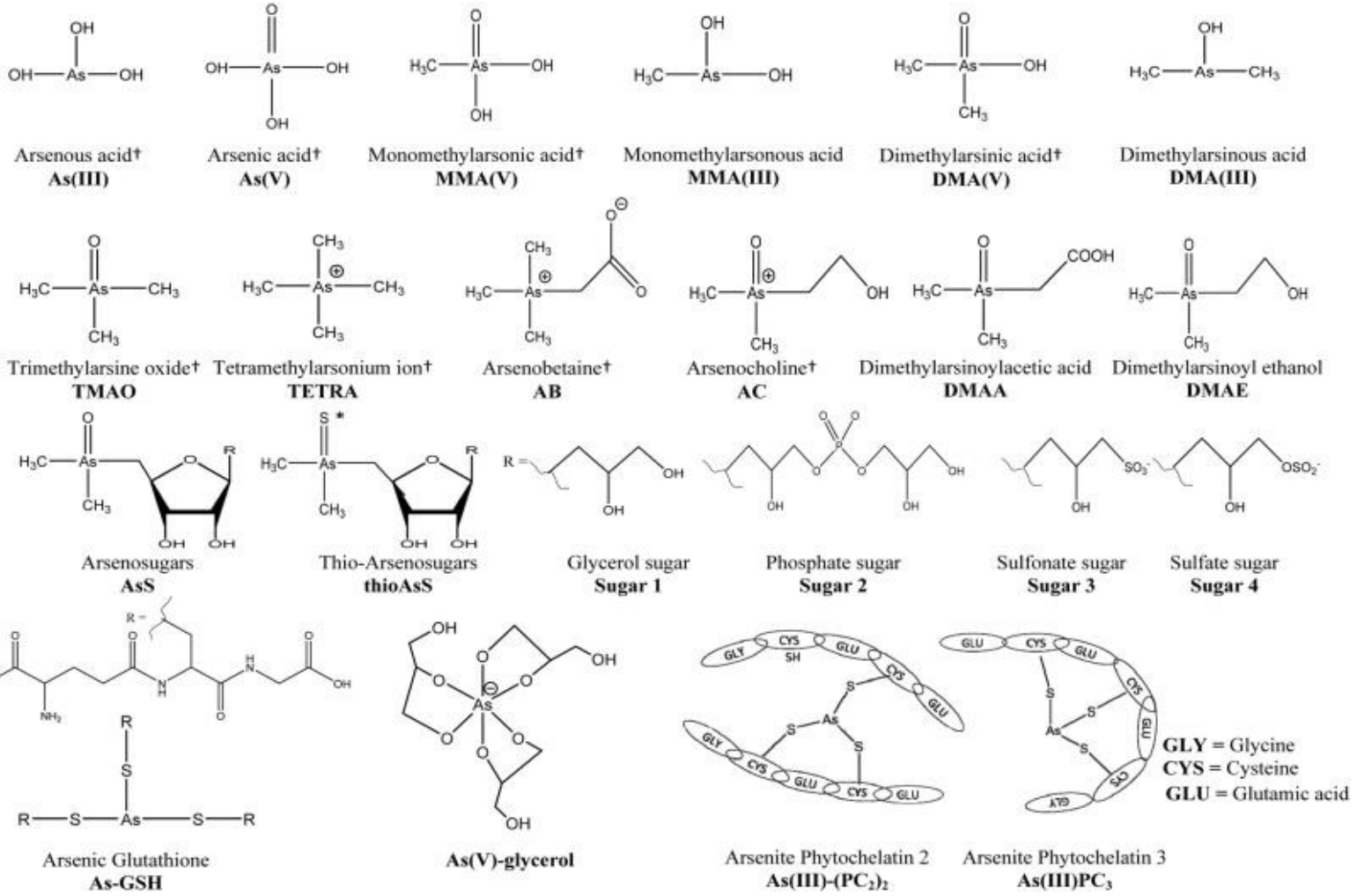
Another use area is in the glass industry. Arsenic is added to glass to increase the transparency of the material especially in high quality optical components and chalcogenide glasses [21–23]. However, due to the risk of arsenic leaching especially for glass containers, its use is restricted in some regions as glass may be in contact with food [21].

1.1.2 Arsenic compounds

There are many arsenic compounds present in the nature. Some of them are already mentioned in the previous section. Compounds containing arsenic could be divided into two main categories as organic and inorganic ones. Most common inorganic arsenic forms are trivalent arsenic trioxide (As_2O_3), arsenous acid (H_3AsO_3), arsenic trichloride ($AsCl_3$), sodium arsenite ($NaAsO_2$) and pentavalent arsenic pentaoxide (As_2O_5), arsenic acid (H_3AsO_4), arsenate containing compounds such as lead arsenate ($PbHAsO_4$) or calcium arsenate ($Ca_3(AsO_4)_2$). Elemental arsenic is rarely seen in environment since it is spontaneously oxidized when in contact with oxygen and water. Arsine (AsH_3) is a gaseous compound that is formed under reducing conditions.

There is a variety of different organoarsenic compounds. Dimethyl arsenic acid, also known as cacodylic acid, is the oldest known organoarsenic compound to humans [24]. Later on, different compounds were discovered and isolated. One large group is the methylated arsenic compounds. This group includes monomethyl arsenate (MMA), dimethyl arsenate (DMA) or their acidic forms, [25–27] .These compounds are usually formed under the influence of microorganisms. More complex arsenic compounds, such as arsenobetaine, arsenocholine, arsenolipids and arsenosugars are found in marine organisms [27–30]. Some of the common organic and inorganic arsenic compounds are given in Figure 1.1.

Figure 1.1 Structures of some arsenic compounds found in nature, adapted from reference [26].



1.1.3 Toxicity

Arsenic and most of the arsenic containing compounds are highly toxic and their toxicity is strongly dependent on the chemical form [31]. In human metabolism, more than 200 enzymes are known to be inactivated by arsenic compounds [32]. Some of these enzymes take active part in energy transfer and DNA replication.

Toxic effect may be acute or chronic. Acute toxic effects are observed if large doses are consumed in a short period of time. Symptoms may be seen within hours or may be several days after exposure. LD_{50}, which is the dose of a compound that will kill 50% of a population, is a good indication of acute toxicity. LD_{50} values of common arsenic compounds are given in Table 1.1. It is easily seen that inorganic arsenic species, namely As(III), As(V) and arsine (AsH_3), are more toxic compared to organoarsenic derivatives [33,34]. Symptoms of acute toxicity involves vomiting, diarrhea, garlic odor in breath and body tissues, hyper salivation nausea and colicky abdominal pain [35]. In severe cases the reason of death is usually hypovolemic shock and cardiovascular collapse.

Table 1.1 LD_{50} values of common arsenic compounds in laboratory animals.

Chemical	**Species**	**LD_{50} (mg As/kg)**	**Reference**
Arsenic trioxide	Mouse	26	[36]
Arsenic trioxide	Mouse	26–48	[37]
Arsenic trioxide	Rat	15	[37]
Arsenite	Mouse	8	[38]
Arsenite	Hamster	8	[39]
Arsenate	Mouse	22	[38]
MMAIII	Hamster	2	[39]
MMAV	Mouse	916	[40]
DMAV	Mouse	648	[40]
TMAOV	Mouse	5500	[40]
Arsenobetaine	Mouse	>4260	[36]

Chronic arsenic poisoning is more insidious and difficult to diagnose at an early stage since it is not a very common type. Most affected systems and organs include lungs, liver, skin and blood systems [41]. Deficiency of vitamin A is also observed [42]. Skin lesions and depigmentation take place; keratosis on the palms, hands and feed are seen after few months of continuous exposure. Peripheral neuropathy, cardiovascular disorder, diabetes and cancer are other common effects but these may change depending on the frequency and dose of exposure, chemical form, gender and age of the patients [43,44].

Diagnosis of chronicle arsenic poisoning is not an easy task. Basic diagnostic includes examination of skin and neuropathy of the patient but these symptoms are not reliable, may change from one patient to another and does not give a clue regarding how severe is the poisoning [43,45–47]. Chemical analysis of body fluids is a better choice. Blood, plasma and urine samples are analysed. However, presence of arsenic in body fluids depends on the dose and exposure time. Arsenic is rapidly cleared from blood and transferred to tissue since it has high affinity towards tissue proteins [48]. Half-life of arsenite and arsenate in blood is in the order of hours, whereas organoarsenic derivatives, such as MMA and DMA, may survive up to a day. As a result, blood is not a good indicator for arsenic exposure. Urine analysis is used for ongoing exposure to arsenic since discharging of arsenic takes place for few days. However, urine analysis is not an indication of long term exposure [45]. Arsenic is deposited in hair and nails, hence long time exposure may be monitored by the analysis of these samples [49,50]. Frequency and dose of long time exposure may be monitored by segmental analysis of hair [51,52] and nail samples [53].

1.1.3.1 Toxicity mechanism

Mechanism for toxicity strongly depends on the oxidation state. Arsenate is a phosphate analogue, and therefore it can compete with phosphate in the cytoplasm, replacing phosphate in ATP, leading to the disruption of energy flows in cells [54].

Mechanism is named as arsenolysis. Arsenate can also disturb Krebs cycle and consequently resulting in further loss of ATP [55].

On the other hand, arsenite reacts with sulfhydryl groups (-SH), or thiols, in enzymes and aminoacids, such as GSH and cysteine, their cofactors and tissue proteins, inhibits their activity [56–58]. Arsenite also inhibits some of the disulfide oxidoreductase enzymes like glutathione reductase [59] and thioredoxin reductase [60]. Binding of arsenic to active sides of proteins is an important toxicity mechanism. However, binding to nonessential sites of a protein may be considered as a detoxification mechanism [61].

1.1.4 Source of exposure

Arsenic is a widely distributed element in water, soil and atmosphere. More than one hundred million people are at risk of high arsenic exposure [62]. Sources are natural and anthropogenic. Humans are exposed to arsenic mainly from drinking water supplies and from their dairy diet.

Arsenic level in sea and oceans is fairly constant, about 1-5 μg/L [63–65]. However, concentration in fresh water changes drastically from one place to other depending on the geology. Drinking water is the main cause of arsenic problem in some countries like Argentina, Chili, Bangladesh and some parts of India where underground arsenic reserves contact with underground water streams. More than 50% of the population in Bangladesh is at risk of arsenic poisoning [66,67]. Arsenic is dominantly found as inorganic arsenite and arsenate in fresh water supplies.

Seafood is another source of arsenic for humans. People consuming large amounts of seafood based products are exposed to arsenic frequently. However, amount of inorganic arsenic in most of the seafood samples is fairly low, in most cases not detectable. Most of the arsenic is found as organoarsenicals of low toxicity such as arsenobetaine and arsenocholine [68–71]. Blue algae, edible seaweed, bivalves, and

some other sea plants, however, may contain appreciable amount of inorganic arsenic and may cause arsenic toxicity [68,72].

Plants may also contain toxic arsenics and humans may be exposed to arsenic via consuming edible plants. Rice is the most studied of all due to its high arsenic deposition capability and worldwide consumption as a staple food [19,73,74]. According to a recent study, contribution of rice to total inorganic arsenic in Chinese adults is almost 60% [75]. Unlike seafood, rice may contain organic and inorganic arsenic at comparable level. Ratio of inorganic to organic arsenic concentration, hence the toxicity may change depending on geographical conditions, origin of seeds and microbial community present in the soil [76].

1.2 Selenium

1.2.1 Occurrence, production and use

Selenium is a metalloid with atomic number 34. It is located between sulphur and tellurium in group VIA of the periodic table. Its concentration in earth crust is relatively low, about 50 μg/kg, making it 67th most abundant element [3]. Selenium is mostly found in combination with heavy metals, some of the common minerals are crookesite ($(CuTlAg)_2Se$), eucairite (CuAgSe), naumanite (Ag_2Se), claustalite (PbSe) and zorgite (PbCuSe). Selenium is also found in sulphide minerals, such as galena (PbS) and pyrite (FeS_2), replacing the native sulphur because of their similar chemical behaviour and size.

Distribution of selenium in soil is not even. In some regions soil is rich in selenium, so called seleniferous soil. In such regions selenium concentration may reach to 5.32 mg/kg level [77]. On the other hand, in some other regions concentration is as low as 0.1 mg/kg. High selenium containing soil is usually formed from shale that is formed in Cretaceous Period that is 146 to 66 million years ago. Seleniferous soils are observed in central part of United States and Canada, Mexico, Colombia, China and Ireland [78]. Selenium concentration is particularly high in organic deposits such as

coal and oil. Some coal deposits may contain as high as 4.0 mg/kg of selenium [79]. It is obvious that main source of selenium in atmosphere is the particulate matter formed during fossil fuel burning. Main inorganic forms are tetravalent selenite, hexavalent selenate, elemental selenium and selenide with "-2" oxidation state. Selenite and selenate are water soluble and can easily be transported to aquatic environment. Consequently, high selenium levels are observed in ground and surface water sources that are in contact with seleniferous soil.

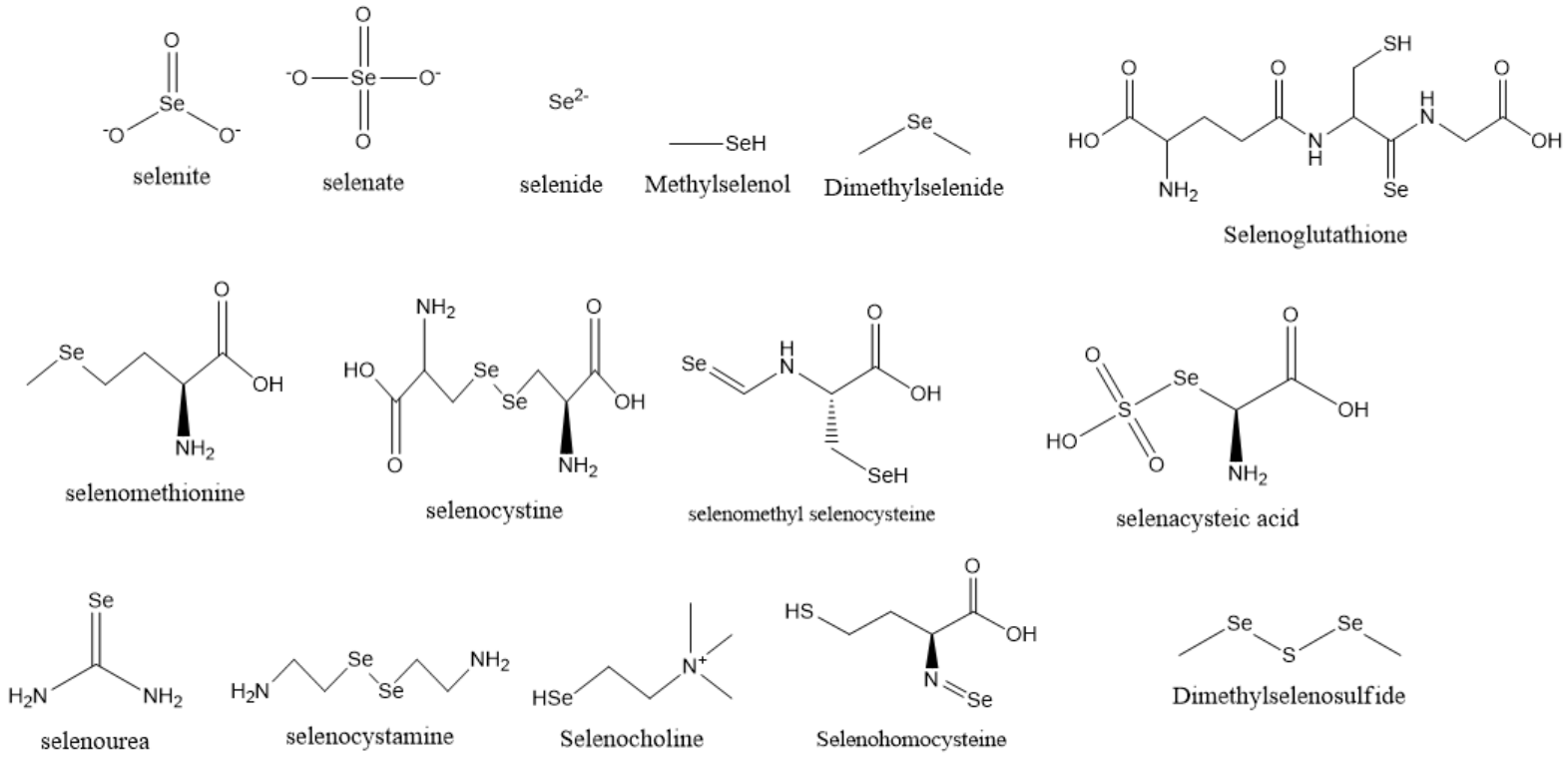
There is no feasible mining area with sufficiently high selenium concentration to be processed economically. As a result, it is produced as a by-product in industry. The anode sludge that is produced during electrolytic refining of copper, nickel and lead is the most important source of selenium. According to United States Geological Survey Research, annual selenium production is about 200 tone. Germany and Japan are, by far, the biggest producers [80].

About 50% of selenium produced is used in glass production industry. Presence of iron oxides produces a green-yellow colour in glass and selenium is added to decolorize the glass. Additionally, selenium is used as pigment for the production of ruby-red glass [81,82]. Its photochemical and photovoltaic properties make it useful in electronic industry, especially photocells, light meters and solar cells [83–85]. Biological applications include food supplements and animal feed for certain diseases [86,87].

1.2.2 Selenium compounds

Selenium occurs mainly as tetravalent selenite, Se(IV), hexavalent selenite, Se(VI), elemental selenium and selenide ions. Some of the common selenium compounds are given in Figure 1.2.

Figure 1.2 Structures of common organic and inorganic selenium compounds.



1.3 Selenium in plants

Selenium is classified as an essential micronutrient for mammals, some bacteria and algae and classified as non-essential for most plants. Selenium is an essential element at a narrow concentration range beyond which deficiency or toxic effects are observed. It is taking part in some important enzymes in antioxidation mechanism of the body and may have a similar mechanism in plants [88].

Most of the plants are non-selenium accumulators and do not have special selenium uptake mechanisms. Selenium is a sulphur analogue and taken up readily by sulphur transport mechanisms [89]. However, selenium hyperaccumulator plants, which are found in seleniferous soils, have differentiation mechanism for S and Se and have specific metabolism for selenium uptake [90].

Selenate and selenite have different intake mechanisms. Selenate is structurally very similar to sulfate. It is believed that selenate uptake is through sulphate transporter genes [91]. Ones up taken, selenate is converted to selenite in the leaf chloroplasts and this is the rate determining step [92] since plants mostly deposited selenate as it is. ATP sulfurylase and APS reductase enzymes actively take part in such conversion.

On the other hand, selenite uptake is not well understood. It is believed that uptake is a passive diffusion process [93,94]. Selenite is then converted to selenide, Se^{2-}, by two different mechanisms. It may be reduced in chloroplast by the help of sulfite reductase enzyme or by nonenzymatic ways through reduced glutathione [95]. Selenide is further coupled to O-acetylserine and transformed to SeCys by thiol lyase enzyme. SeCys replace Cys in protein structure and lead to toxicity. SeCys may be converted to SeMet that is also incorporated into protein structure leading Se toxicity again. MeSeCys, on the other hand, is not taking part in protein structure. Consequently, it can be accumulated safely in the plant structure. Dimethyl selenide, another selenium form converted from SeCys, is volatile. Plants use it as a detoxicifation mechanism by volatilizing it from leaves [89].

1.4 Speciation

At the early stages of trace element determination, total amount of elements were measured and reported. Any chemical, biological, environmental or geological decision was made according to this information only. This is partly due to the fact that researchers believe elements behave similarly in a definite matrix regardless of the chemical form and partly because the technology and instrumentation is not good enough to specify the chemical form of elements since most of the atomic techniques are destructive in their nature. Later in the history, researchers realised that the mobility, distribution and biological availability of chemical elements depend not simply on their concentration but, critically, on the chemical and physical association which they undergo in natural systems. Behaviour, toxicity and bioavailability of different forms of arsenic and selenium, for instance, have already been discussed in the previous sections.

1.4.1 Definition

The term speciation was first introduced by biologist to explain the formation process of two or more different species in a definite ecosystem. Chemist, later on, barrowed and modified the term. Referring to the same idea, chemists developed the term *chemical speciation* referring to different species of an element in a definite environment [96].

Many scientists defined the term according to their point of view. In order to eliminate the confusion, The International Union of Pure and Applied Chemistry (IUPAC) published a guideline on chemical speciation in 2000 [97].

In the guideline, speciation analysis is defined as;

> *"The analytical activity of identifying and/or measuring the quantities of one or more individual chemical species in a sample"*

Guideline also differentiates the terms speciation and fractionation. Definition of speciation is given as;

> *"The speciation of an element is the distribution of an element amongst defined chemical species in a system."*

Whereas, fractionation is defined as;

> *"Fractionation is the process of classification of an analyte or a group of analytes from a certain sample according to physical (e.g. size, solubility) or chemical (e.g. bonding, reactivity) properties."*

Difference between speciation and the fractionation is the target molecules or ions. Speciation refers to a specific molecule or ion whereas fractionation is pointing not a single species but a group of molecules/ions [98].

These definitions are well accepted and used by the chemistry community.

1.4.2 Speciation analysis

In total element analyses, the main goal is conversion of all chemical species of a specific element to a single form, so called *isoformation*, since behaviour of different forms may differ even in very harsh atomic techniques like plasma or arc [99]. In speciation studies, on the other hand, the opposite is true. Species must stay as they are and inter/intramolecular conversions must be avoided. This necessity makes the speciation studies more complex compared to total element determination. Another difficulty is that most of the atomic techniques are destructive and any molecular information is lost during atomization process. As a consequence, for most cases a separation of species prior to detection is needed.

1.4.2.1 Sampling

Sampling in speciation analysis is one of the most critical steps. Regular steps needed for total element analysis, like homogeneity [100,101], sampling place [102,103] and sampling time [104] apply here [105]. Additionally, more precautions must be taken so as to keep the species at their original state. Sampling equipment should be selected properly. Use of metal equipment, such as diggers, spoons, syringes etc., is not recommended, alternatively high density and low porosity polymeric materials will be a better choice [106,107]. Increase in the concentration of Ni and Cr was observed if metal syringes were used for blood sampling since blood proteins form complexes and solubilise those metals from syringe needle [108].

Sampling equipment should also be cleaned before use. In addition to conventional cleaning with detergent and water, all plastic and glass equipment should be soaked in 5-10% (v/v) HNO_3 solution and rinsed with plenty of deionised water so as to desorb any metal residue adsorbed on the surface.

Liquid samples are collected in high density polymeric containers, addition of acid is not allowed since change in pH may disturb ionic equilibria. High volume containers will be advantageous since ratio of contact surface to sample volume is reduced, hence loss due to surface absorption is minimised. It is a good practice to completely fill the container since volatile forms may be transferred to the gas phase disturbing the equilibrium [109].

1.4.2.2 Storage

Storage of the samples from the sampling till time of analysis is also important. Freezing samples will decrease the activity hence possibility of degradation of the analytes is minimised. Storage at -80 °C is recommended to stop any activity caused by microorganisms. Water content of samples is very critical. Presence of water eases most of the ionic and molecular reactions. Removal of excess water, if

possible, is advantageous. Drying in a conventional laboratory oven is not recommended since some of the metastable compounds may decompose or excess heat may lead to undesirable reactions. Instead, samples may be freeze dried under vacuum and liquid nitrogen atmosphere. By freeze drying, storage life of samples is extended significantly.

1.4.2.3 Sample preparation

Sample preparation covers the extraction of the analyte species from the sample medium as well as necessary derivatization and clean up stages. Several methodologies are used to extract analyte from the matrix. Most popular techniques are classical liquid-liquid (micro)extraction [110], cloud point extraction [111–114], microwave assisted extraction [115–117], solid phase extraction [118–120], solid phase microextraction [121–123] and enzymatic extraction [124–126]. Selection of proper extraction method is strongly dependent on the sample and the analyte species. Enzymatic extraction, for instance, is necessary to extract protein bonded species.

Among these methods, classical liquid-liquid extraction is the most popular one. It includes mixing of two or more liquids that are immiscible. Transfer of analyte to extraction phase is aimed since target molecules/ions have different solubility's in different phases. Usually the volume of the extracted phase is smaller so that extracted analytes are enriched in the new phase. Different complexing agents and other mediators, such as ionic liquids, are used to increase the transfer efficiency [111,127–129].

Main goal in speciation analysis is to extract as much target species as possible without interfering species. In most cases extracting the whole analyte molecules/ions is not possible. In that case, extraction efficiency is precisely calculated and used for conversion. There are several methodologies in the literature for the estimation of extraction efficiency. Among them, analyte spiking, use of certified reference materials, comparison with total element determination, isotope

labelling and surrogate standards are the most popular ones. However, none of the methodologies is perfect, each one having a weak point.

Certified reference materials (CRM) are good for the extraction studies but number of CRMs available for speciation analysis is limited due to stability problems. Another alternative is conducting spiking experiments for analyte species. This methodology well suits to simple matrices, such as natural water samples, but will not be useful for complex samples like blood, tissue and most biological samples. This is because spiked samples may not bond to the sample matrix as the same way with analyte species.

1.4.2.4 Extraction of arsenic and selenium from plant matrix

Arsenic and selenium are among the most widely studied metalloids due to their toxic nature and biological activities. Several different methods have been tried in the literature for highest extraction efficiency. Some of the extraction methods, solvents and target species of arsenic and selenium are listed in Table 1.2 and Table 1.3, respectively. Generally arsenic is extracted from plant matrix with moderate to high efficiency using water based solvent and buffers. Addition of complexing agents, such as EDTA, TFA, ascorbic acid, increases the extraction efficiency by forming stable arsenic complexes [130–132]. However, these complexing agents may cause problem by disturbing the mobile phase composition and pH if analysis of arsenic species are carried out by liquid chromatography. Arsenic is mostly found as inorganic arsenite, arsenate and organic MMA and DMA in plant matrix. Hence, use of enzymatic hydrolysis is not crucial. Selenium, on the other hand, may be present as bonded amino acids in proteins, therefore enzymatic extraction process is necessary to increase the efficiency of Se extraction from the sample matrix.

Table 1.2 Selected extraction methods, solvents and target molecules for *arsenic* speciation in plant matrix.

Sample	Target molecules	Extraction method	Extraction solvent	Extraction efficiency, %	Reference
Carrot, lettuce and spinach	As(III), As(V)	Solid-liquid ultrasonic extraction	10 mL of *methanol/water* (1:1) mixture followed by 0.1 M HCl	3 to >100	[133]
Rice	As(III), As(V), MMA, DMA	Solid-liquid extraction at 100 °C, 6 h	2.0 mL of 2.0 M trifluoroacetic acid	5 to 91	[134]
Rice	As(III), As(V), MMA, DMA	Protein extraction Microwave assisted extraction at 90 °C, 20 min	0.33 M sucrose, 50 mM MES, 5 mM EDTA, and 5 mM 0-ascorbate mixture, pH 5.50	78 to 150	[134]
Chinese herbal medicines	As(III), As(V), MMA, DMA	Microwave assisted extraction at 55 °C for 10 min, 75 °C for 10 min, 95 °C for 30 min.	*1% HNO_3 and 1% HNO_3 + 1% H_2O_2*	51 to 128	[135]
Pteris vittata	As(III), As(V)	*Solid-liquid extraction, mechanical shaking for 2 h*	*5.0 ml of 1:1 methanol/water mixture*	60 to 100	[136]
White mustard	As(III), As(V), MMA, DMA	*Solid-liquid extraction, ultrasonic shaking for 1 h*	*10 mL Milli-Q water*	9 to 91	[137]

Table 1.3 Selected extraction methods, solvents and target molecules for *selenium* speciation in plant matrix.

Sample	Target molecules	Extraction method	Extraction solvent	Extraction efficiency, %	Reference
Stanleya pinnata	TMSe, Se(IV) DMSeO, Se(VI), SeMet	Solid-liquid extraction, mechanical shaking, 4 h	30 mL deionised water	60.4–72.6	[138]
Green onion	Se(IV), MeSeCys, SeMet,$SeCys_2$,Se(VI)	Solid-liquid extraction, mechanical shaking, 4 h	3 mL of 0.10 mol/L NaOH	55	[139]
Green onion	Se(IV), MeSeCys, SeMet,$SeCys_2$,Se(VI)	Enzymatic extraction, mechanical shaking, 24 h	5.0 mL deionised water and 0.005 g of Pronase E	80	[139]
Garlic	Se(IV), MeSeCys, SeMet, Se(VI)	Enzymatic extraction, mechanical shaking, 20 h and ultrasonic probe, 3 min	2.5 mL of 0.1 M HCl	62-96	[140]
Garlic	Se(IV), MeSeCys, SeMet, Se(VI)	Solid-liquid extraction, mechanical shaking, 20 h and ultrasonic probe, 3 min	2.5 mL of 25 mM ammonium acetate buffer, pH 5.60	80-107	[140]
Garlic	Se(IV), MeSeCys, SeMet, Se(VI)	Solid-liquid extraction, mechanical shaking, 20 h and ultrasonic probe, 3 min	2.5 mL of Milli-Q water, 0.05 g of protease	103-127	[140]

1.4.3 Quantification

Arsenic and selenium extracts are quantified by several different methodologies. Most of the atomic techniques are destructive and any molecular information is lost during quantification. This property makes the separation prior to detection necessary. However, in some cases non-chromatographic speciation is also possible. Speciation can be classified as non-chromatographic and chromatographic speciation depending on the separation technique used.

1.4.3.1 Non-chromatographic speciation

Non-chromatographic speciation relies on the different chemical, physical or kinetic properties of species [141]. One example is the formation of volatile species of arsenic species. Upon reaction with a suitable reducing agent, in most cases $NaBH_4$, As(III) forms volatile AsH_3. Formation of arsine gas from As(V), on the other hand, is not kinetically favoured at the same conditions and does not take place during the course of hydride generation process [142,143]. In such studies speciation strategy is based on this idea. First quantification of kinetically favoured species, in the above example As(III), was performed simply by reacting the acidified sample with a reducing agent. Then, kinetically unfavoured species, in above example As(V), are reduced or oxidised to the kinetically favoured one and total amount of the element is determined. The difference between two results gives the amount of kinetically unfavoured species. Same procedure can be used for the speciation of inorganic As, Se, Te, Sb and Hg [142,144–146]. Sb(V) and As(V) may be reduced to lower oxidation states by using ascorbic acid-potassium iodide mixture [142,145,147,148] whereas reduction of Te(VI) and Se(VI) requires heating in concentrated acids, such as HCl, or thiourea [149,150]. In order to use the hydride generation for speciation purposes, at least one of the species must have higher hydride generation efficiency compared to others.

Another non-chromatographic speciation approach is selective separation of one species by a physical process. Preparation of solid phase disks or microcolums that

are selective or even specific to one species is possible. These SPE devices may be used to separate species prior to detection. Garbos et al. used ammonium pyrrolidine dithiocarbamate (APDC) as a chelating agent and a C16-bonded silica microcolumn to selectively sorb Sb(III) prior to graphite furnace detection [151]. Adsorbed Sb(III) is then desorbed using ethanol. Total Sb was determined by pre reduction of Sb(V) to Sb(III) using L-cystine. In another study, Lopes-Garcia et al. used cloud point extraction (CPE) for Cr speciation using silver nanoparticles, EDTA and Triton X-114 [112]. In the presence of silver nanoparticles, EDTA and Triton X-114, Cr(III) was transferred to surfactant rich phase whereas Cr(VI) was bonded to Ag nanoparticles. When EDTA was not used, both Cr(III) and Cr(VI) were bonded to Ag nanoparticles, hence total Cr was quantified. Amount of Cr(III) was calculated as the difference between total Cr and Cr(VI).

Non-chromatographic speciation is fast, reliable and a low-cost method but it is strongly limited to the type of chemicals used and the sample matrix. This approach is not suitable for elements having variety of organic species present in the same medium. They are used for simple matrices, such as water, and in the presence of dominating species [146,152].

1.4.3.2 Chromatographic speciation

Chromatographic speciation requires coupling of a separation unit with a detector system. In the early days of speciation, separation and detection procedures were done off-line, i.e. performed in different time intervals. This type of coupling resulted in many difficulties regarding the analytical procedures. Today hyphenated techniques couple the separation technique on-line with the detection technique resulting in high resolution and short analysis times. Many combinations are possible depending on the nature of sample and analytes as seen on Figure 1.3. Most of the chromatographic separations rely on HPLC and GC since they are suitable for online coupling to atomic techniques. By this way molecular information is coded to the elution time by the help of chromatography and quantitative judgment is done by using the data obtained from atomic or molecular detector [153].

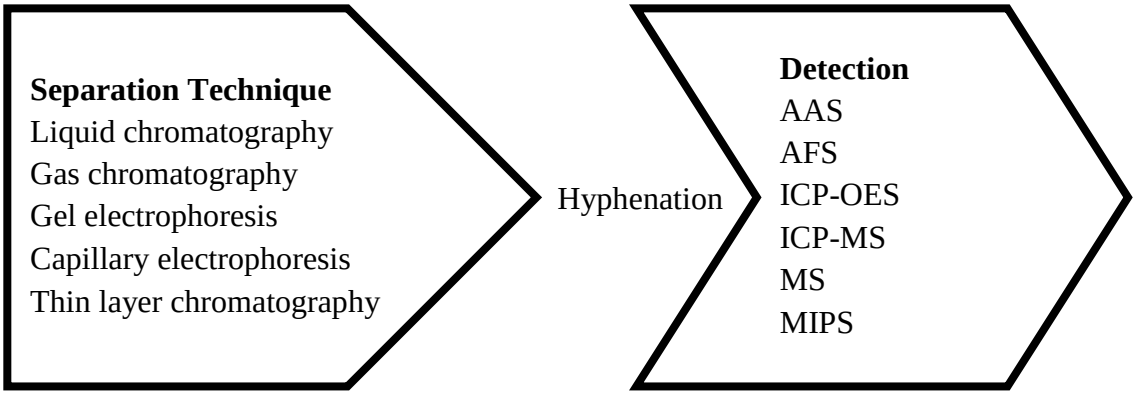


Figure 1.3 Hyphenated techniques for speciation analysis.

HPLC-ICP-MS coupling is one of the most popular combinations since it offers several advantages. First of all, liquid samples can be studied and the requirement of being volatile without any decomposition is not needed anymore as it was the case for gas-liquid chromatography. Moreover, HPLC system is very flexible and numerous combinations of mobile phases can be applied for the separation process. Coupling of HPLC to ICP-MS is an easy operation since both of the systems have compatible flow rates, around 1.0 mL/min. By the help of a simple inert tubing outlet of the column is connected to the nebulizer system of ICP. The critical point is the tubing diameter. If it is too wide, then it causes loss of resolution and may lead to peak overlapping as well as creating extra dead volume. Ideally it should match the diameter of column outlet tubing [140,153].

1.4.4 Multielement speciation

Speciation studies provide useful information about the transport, mobility, distribution and biological activity of a specific element in nature. A new trend in speciation analysis is the simultaneous speciation of different elements to see the relationship between different forms of different elements in a specific matrix or to have more comprehensive information about a definite sample. Multielement speciation is mostly performed as simultaneous speciation of different element because simultaneous speciation provides more information with a relatively low

cost and less time. Initial studies mostly concentrated on inorganic species in water, soil or mineral extracts [154–156].

Simultaneous speciation studies are especially important for biological systems where behaviour of elements are not only effected by the biological system, but also effected by the presence or absence of other chemicals. However, number of studies are limited in this area since biological matrices are complex and number of species involved are higher in number.

1.4.5 Arsenic-selenium interaction and simultaneous speciation

In literature, few studies are present dealing with the interactive effect of As and Se. In their study with mung bean, Malik et al. found that Se (2.5 or 5.0 μM) application stimulated plant growth, As (10 μM) application inhibited plant growth and simultaneous application of Se (5μM) and As (10 μM) stimulated plant growth. Additionally, presence of Se decreased the amount of As deposited while amount of Se increased in the presence of As [157]. This study indicates that presence of Se decreased the toxic effect of As.

In another study, As and Se were applied to Chinese bracken fern *(Pteris vittata)* plant. Results revealed that if Se level is lower than 2.5 mg/L, presence of As increased the Se uptake while if Se level is higher than 2.5 mg/L, presence of As decreased the Se uptake [158].

These results indicate that As and Se interact and affect one another within plant matrix. However, these studies are based on total element determination. Up to our current knowledge, there is one study in the field of simultaneous speciation of As and Se in plant matrix published by Afton et al. [159]. In that study, As(III), Se(IV) and Se(VI) were applied to spider plant (*Chlorophytum comosum*). Antagonistic and synergic relations were studied. A synergic relation was found between As and Se considering total element results. However, a detailed distribution scheme of the

species, i.e. portion remained in the feed solution and percent distribution within the plant, could not be done since plants were grown in soil medium.

1.5 Aim of the study

Aim of this study is the development of a fast and reliable HPLC-ICP-MS method for the simultaneous speciation of five selenium species, namely Se(IV), Se(VI), selenomethionine, selenocystine, selenomethyl selenocysteine, and two arsenic species, As(III) and As(V), and application of the developed methodology for the speciation of As and Se in hydroponically grown green onion plants in order to observe any synergic or antagonistic effect between these elements..

CHAPTER 2

EXPERIMENTAL

2.1 Chemicals and reagents

All chemicals used throughout the study were of reagent grade. 1000 mg/L stock solutions of Se(IV), Se(VI), seleno-DL-methionine, seleno-DL-cystine and selenomethyl selenocysteine were prepared by dissolving appropriate amounts of sodium selenite (Ventron, Karlsruhe, Germany), sodium selenate (Ventron), Seleno-DL-methionine (>99%, Sigma-Aldrich, St. Louis, MO, USA), seleno-DL-cystine (Sigm-Aldrich) and Se-(Methyl)selenocysteine hydrochloride (>95, Sigma-Aldrich), respectively, in deionised water (18 MOhm) obtained from TKA water purification system (Niederelbert, Germany). 1000 mg/L stock solutions of As(III), As(V), monomethyl arsenic and dimethyl arsenic were prepared by dissolving sodium arsenite (Fisher Scientific, New Jersey, USA), sodium arsenate (Fisher Scientific), cacodylic acid (Sigma-Aldrich) and disodium methyl arsenate (Supelco, Bellefonte, Pa, USA) in deionised water, respectively. Main and intermediate stocks were kept at -18 °C. Working standards were prepared weekly from intermediates and kept at 4 °C.

Protease type XIV, Trypsin, Proteinase K, Drisilaze and Pancreatin enzymes used in the enzymatic extraction process were obtained from Sigma-Aldrich.

Digestion procedure was validated by using DOLT-4 Dogfish Liver (NRCC, Ottawa, Canada) and 1566b Oyster Tissue (NIST, Maryland, USA) standard reference materials.

Nutrient solution recipe proposed by Hoagland and Arnon was used in the study [160]. This solution contains the necessary inorganic chemicals for proper plant growth. In order to prepare the nutrient solution, 100.0 mL stock solutions of KNO_3 (Acros Chemicals, Geel, Belgium), $Ca(NO_3)_2.4H_2O$ (Riedel-de Haen, Seelze, Germany), $MgSO_4.7H_2O$ (Kimetsan, Ankara, Turkey), NH_4NO_3 (Riedel-de Haen, Seelze, Germany), H_3BO_3 (Merck, Darmstadt, Germany), $MnCl_2.4H_2O$ (Riedel-de Haen, Seelze, Germany), $ZnSO_4.7H_2O$ (Fisher Scientific), $CuSO_4.5H_2O$ (Riedel-de Haen, Seelze, Germany), $Na_2MoO_4.2H_2O$ (Fisher Scientific, USA), KH_2PO_4 (Riedel-de Haen, Seelze, Germany) were prepared at concentrations given in Table 2.1. Indicated volumes of stock solutions were pipetted in a 2.0 L volumetric flask and diluted to the mark with deionised water in order to prepare half strength Hoagland nutrient solution.

Table 2.1 Concentration of stock solutions used for Hoagland nutrient solution.

Chemical	Concentration, mol/L	Volume of stock taken for 1.0 L Hoagland solution, mL
KNO_3	2.0	2.5
$Ca(NO_3)_2.4H_2O$	1.0	2.5
Iron chelate*	0.05	1.5
$MgSO_4.7H_2O$	1.0	1.0
NH_4NO_3	1.0	1.0
H_3BO_3	0.05	1.0
$MnCl_2.4H_2O$	0.01	1.0
$ZnSO_4.7H_2O$	0.001	1.0
$CuSO_4.5H_2O$	$2.0x10^{-4}$	1.0
$Na_2MoO_4.2H_2O$	$2.0x10^{-4}$	1.0
KH_2PO_4	1.0	0.5

*Iron EDTA chelate was synthesized using the procedure proposed by Beale [161]. 146.0 g disodium salt of EDTA (Merck) and 53.5 g $Fe(OH)_3$ (Merck) were dissolved in 500 mL deionised water and refluxed 3 h at 90 °C. Cooled mixture was filtered through Whatman No. 1 filter paper. Precipitate was washed with ice cold water and dried overnight at 80 °C in conventional laboratory oven. Synthesized yellow precipitate was homogenised and stored at dark.

5.0 mmol/L citrate buffer in 2.0% (v/v) methanol was used as mobile phase for anion exchange chromatography. Mobile phase was prepared by dissolving appropriate amounts of citric acid (Fisher, USA) and chromatographic grade methanol (Merck, Germany) in deionised water. pH of the solution was adjusted to 5.00 using 0.1 mol/L NH_3 (Merck). Prepared mobile phase was filtered through 0.20 μm nylon net membrane filter (Millipore) by the help of a Pall vacuum filtration unit in order to remove any particle. Finally, solution was degassed 30 minutes by ultrasonic water bath.

2.2 Instrumentation

2.2.1 Inductively coupled plasma mass spectrometry (ICP-MS)

Thermo Scientific X series ICP-MS instrument with a concentric nebulizer and Peltier effect cooled spray chamber was used for total element determination and speciation studies. Instrumental working parameters were optimized using "Tune A" mixed standard solution with a concentration of 10 μg/L for each element as suggested by the manufacturer. Tune A solution contains Li(I), Co(II), In(III), Pb(II), Bi(V) and U(IV) ions in 0.5 mol/L nitric acid solution. Furthermore, a mixed standard solution of 10 μg/L As and Se was used for the fine tuning of the instrument to get the best signal to noise ratio with ^{75}As, ^{78}Se and ^{82}Se isotopes. Continuous flow mode with 20 ms dwell time was used for total element determination and time resolved transient signal mode with 10 ms dwell time was used for chromatographic runs.

Plasmalab, OriginLab and Microsoft Excel softwares were used for the evaluation of data. As and/or Se concentrations reported in this study are not for ions or molecules but for the elements in these species.

2.2.2 High performance liquid chromatography (HPLC)

Chromatographic separations were performed using a Dionex 3000 HPLC pump with online degasser system. A Rheodyne 8125 flow injection system with 100 μL sample loop was used for sample introduction. Species were separated using Hamilton PRP-X100 (4.6 X 250 mm) strong anion exchange column.

HPLC system was hyphenated to ICP-MS by use of 40 cm 0.56 mm i.d. PTFE tubing. By this way, column outlet was connected directly to the nebulizer bypassing the peristaltic pumps. Tygon connections were used at the junctions. Summary of the instrumental parameters are given in Table 2.2.

Table 2.2 HPLC instrument working conditions.

SAX-HPLC parameters	
Column	Hamilton PRP-X100 (4.6 X 250 mm)
Mobile phase	5.0 mmol/L citrate buffer, 2% (v/v) methanol, pH 5.00, isocratic elution
Flow rate	1.0 mL/min
Injection volume	100 µL

An Elma S 40H (New Jersey, USA) ultrasonic water bath was used for the degassing of mobile phase and the dissolution of solids. Lyophilisation of samples was performed at -47 °C and 1.4 mbar using Christ LDC-1 (Osterode am Harz, Germany) freeze drying instrument. Samples were digested using Milestone Ethos Plus (Sorisole, Italy) microwave digestion system.

2.3 Procedures

2.3.1 Controlled cultivation of onion samples

Onion samples were cultivated hydroponically in laboratory made cultivation containers; for this purpose 400 mL plastic containers were used. Styrofoam covers were cut in the desired form to close the top surface of the containers. Containers were filled with 1:1 diluted Hoagland solutions [17]. Final concentrations of each element are provided in Table 2.3. Nine different pots with different As and/or Se spikes were prepared, as stated in Table 2.4, in order to see the effect of individual species as well as combination of different forms. Two shallots obtained from a local market were planted into each container dipping the roots inside the nutrient solution while keeping the body above the liquid level. Nutrient solutions were aerated daily for 5 min using an external air pump with an output of 5 L/min. Containers were put in a controlled growth chamber. Plants were grown at ambient temperature (20-25

°C) under constant light intensity (100 µmol photons.m^{-2}.s^{-1}) provided by fluorescent lamps with a cycle of 16 hours illumination followed by 8 hours night period. After 25 days, plants were collected and separated into parts as root, bulb and leave.

Table 2.3 Final concentration of element in Hoagland nutrient solution*.

Element	**Concentration, mg/L**
Nitrogen	210
Potassium	235
Calcium	200
Phosphorus	31
Sulphur	64
Magnesium	48
Boron	0.5
Iron	1.0
Manganese	0.5
Zinc	0.05
Copper	0.02
Molybdenum	0.01

* Hoagland solution used for onion samples was diluted two fold.

Table 2.4 Composition of feed solutions.

Pot Number	Feed Solution, 1.0 mg/L each
1	Control
2	As (III)
3	As (V)
4	Se (IV)
5	Se (VI)
6	As (III) + Se (IV)
7	As (III) + Se (VI)
8	As (V) + Se (IV)
9	As (V) + Se (VI)

2.3.2 Sample preparation

Collected samples were washed with deionised water and separated into parts as root, bulb and leave as shown in

Figure 2.1, and then, frosted with liquid nitrogen. Frozen samples were ground with pestle and mortar. Homogenized samples were freeze dried for 48 h at -47 °C and 1.4 mbar. A Christ LDC-1 lyophilizer (Osterode am Harz, Germany) was used for this purpose. Prepared samples were stored at -18 °C till analysis.

Nutrient solutions left in the containers were also collected so as to determine the As and/or Se content. For this purpose, plants were removed and volume of the nutrient solution was completed to 400 mL using deionised water. Resultant solution was filtered.

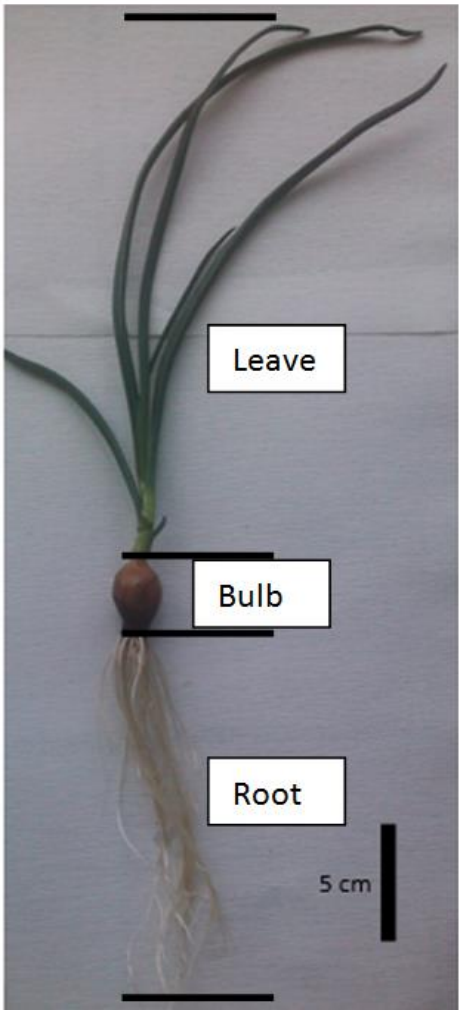


Figure 2.1 Parts of onion plant.

2.3.2.1 Total selenium and arsenic determination

A milestone Ethos Plus microwave system was used for sample digestion. Approximately 0.10 g of sample was weighed and transferred into Teflon digestion vessels. 3.0 mL of concentrated nitric acid and 3.0 mL of 30% (v/v) hydrogen peroxide were added. Temperature program in Table 2.5 was applied for the digestion of samples. When the digestion was complete, digested samples were filtered through 0.45 µm membrane filters and transferred to 25.0 mL volumetric flask. Samples were diluted to the mark with deionised water and stored in polyethylene flasks till time of analysis at 4.0 °C.

Table 2.5 Temperature program for sample digestion.

Temperature, °C	Ramp time, min	Hold time, min
100	5.0	10.0
150	5.0	10.0
Ventilation	-	10.0

2.3.2.2 Extraction procedure

0.10 g of dried sample was taken into 15 mL centrifuge tube for the non-enzymatic extraction of selenium and arsenic from plant matrix. Then, 7.0 mL of the selected extraction solvent was transferred into the tubes and resultant mixtures were mechanically shaken for 15 min followed by a 20 min ultrasonication. Mixtures were centrifuged at 5000 rpm for 15 min. The supernatant was decanted and filtered through 0.2 μm filters.

For the enzymatic extraction, 0.10 g of sample was placed in a 15 mL centrifuge tube. 30 mg of enzyme and 7.0 mL of 25 mmol/L phosphate buffer at pH 7.50 were added. Resulted mixture was mechanically shaken for 15 min and ultrasonicated. Solution was placed on a mechanical elliptic shaker and allowed to mix over 24 h at 37 ^{o}C. Then, resultant mixture was centrifuged at 5000 rpm for 15 minutes. Supernatant was decanted and filtered through 0.2 μm filters.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Optimization of ICP-MS system

Sensitivity and precision of ICP-MS instrument is strongly dependent on the optimization of instrumental working conditions. Three main groups of parameters are optimized in ICPMS instrument. First group covers the sample introduction, second group includes plasma conditions and ion sampling and the third group includes ion optics and detector parameters. These were optimised regularly by using the auto tune sequence of the instrument with 10.0 µg/L "Tune A" mixed standard solution recommended by the manufacturer. Tune A solution contains Li(I), Co(II), In(III), Pb(II), Bi(V) and U(IV) ions in 0.5 mol/L nitric acid solution. Performance of the instrument was regularly monitored using this solution; if there is a change in the instrument sensitivity with time, autotune sequence was run again. Furthermore, a fine tuning of the target isotopes was performed using 10.0 µg/L As(III) and Se(IV) mixture. A typical set of optimised parameters can be seen in Table 3.1, but these values prone to change on time.

Table 3.1 Optimization results of ICP-MS instrument.

Parameter	**Optimum value**
Extraction lens voltage, V	-158
Lens 1, V	-7.6
Focus, V	10.9
D1, V	-47.6
Pole bias, V	-7.2
Hexapole bias, V	0.3
Nebuliser pressure, psi	0.9
Sampling depth*, mm	28
Lens 2, V	-80
Lens 3, V	-168.9
Forward power, W	1400
D2, V	-160
Diffraction aperture, V	-29.8
Coolant gas flow, L/min	13
Auxiliary gas flow, L/min	0.8

*Sampling depth is the distance from torch tip to sampling cone

One problem with mass detectors equipped with low resolution quadrupole mass analyser is the spectral interferences caused by elemental isobaric ions, multiple charged ions or polyatomic species. Most abundant selenium isotope is ^{80}Se with relative abundance of 49.96%. However, $^{40}Ar^{40}Ar^{+}$ molecular peak interferes at the same mass to charge ratio. In the current study all of the selenium isotopes, namely $^{74}Se^{+}$, $^{76}Se^{+}$, $^{77}Se^{+}$, $^{78}Se^{+}$ and $^{82}Se^{+}$, except $^{80}Se^{+}$ were monitored to eliminate the interference from Argon related ions and $^{78}Se^{+}$ and $^{82}Se^{+}$ ions were used for the quantification. Other ions, $^{74}Se^{+}$, $^{76}Se^{+}$, $^{77}Se^{+}$, were monitored for information purpose only.

In case of arsenic, interference problem is more severe since arsenic has only one stable isotope, ^{75}As, which has the same nominal mass with $^{40}Ar^{35}Cl^{+}$ molecular ion. PlasmaLab software has a prebuilt interference equation library that helps to eliminate isobaric interferences using mathematical algorithms. Correction equations for $^{75}As^{+}$ may be seen below, where M designates the total count at the indicated mass.

Equation 1 $^{82}Se^{+} = {}^{82}M^{+} - 1.001 \times {}^{83}Kr^{+}$

Equation 2 $^{77}ArCl^{+} = {}^{77}M^{+} - 0.860 \times {}^{82}Se^{+}$

Equation 3 $^{75}As^{+} = {}^{75}M^{+} - 3.12 \times {}^{77}ArCl^{+}$

According to the algorithm, first $^{82}Se^{+}$ is corrected with respect to $^{83}Kr^{+}$ to eliminate the $^{82}Kr^{+}$ interference. Second step includes correction of $^{77}ArCl^{+}$ with respect to $^{82}Se^{+}$ that was corrected at the previous step. Finally $^{75}As^{+}$ is corrected using corrected $^{77}ArCl^{+}$ mass.

However, during the study it was observed that in some cases this algorithm is not working properly; unexpected counts were obtained. In order to see the effect of Cl^{-} on $^{75}As^{+}$ signals, series of standards containing Cl^{-} with different concentrations were prepared and signals at m/z 75 were monitored. Relative effect of Cl^{-} concentration on counts at m/z 75 is given in Figure 3.1 with and without software correction applied. In order to see the extend of interference effect, raw and corrected counts were normalized with respect to 0.10 mg/L As signal by dividing corresponding signals to 0.10 mg/L As signal at the same conditions. 0.10 mg/L As solution does not contain Cl^{-} ions, hence no interference effect was observed. It can be seen that if software correction is not applied, no interference effect was observed up to 100 mg/L Cl^{-} level. Above that level positive deviation, less than 6%, was observed. This result indicates that Cl^{-} interference is not significant up to 100 mg/L Cl^{-} level.

However, if software correction was applied to raw data, negative error, as high as 70%, was resulted at 10 g/L Cl^{-} concentration. This is a strong indication of

overcorrection. The reason for overcorrection is not clear but is believed that resolution if the instrument, i.e. 1 amu, is not enough for such precise measurements.

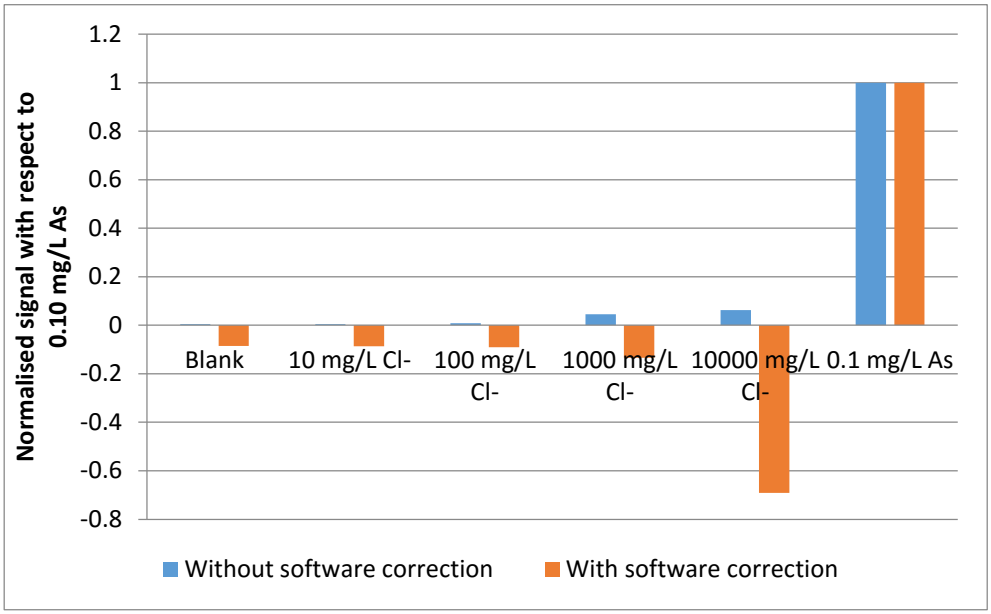


Figure 3.1 Relative effect of Cl^- concentration on ^{75}As counts with and without software correction.

Another problem is the presence of selenium. Se does not have an isotope at m/z 75 so it does not have a direct spectral interference at the target mass. Yet, it is included in the correction algorithm as can be seen in Equation 2, so it may have an indirect effect. In order to test the effect of Se on As signal, solutions with different Se concentrations were prepared. Relative signals at m/z 75 with respect to 0.10 mg/L As were given in Figure 3.2. Interference effect is not observed, as expected, without software correction. However, very high, up to 750%, positive error is observed in the presence of Se with respect to 0.10 mg/L As signals. It is clear that presence of Se at high concentrations drastically affect the As signal at m/z 75.

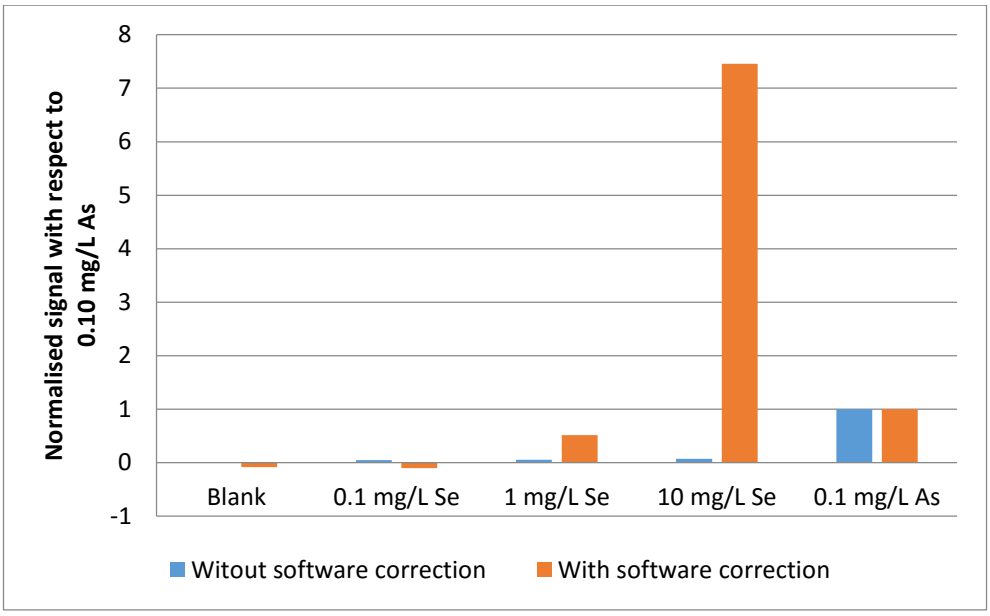


Figure 3.2 Relative effect of Se on ^{75}As counts with and without software correction.

Total amount of Cl^- in the onion samples were estimated by argentometric titration using Fajan's method. 0.10 g of dried sample was collected and digested as indicated in the experimental section. pH of the resulted mixture was adjusted to 5-7 range by using $NaHCO_3$ and titrated with standard 0.010 mol/L $AgNO_3$ solution in the presence of dextrin and dichlorofluorescein indicator. Result revealed that onion samples contain average of 10 mg/g Cl^-. In this case Cl^- concentration in the final solution is about 40 mg/L which is well below the interference level. This result is consisting with the literature. Chang and Randle added NaCl to nutrient solution at different concentrations and showed that at 100 mmol/L NaCl concentration, Cl^- level in bulb and leave was below 10.0 mg/g level [162]. Interference of $^{75}ArCl^+$ on As signal is negligible at this level as seen from Figure 3.1. As a result, software correction function was disabled for the rest of the experiments.

3.2 Plant cultivation

Green onion plant was selected as target because;

- It grows fast,
- It is a natural selenium accumulator,
- It is resistive to chemical stress,
- It can be grown under hydroponic conditions,
- It is widely consumed in Turkey.

Cultivation was conducted as stated in the experimental section. Nine pots were prepared and different combinations of As and/or Se was spiked into feed solutions. In the literature, there are two different approaches in controlled plant experiments; some studies spike the target element(s) at the beginning of planting [163] while others spike after a certain growing period [164]. In this study spiking was done at the time of planting in order to decrease the chemical stress caused by As and/or Se ions.

First set was spiked with 10.0 mg/L of As and/or Se ions. After a 25 day growing period plant were harvested, separated as root, bulb and leave. Mass of each part was recorded and reported in Table 3.2.

Table 3.2 Wet mass of root and leave parts after 10.0 mg/L As and/or Se treatment.

Treatment	Wet mass of root, g*	Wet mass of leaves, g*
Control	5.55	13.8
As(III)	1.36	10.8
As(V)	1.69	10.3
Se(IV)	0.92	7.42
Se(VI)	7.15	14.1
As(III) + Se(IV)	0.15	4.85
As(III) + Se(VI)	0.22	1.02
As(V) + Se(IV)	0.31	2.17
As(V) + Se(VI)	0.27	3.14

*RSD < 3%, N=3

As seen from the table, plant growth for spiked samples were significantly lower, except for Se(VI) supplementation, when compared with the control plant. Pictures of control plant and plant supplemented with 10.0 mg/L As(III) and 10.0 mg/L Se(IV) at the time of harvesting are given in Figure 3.3a and 3.3b, respectively. Results indicate that both As and Se has toxic effect on plant growth at this specific concentration value except for Se(VI) supplementation.

(a) **(b)**

Figure 3.3 Control plant (a), and plant supplemented with 10.0 mg/L As(III) and 10.0 mg/L Se(IV) feed solution (b).

Concentration values of As and Se were lowered to 5.0 mg/L for the second set of plants. Wet masses of root and bulb after 25 days were recorded and given in Table 3.3. Plant growth was better compared to the first set if we compare single element spike, but it is significantly lower in case As and Se spiked together. This is an indication of combined toxic effect. Pictures of control plant, plant supplemented with 5.0 mg/L As(III) and plant supplemented with 5.0 mg/L As(III) and 5.0 mg/L Se(IV) were given in .

Table 3.3 Root and leave mass of plants after 5.0 mg/L As and/or Se treatment.

Treatment	Wet mass of root, g*	Wet mass of leave, g*
Control	6.17	14.2
As(III)	5.14	12.8
As(V)	5.72	11.1
Se(IV)	4.91	10.9
Se(VI)	6.42	13.6
As(III) + Se(IV)	0.97	8.82
As(III) + Se(VI)	1.21	7.63
As(V) + Se(IV)	1.05	8.12
As(V) + Se(VI)	0.88	8.14

*RSD < 3%, N=3

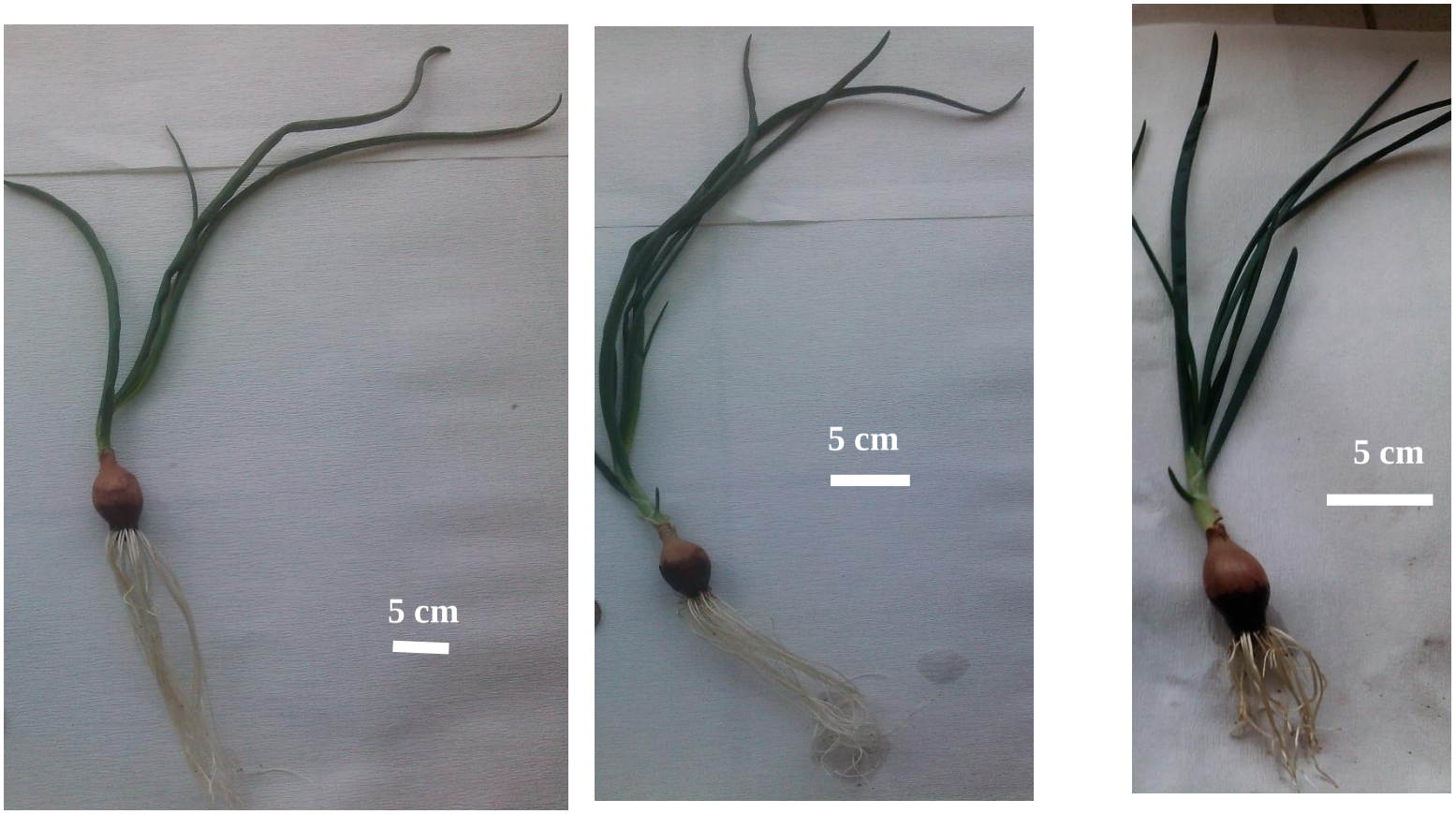


Figure 3.4 Control plant (a), plant supplemented with 5.0 mg/L As(III) (b) and plant supplemented with 5.0 mg/L As(III) and 5.0 mg/L Se(IV) (c) feed solution.

Third set of samples were grown by spiking 1.0 mg/L of As and/or Se. In this case root and leave growth was comparable to that of the control plant as seen from Table 3.4. It is concluded that at 1.0 mg/L concentration level, toxic effects of As and Se were negligible. 1.0 mg/L spiked samples were grown and used for the rest of the study.

Plants were grown as explained in the experimental section. All plants were green without any observable defect at the time of harvesting. Wet mass of individual parts of plants and the total masses are shown in Figure 3.5. Control plants have the highest total mass compared to plants fed with As and/or Se. Plants supplemented with Se(IV) have the lowest mass. However, the total mass of plants supplemented with Se(IV) together with As(III) or As(VI) is higher as compared to Se(IV) only supplementation.

Table 3.4 Root and leave mass of plants after 1.0 mg/L As and/or 1.0 mg/L Se treatment.

Treatment	**Wet mass of root, g***	**Wet mass of leave, g***
Control	4.77	18.2
As(III)	4.56	12.5
As(V)	4.13	11.5
Se(IV)	1.93	10.3
Se(VI)	4.49	11.9
As(III) + Se(IV)	4.07	11.0
As(III) + Se(VI)	2.21	11.1
As(V) + Se(IV)	4.07	11.2
As(V) + Se(VI)	3.66	12.6

*RSD < 3%, N=3

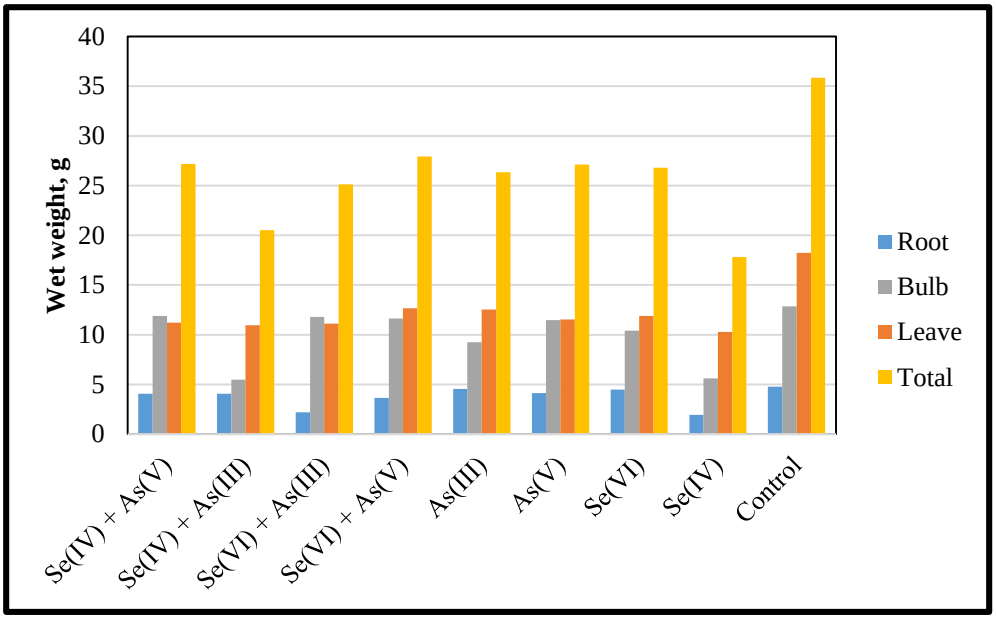


Figure 3.5 Total mass of plant parts supplemented with 1.0 mg/L As and/or Se solution.

Total wet masses of plants supplemented with 10.0 mg/L, 5.0 mg/L and 1.0 mg/L solution of As and/or Se are given in Figure 3.6.

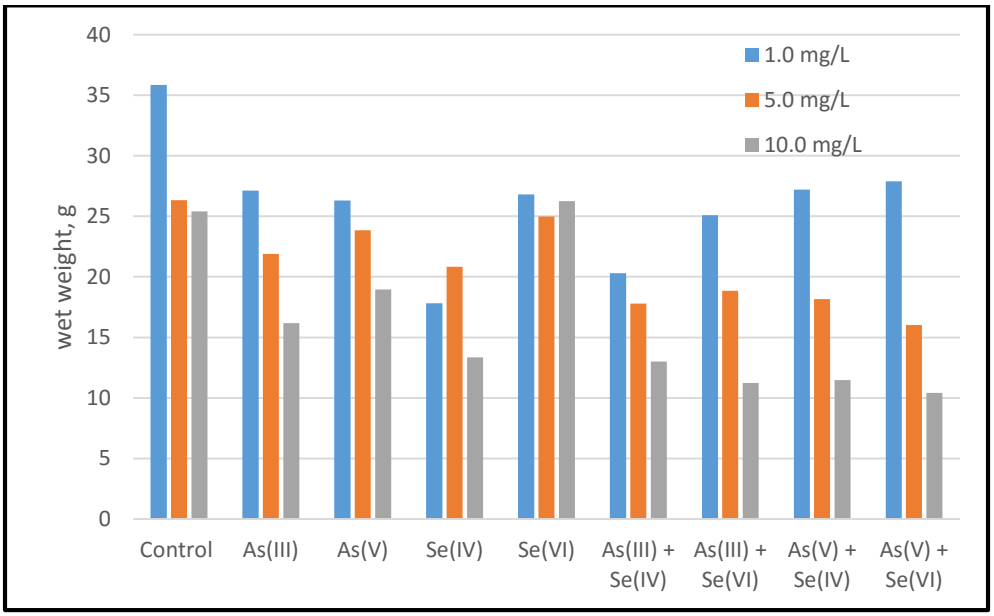


Figure 3.6 Comparison of the total mass of plants supplemented with 10.0 mg/L, 5.0 mg/L and 1.0 mg/L As and/or Se solutions.

Plants were lyophilized to remove the moisture. This is an essential step since in water media enzyme activities or microorganisms' may lead to decomposition of large molecules as well as intermolecular or intramolecular conversion. Lyophilized samples were stored in airtight containers since these samples were highly hydroscopic.

Average moisture content of root, bulb and leaves were calculated by subtracting the dry mass from wet mass and reported in Table 3.5. These results are higher when compared with National Food Composition Database. Average moisture content in the database is given as 91.42% [165] for green onion. Higher results may be attributed to the hydroponic growing conditions where water is readily available when compared to soil media.

Table 3.5 Moisture content of root, bulb and leaves of green onion plants.

	Water content*, % (w/w)
Root	96.0 ± 2.1
Bulb	94.0 ± 1.2
Leave	95.1 ± 1.3

*N=3

3.3 Arsenic and selenium determination

Concentration of total Se and total As in the samples were determined by ICP-MS following microwave digestion. Optimized parameters given in section 3.1 were used for ICP-MS instrument.

Sample digestion is a crucial step in total element determination because improper digestion may lead erroneous quantification. For the digestion of onion samples, digestion method optimized previously in our research group was used [166].

Method was developed for determination of As, Se and Cd in Turkish spices which is very similar to samples in this study. In summary, 0.10 g onion sample was digested using 3.0 mL concentrated HNO_3 and 3.0 mL of 30% (v/v) H_2O_2 mixture and the temperature program given in Table 2.5 was applied. No visible residue remained in the digestion vessels, nonetheless digests were diluted with few mL of water and filtered through 0.45 μm membrane filters. Final volume was completed to 25.0 mL with deionised water. Matrix matched standards were prepared so that the final acid and peroxide concentration in the standards were same with that of the samples. Calibration graphs of As and Se are given in Figure 3.7 and Figure 3.8, respectively. All isotopes of selenium, except ^{80}Se, were monitored. Quantification was done both with ^{78}Se and ^{82}Se isotopes.

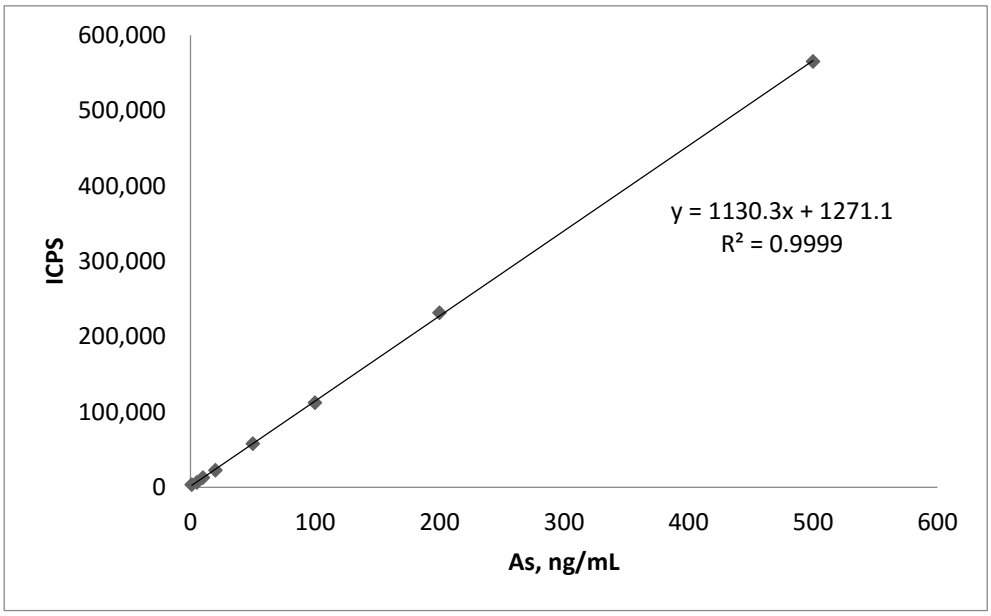


Figure 3.7 Calibration graph and the best line equation for ^{75}As by ICP-MS.

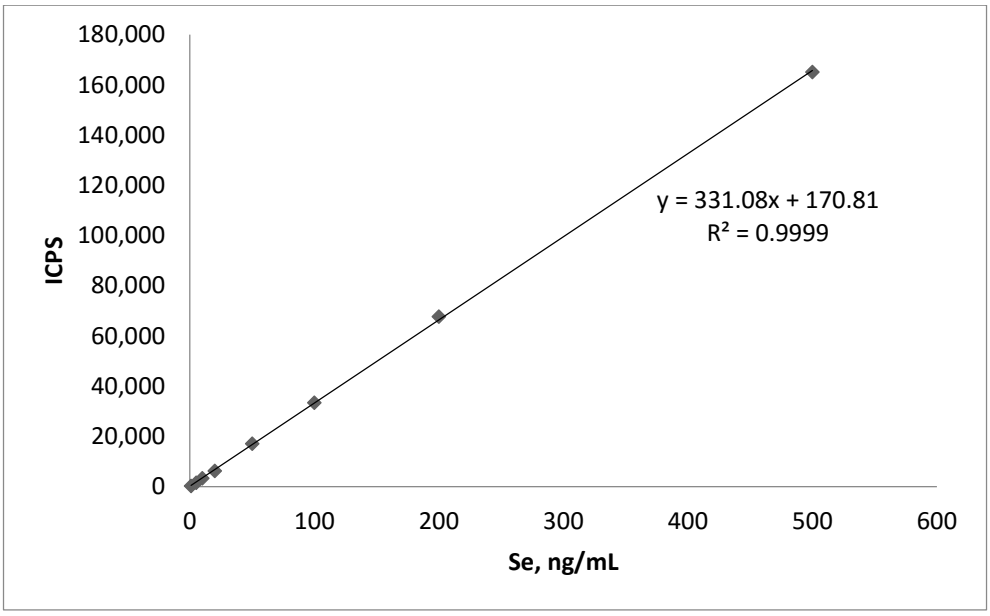


Figure 3.8 Calibration graph and best line equation for ^{78}Se by ICP-MS.

LOD and LOQ of ICP-MS technique were calculated and reported in Table 3.6. LOD and LOQ values were calculated using the formula 3s/m and 10s/m, respectively. In the equations s indicates the standard deviation of the lowest standard, i.e. 1.0 ng/mL, (N=10) and m is the slope of calibration plot.

Table 3.6 Analytical figures of merit.

	As	**Se**
LOD, mg/kg	0.21	0.28
LOQ, mg/kg	0.70	0.93
Working range, ng/mL	1.0-500	1.0-500

NRC DOLT-4 Dogfish Liver and NIST 1566b Oyster Tissue standard reference materials were used to test the accuracy of the system. Both reference materials were digested and prepared with the same procedure applied for onion samples. Results

are given in Table 3.7. No significant difference was found between the certified values and experimental values when t-test was applied at 95% confidence level.

Table 3.7 Certified values and experimental results of standard reference materials used for validation.

	Certified value[b], mg/kg		**Experimental result[a], mg/kg (N=3)**	
	As	**Se**	**As**	**Se**
NRC DOLT-4	9.66 ± 0.62	8.3 ± 1.3	9.01 ± 0.39	8.64 ± 0.52
NIST 1566b	7.65 ± 0.65	2.06 ± 0.15	8.31 ± 1.30	2.33 ± 0.35

[a]Mean ± standard deviation

[b]Mean ± expanded uncertainty (U_{CRM})

3.3.1 Arsenic distribution in Onion

As and Se concentration in root, bulb and leave parts of the plant was determined and As concentrations were reported in Table 3.8. Control plant is not included since the concentration of Se and As was below LOD values. All calculations in the study are based on dry weight of the sample. Approximately 0.100 g sample was digested for bulb and leaves and ca. 0.050 g sample was digested for roots since the mass of root part is smaller compared to the other part of the plant.

Table 3.8 Concentration of As in root, bulb and leave parts of the plants.

		$C_{As} \pm s$, mg/kg
As(III)	Root	2468 ± 239
	Bulb	34.9 ± 3.4
	Leave	50.7 ± 4.9
As(V)	Root	1504 ± 146
	Bulb	20.2 ± 2.0
	Leave	45.6 ± 4.4
Se(IV) + As(III)	Root	2027 ± 197
	Bulb	n.d.*
	Leave	36.9 ± 3.6
Se(IV) + As(V)	Root	2271 ± 220
	Bulb	25.3 ± 2.5
	Leave	29.9 ± 2.9
Se(VI) + As(III)	Root	3362 ± 326
	Bulb	13.9 ± 1.3
	Leave	47.0 ± 4.6
Se(VI) + As(V)	Root	2345 ± 228
	Bulb	31.9 ± 3.1
	Leave	83.6 ± 8.1

*n.d: Not detected

In order to have a better composition, As concentration was plotted against feed composition in Figure 3.9 and percent mass distribution of As in different parts of the plant is given in Figure 3.10. It is clear from the figures that most of the As was deposited in the root parts of the plant and very small percentage was found in bulb and leave parts. It may be concluded that green onion plant tent to deposit As in root

part mainly. This behaviour may be attributed to the toxic nature of As and it may be considered as a defence mechanism. Plant somehow bind the As in the root part to preserve bulb and leaves. This behaviour is not unique to green onion plant. In the literature other plants, such as *Camellia sinensis L.*, *Dryopteris filix-mas, Calluna vulgaris (L.) Hull, Alternanthera philoxeroides, Zea mays* and *Helianthus annuus* with similar As intake and accumulation profiles were reported [167–171]. In those plants, As was reported to be accumulated mainly in the root part.

When As(V) was administered together with Se(IV) or Se(VI), arsenic concentration in the roots increases. This is an indication of synergism. Presence of either Se(IV) or Se(VI) increases the intake and concentration of arsenic if it is administered in pentavalent oxidation state. Similar synergic effect was also observed between As(III) and Se(VI).

On the other hand, As hyperaccumulators, such as *Pteris vittata L.* and *Agrostis capillaris L.*, shows different distribution scheme; having higher As concentration in leaves compared to roots [56,136,158,172–175]. It is understood that distribution scheme of As differs among plants. However, it is important to note that only As spike was done in the above studies and none of them contains As-Se interaction.

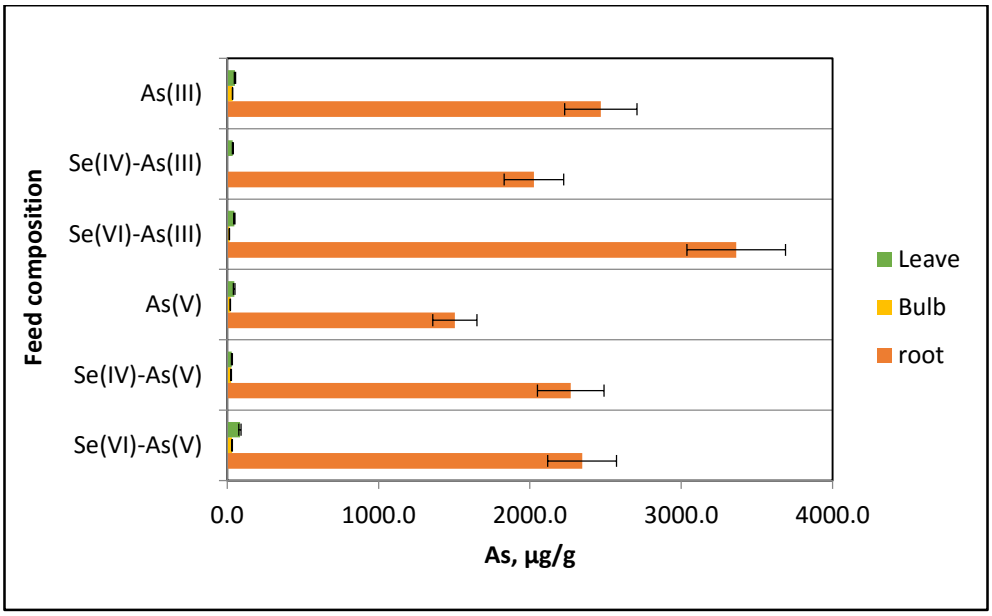


Figure 3.9 Concentration of As in root, bulb and leave of plants that are grown by spiking 1.0 mg/L As and/or 1.0 mg/L Se.

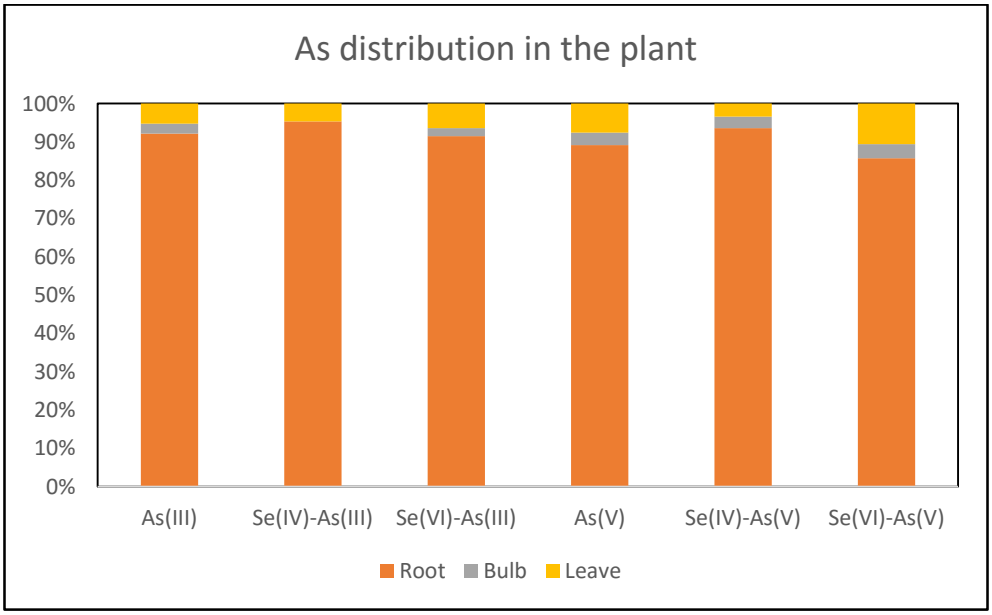


Figure 3.10 Percent distribution of As within plant.

Amount of As left in the feed solution at the time of harvesting was determined by removing the plants and completing the volume of remaining feed solution to 400.0 mL with deionised water. Results are given in Figure 3.11. Concentration of As remaining in the feed solution was very low compared to initial concentration, ie 1.0 mg/L. It is an indication that most of the As was absorbed by the plants and 1 to 8% remains in the solution. Even though As is a toxic element, its intake by plant is not restricted. It was also observed that presence of Se did not affect the uptake of As. Highest concentration was in Se(VI)-As(III) spiked solution which is about 7 times more than the others as shown in Figure 3.11 but still much lower compared to the initial concentration (1.0 mg/L).

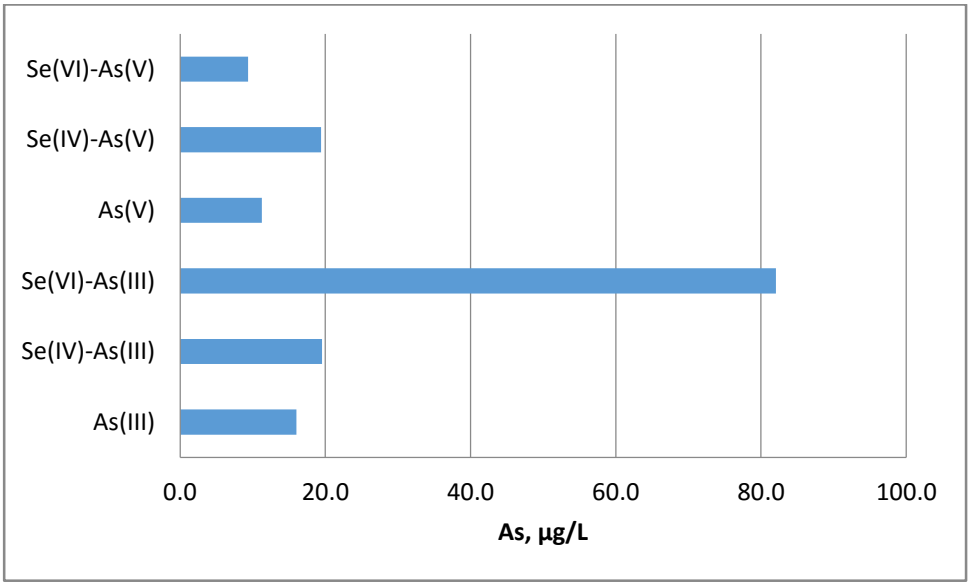


Figure 3.11 Concentration of As remaining in the feed solution.

Mass distribution of As between plant and feed solution at the time of sampling was plotted in Figure 3.12. Mass of arsenic in plant was calculated by multiplying the dry weight with the concentration in different parts of plant. During the lyophilisation and homogenisation processes, some of the sample was lost, and the measured dry weight was not representing the actual weight. In order to minimize the error caused by sample loss, dry mass was calculated by precisely measuring the water content of

a representative sample and converting the wet weight measured at the time of sampling to dry weight.

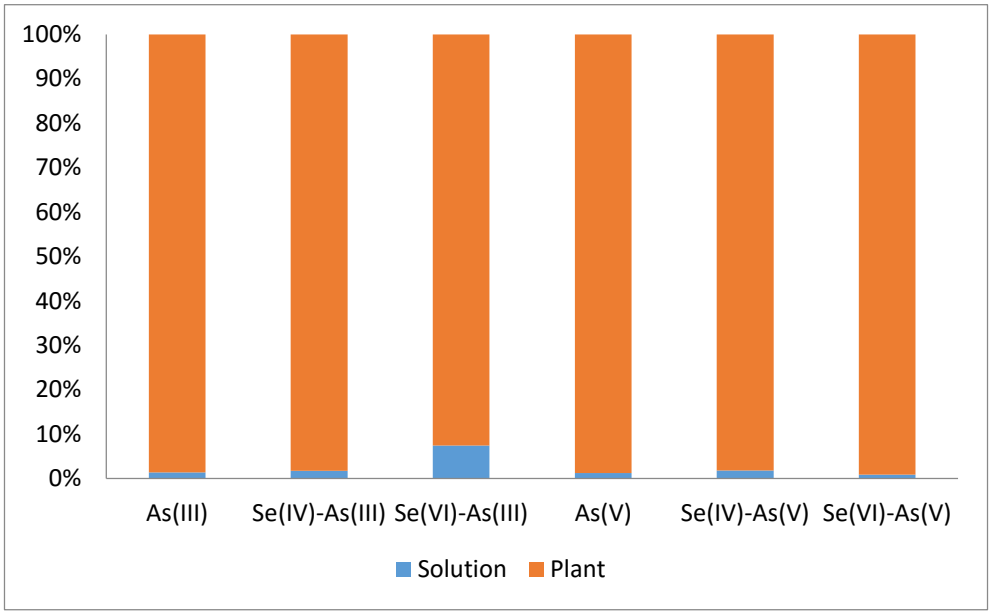


Figure 3.12 Distribution of As between plant and feed solution at the time of harvesting.

Uptake mechanism of arsenic can be mediated in two different mechanisms depending on the oxidation state.

Arsenate uptake may be related to its similarity with phosphate (Figure 3.13). Phosphorus is usually the limiting component in plant growth. Most of the organophosphorous compounds cannot be taken and used by plants and inorganic phosphate is the most convenient form [176,177]. Thus plants have high affinity towards phosphate molecule. Phosphate molecule uptake is facilitated by a group of proteins called PHT [175]. Because of the structural similarity, As(V) is taken up by the plants with the same mechanism as phosphate. This structural similarity may be used to explain the high As(V) uptake. Once As(V) was in the root system of the green onion plant, molecular recognition system differentiate the arsenate from phosphate and further transport of As(V) to bulb and leaves was restricted.

Arsenate Phosphate

Figure 3.13 Chemical structures of arsenate and phosphate.

Uptake of As(III) is facilitated with a class of proteins called NIPs (nodulin-26-like intrinsic proteins) [175]. Those proteins form channel on cell membranes called NIPs channels. NIPs channels facilitate the passage of small structured uncharged molecules such as urea, ammonia, silicic acid and glycerol. Arsenite has a pKa value of 9.2, so it present as uncharged molecule in most biological environments. Having similar size with target molecules, NIPs channels allow the passage of arsenite into cell [74].

3.3.2 Selenium distribution

Concentration of total selenium in root, bulb and leaves is reported in Table 3.9 and Figure 3.14. Distribution of total selenium is affected by the composition of feed solution. Se distribution follows a different trend when compared with As. If feed solution contains selenite, Se(IV), highest Se concentration was measured in roots. On the other hand, if selenium was introduced to plant in the form of selanate, Se(VI), highest selenium concentration was measured in leaves. Similar trends with other plants were also reported for bean plant, rice, werat, indian mustard in the literature [178–181]. For instance, if bean plants (*Phaseolus vulgaris*) were supplemented with selenate, 50% of the absorbed selenium was transferred to aerial organs within 3 hours. However, if selenium was supplemented as selenite, major part was deposited in the root part [178]. Explanation for such behaviour is that if selenium is introduced in the form of selenite, it can easily be converted to organoselenium species in the roots. Transport of organoselenium species is lower

compared to inorganic ones since they are mostly incorporated with large biomolecules, such as proteins and peptides. On the other hand, rate of conversion of selenate to organoselenium species were lower, it is translocated to leaves where it is slowly converted to organoselenium species [182]. Such a conclusion is going to be more supported in the preceding sections.

Table 3.9 Concentration of Se in root bulb and leave parts of the plants.

		$C_{Se} \pm s$, µg/g
Se(IV)	Root	362±29
	Bulb	36.8±3.0
	Leave	145±11
Se(VI)	Root	182±14
	Bulb	30.0±2.4
	Leave	334±27
Se(IV) + As(III)	Root	301±24
	Bulb	n.d.*
	Leave	206±16
Se(IV) + As(V)	Root	289±23
	Bulb	21.5±1.7
	Leave	269±21
Se(VI) + As(III)	Root	251±20
	Bulb	21.9±1.8
	Leave	362±29
Se(VI) + As(V)	Root	228±18
	Bulb	26.3±2.1
	Leave	376±30

*n.d: Not detected

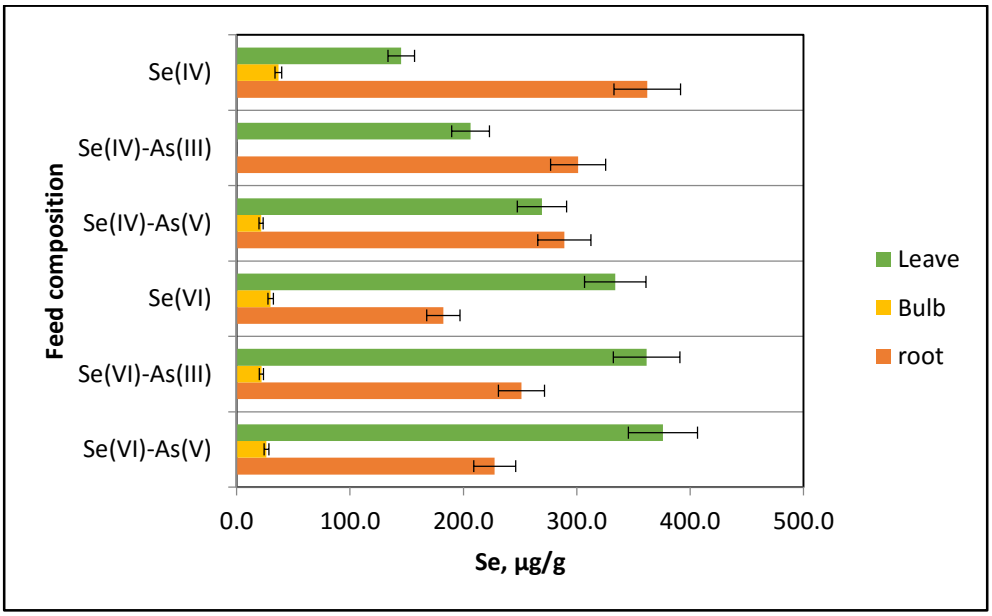


Figure 3.14 Concentration of Se in root, bulb and leave of plants that were fed by spiking 1.0 mg/L As and/or 1.0 mg/L Se.

Selenium concentration decreases in roots and increases in leaves if Se(IV) was administered together with As(III) or As(V) as seen from Figure 3.14. It may be concluded that presence of As decreases the conversion efficiency of Se to organoselenium forms since mobility of organoselenium compounds are much lower as discussed in the above paragraphs. In this case increasing inorganic selenium concentration resulted in relatively easy transportation to bulb and leaves, where As concentration is much lower, and Se is converted to organoselenium forms in leaves.

Percent mass distribution of Se in different parts of the plant is given in Figure 3.15. It is clear that most of the Se is concentrated in the leave part compared to bulb and roots.

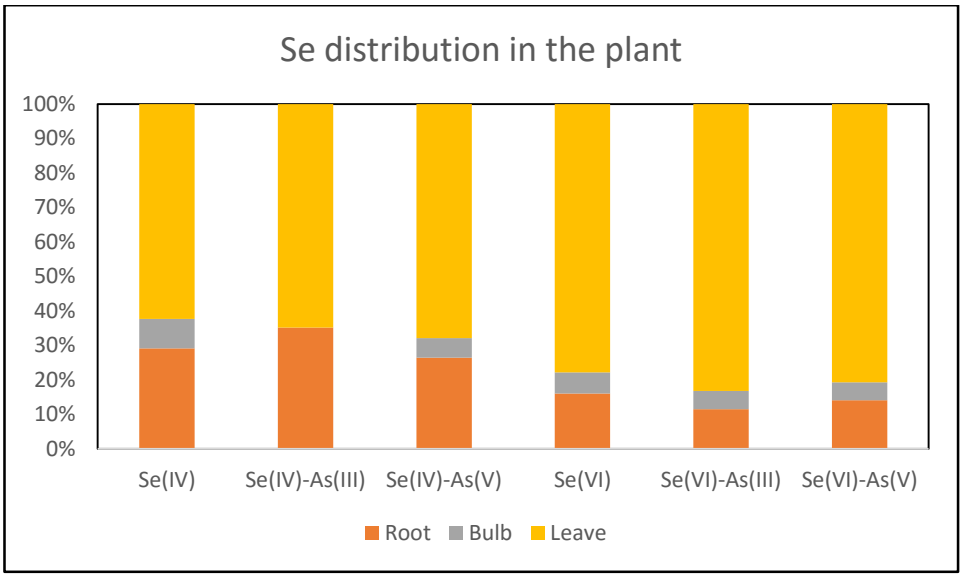


Figure 3.15 Percent mass distribution of Se in root, bulb and leave of onion plant.

Concentration of Se in feed solution at the time of harvesting is given in Figure 3.16 and percent mass distribution of selenium between plant tissue and feed solution is given in Figure 3.17. Selenium uptake is much less compared to arsenic uptake. In the presence of As(III) and As(V), Se(IV) intake is higher while Se(VI) intake is not affected by the presence of neither As(III) nor As(V). This result may be linked with the previous observation that presence of As species drive Se from roots to leaves (Figure 3.14). Since more Se was transported to the leaves, Se concentration in roots decreased and more Se was absorbed from the feed solution.

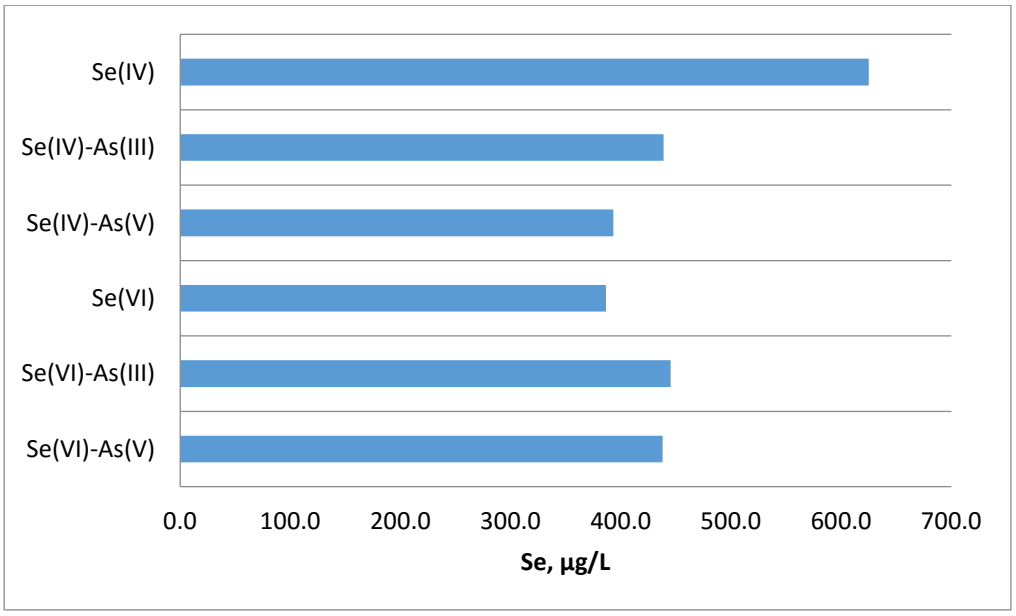


Figure 3.16 Concentration of Se in feed solution at the time of harvesting.

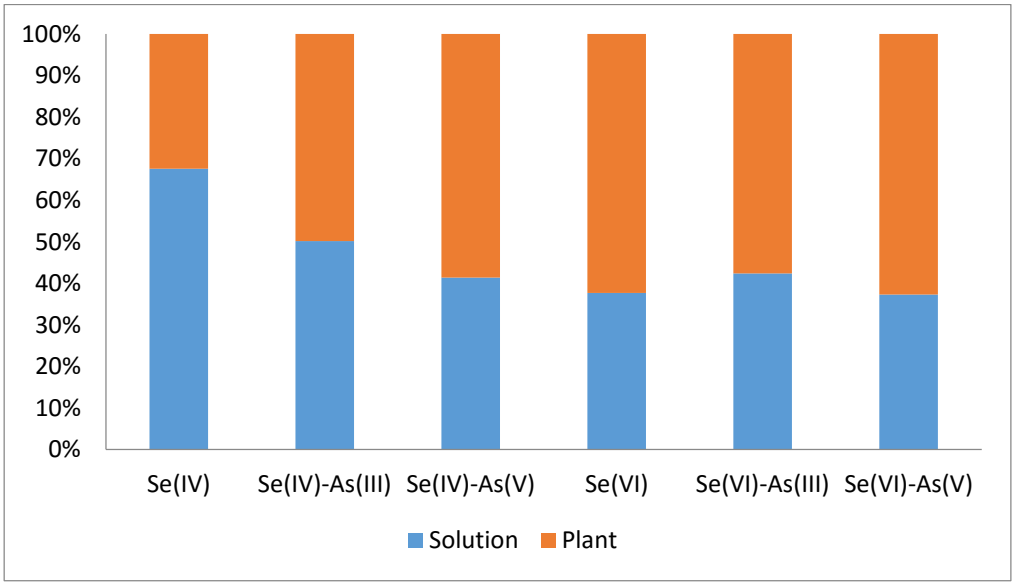


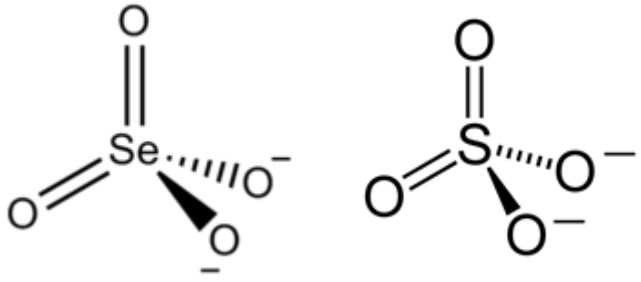
Figure 3.17 Percent mass distribution of Se between plant tissue and feed solution at the time of harvesting.

Selenium uptake is explained by two different mechanisms for selenate and selenite. Selenate is structurally very similar to sulfate as seen from Figure 3.18. It is believed that selenate uptake is through sulphate transporter genes [91]. However, some of the

plants, such as barley, rice and cabbage, have been shown to developed a discriminatory process between Se and S by selective absorption of the species. Mechanism is not fully understood but it is believed that some plants has developed more sophisticated sulphate genes that can recognise the difference between sulphate and selenate [183–186]. It was also shown that sulphate transporters have different characteristics in Se accumulating and nonacumulating plants [183].

Unlike selenate, selenite uptake is not well understood. It is believed that uptake is a passive diffusion process [93,94]. However, a recent study identified an inorganic phosphate transporter (OsPT2) that can actively uptake selenite [187]. General conclusion is that selenite transport is mainly a passive process but some transporters increase the rate of uptake.

This may also account for the low selenite intake in the present study. It is possible that presence of arsenic increases the activity of active transporters resulting in higher uptake.



Selenate Sulfate

Figure 3.18 Chemical structures of selenate and sulfate.

3.4 Simultaneous extraction of arsenic and selenium

Extraction is a curtail step of a successful speciation study. An ideal extraction procedure should quantitatively take all analytes without alteration of chemical composition in a minimum amount of time and effort. Such an extraction system can never be achieved but optimisation of parameters will provide a balanced composition of such parameters. After a comprehensive literature review, set of

solvents or mixture of solvents were studied to find the best composition. A reference sample should be used for the optimization of extraction procedure in order to calculate extraction efficiency. Since the amount of individual samples was limited, a mixed reference sample was prepared by mixing 100-200 mg of sample from hydroponically onion plants grown by spiking 1.0 mg/L As and/or Se. This reference sample was then homogenised and used throughout the optimization study. Total concentration of As and Se in the reference sample was determined by digesting 100 mg of sample with 3.0 mL concentrated HNO_3 and 3.0 mL of 30% (v/v) H_2O_2 mixture in microwave assisted digestion system as described in the experimental section. Results were given in Table 3.10. Extraction efficiencies of different solvents were calculated based on the total As and Se in the reference sample using the equation given below.

$$\text{Extraction efficiency} = \frac{\text{Extract concentration}}{\text{Total concentration}} \text{x} 100\%$$

Table 3.10 Total As and Se in reference sample used for the optimization of extraction procedure.

	As, mg/kg*	**Se, mg/kg***
Reference sample	41 ± 7	193 ± 12

*N=3, mean ± standard deviation

In the present study hot water, NaOH solution, methanol/water mixture, phosphate buffer solution, Tris HCl buffer solution, phosphate buffer solution in the presence of protease type XIV enzyme were studied at the first stage. Extract were further digested in the presence of HNO_3 and H_2O_2 with microwave assisted digestion system in order to minimize matrix effects. Results are reported in
Table 3.11.

Table 3.11 Extraction efficiencies of different solvents.

Solvent	As extraction efficiency, %*	Se extraction efficiency, %*
Hot water	81	38
0.10 mol/L NaOH	86	46
metanol-water mixture (1/3, v/v)	94	59
25 mmol/L phosphate buffer, pH 7.50	86	30
50 mmol/L tris-HCl buffer, pH 7.50	88	34
25 mmol/L phosphate buffer + Protease type XIV enzyme, pH 7.50	99	83

* N=2, RSD < %3.5

Results revealed that arsenic can be quantitatively extracted from samples with high efficiency and the highest extraction was obtained in the presence of Protease type XIV enzyme. This is an indication that most of the arsenic presents as water soluble forms and small percentage was present bonded to protein structure. 25 mmol/L phosphate buffer extracts with and without enzyme were analysed with HPLC-ICP-MS system. Resulting chromatograms are given Figure 3.19.

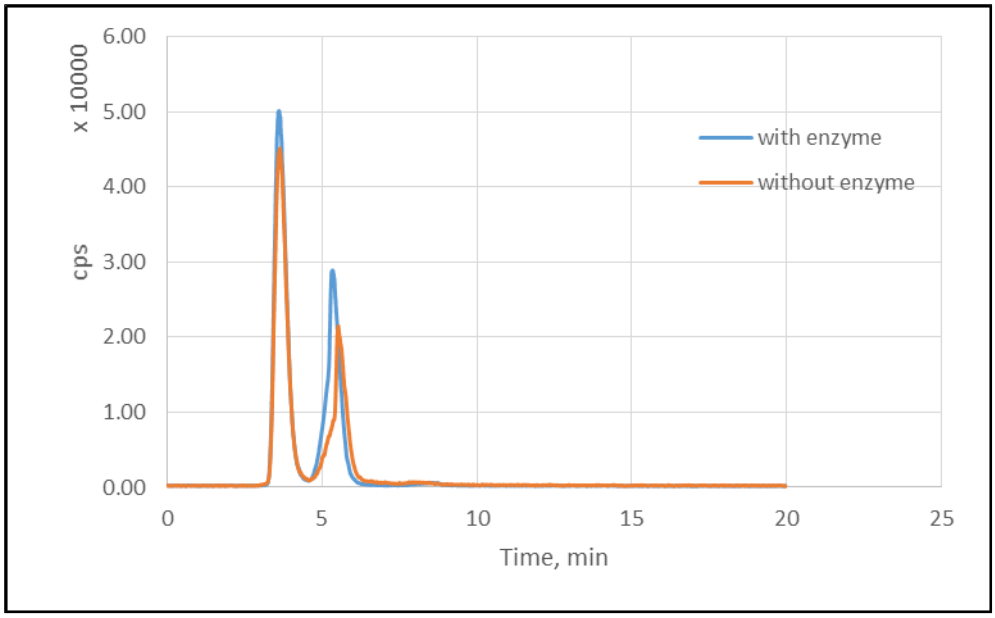


Figure 3.19 [75]As chromatograms of onion extracts with and without enzyme protease type XIV.

On the other hand, extraction efficiencies of selenium were lower. Hot water and buffers yielded the lowest efficiency indicating that small percentage was found as water soluble free molecules. From the literature it is known that basic solutions are used for the extraction of high molecular weight fractions, proteins, peptides, enzymes etc., whereas acidic solutions are used for the extraction of lower molecular weight fractions, species with a molecular weight lower than 1000 amu [124, 167]. This may explain the comparably higher extraction efficiency for Se using 0.10 mol/L NaOH.

Highest efficiency was obtained in the presence of protease type XIV enzyme implying that about half of the selenium was present bonded to protein structure. In order to prove this hypothesis, extracts of 25 mmol/L phosphate buffer with and without enzyme were analysed by HPLC-ICP-MS system. Resulting chromatograms are given in Figure 3.20.

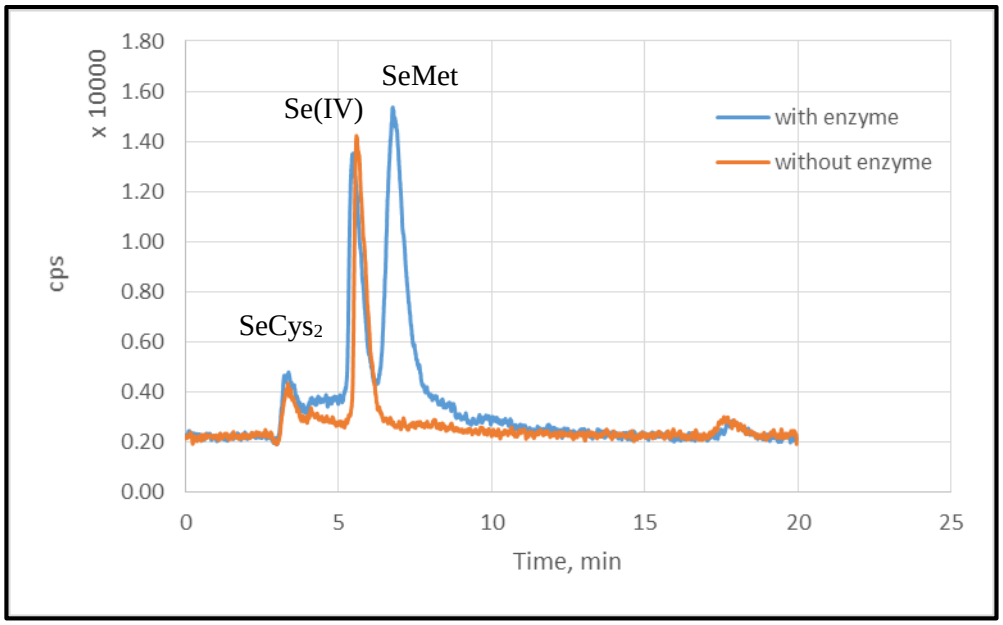


Figure 3.20 ^{78}Se chromatograms of onion extracts with and without using Protease type XIV enzyme.

It is clear from the chromatograms (Figure 3.20) that selenomethionine can only be extracted in the presence of enzyme implying the fact that selenomethionine was present bonded to water insoluble protein structures. Similarly, in their study with selenium enriched plants, Montes-Bayon et al. stated that selenomethionine can only be extracted in the presence of protease enzyme [140].

HPLC-ICP-MS experiments in the extraction stage were done by using samples grown with 10 mg/L As and/or Se spikes in order to have higher signals. As a result, species given in Figure 3.19 and Figure 3.20 are not representing the studied sample set. However, molecular behaviour was supposed to be similar in nature.

3.4.1 Selection of proper enzyme

It is clear from the previous section that enzymatic extraction must be used in order to extract protein bonded amino acids. Extraction efficiencies of different enzymes, protease type XIV, trypsin, proteinase K, pancreatin and driselase, were tested. Extraction efficiencies were reported in Table 3.12. Extractions were performed as

described in the experimental section; 30 mg enzyme was used in 7.0 mL of 25 mmol/L phosphate buffer solution. Protease type XIV enzyme has 3.5 units/mg enzyme.

Use of trypsin and Proteinase K was resulted in increase in baseline as seen from Figure 3.21. Highest extraction efficiencies were achieved in the presence of pancreatin and protease type XIV. Pancreatin contains trypsin type of enzymes whereas protease type XIV contains different types of protein enzymes such as Streptomyces griseus Protease A, Streptomyces griseus Protease B and Streptomyces griseus Trypsin [189–192]. Protease type XIV was used throughout the study due to its ability to digest different type of proteins and it is widely used for speciation studies in plant matrix in the literature [140,189].

Table 3.12 Extraction efficiencies of different enzymes.

Enzyme	**Extraction efficiency for Se, % ***
Protease type XIV	82
Trypsin	28
Proteinase K	55
Driselase	26
Pancreatin	85

* N=2, RSD < %4.5

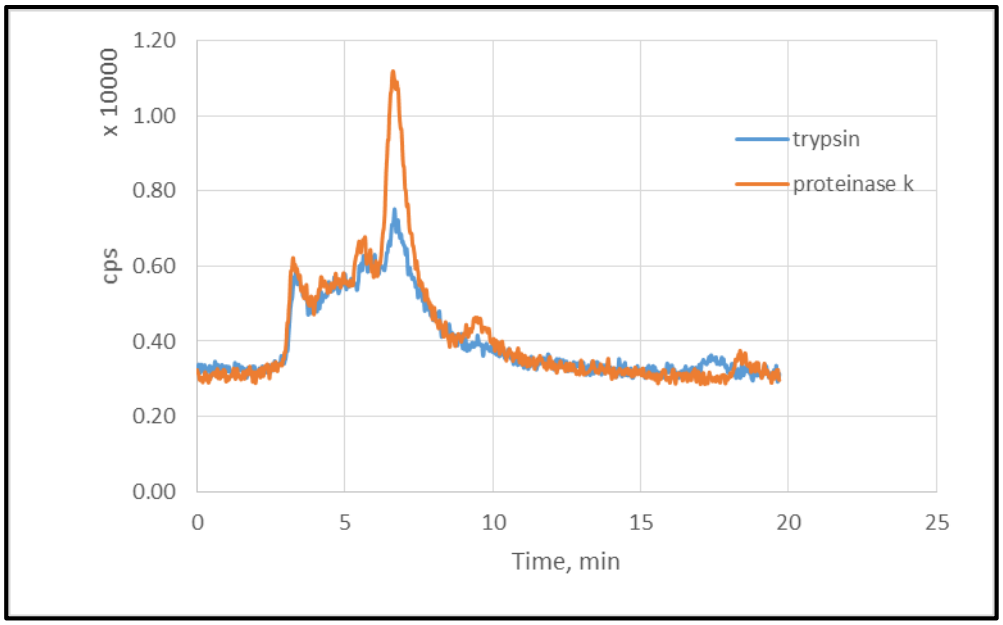


Figure 3.21 Background shift in the presence of trypsin and Proteinase K.

3.4.2 Amount of enzyme

Another critical parameter is the amount of enzyme used for the better chopping, or breaking up, of proteins. Amount of protease type XIV enzyme was optimized by preparation of four different samples with 0.100 g of onion in phosphate buffer. Resultant extraction efficiencies are reported in Table 3.13. Grubb's test was applied at 99% confidence level and no significant difference was observed for 10 to 80 mg of enzyme. Protease type XIV enzyme contains 3.5 units/mg enzyme.

Table 3.13 Extraction efficiencies calculated for different amount of enzymes.

Amount of enzyme, mg	**Extraction efficiency, %***
10	83
30	85
50	82
80	83

* N=3, RSD < %5.0

However, as the amount of enzyme increased, increase in the baseline signal in chromatograms were observed as seen from Figure 3.22. Extractions performed using more than 30 mg enzyme resulted in increasing baseline. Hence, 30 mg enzyme was selected as the optimum amount of enzyme.

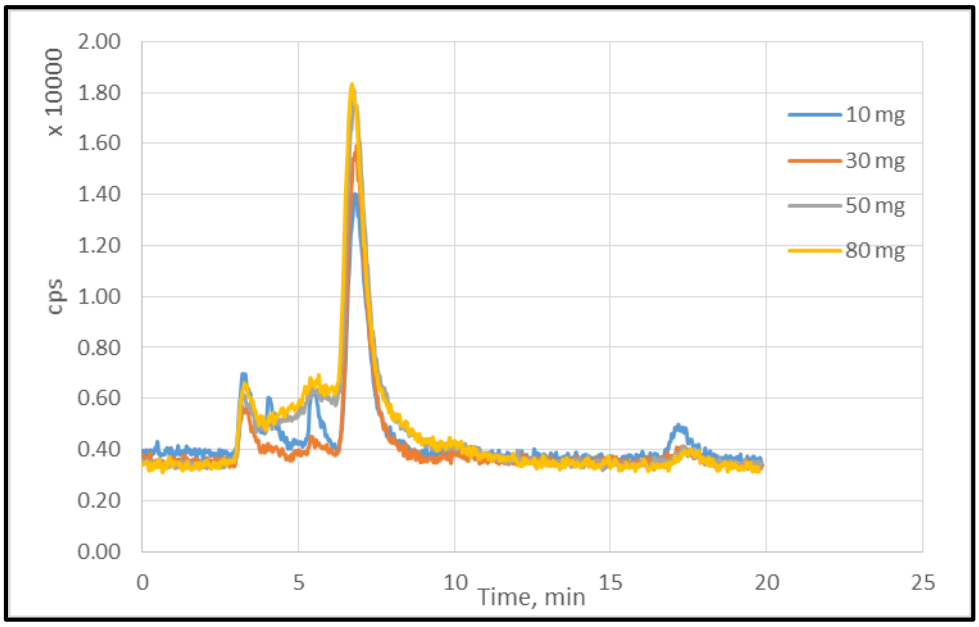


Figure 3.22 Chromatograms of extracts obtained by using 10, 30, 50 and 80 mg of protease type XIV enzyme.

3.5 Optimization of HPLC-ICP-MS

Chromatographic separation of arsenic and selenium species is achieved by using different type of columns, namely, anion exchange, cation exchange and reversed phase. Each column requires different mobile phase composition and pH values for optimum separation. For the column selection and optimization, it is necessary to define analyte species of interest since inclusion of an analyte that is not possibly present in the sample may increase analysis time as well as optimization procedure and effort.

3.5.1 Selection of target selenium and arsenic species

Selenium and arsenic enriched plants are extensively studied in the literature. Chemical form and amount of element deposition is strongly dependent on the type of plant and the supplemented chemical form. Allium group vegetables are classified as lower plants and they are good selenium accumulators. Selenium species that are possibly present in *Allium* group vegetable are selenite, Se(IV), selenate, Se(VI), and seleno amino acids selenomethionine (SeMet), selenocystine ($SeCys_2$) and selenomethyl selenocysteine (MeSeCys) [139,192–196]. These molecules and ions were selected as target selenium species in the current study.

Arsenic in *Allium* group plant is less studied [197,198] and limited information is available in the literature. Other plant studies revealed that arsenic is mostly deposited as inorganic arsenite or arsenate and in rear cases as monomethyl arsenic (MMA) or dimethyl arsenic (DMA) in the plant [168,199–204]. Frequency of MMA and DMA is less in lower plants. High molecular weight arsenic compounds, such as arsenoaminoacids and arsenosugars, are frequently characterised in animals but presence in plants is not common. Consequently, arsenite and arsenate were selected as target molecules and presence of MMA and DMA were monitored but full separation was not aimed since increasing number of analyte species also increase the effort to find optimum conditions for extraction and separation.

3.5.2 Ion pairing chromatography

Reversed phase chromatography is usually applied for the separation of non-ionic species by using C8 and C18 columns. Additionally, anions, cations and molecular species is be separated simultaneously by the introduction of an ion pairing reagent. Different types of columns as well as different composition of mobile phase combinations were tried in this study. studied column-mobile phase combinations are listed in Table 3.14.

Table 3.14 Analytical column and mobile phases studied for ion pairing chromatographic separation.

Column	Flow rate, mL/min	Mobile phase
Altima C8	1.0	0.5% (v/v) Heptafluorobutyric acid 5% methanol, pH 3.60
Altima C8	1.0	0.2% (v/v) Heptafluorobutyric acid 2% methanol, pH2.50
Dionex C18	1.0	0.2% (v/v) Heptafluorobutyric acid 2% methanol, pH 2.50
Dionex C18	1.0	0.1% (v/v) Trifluoro acetic acid 2% methanol pH 5.00
Dionex C18	1.0	0.1% (v/v) Trifluoro acetic acid 50 mM pthalate buffer 2% methanol, pH 5.80
Dionex C18	1.5	4.0 mM Tributylammonium phosphate 4.0 mM diammonium hydrogen phosphate buffer 2% methanol, pH 5.80
Dionex C18	1.0	4.0 mM Tributylammonium phosphate 4.0 mM diammonium hydrogen phosphate buffer 1.5% methanol, pH 6.06

None of the above combinations successfully separated target species simultaneously. Bulky ion pairing reagents, like tributylammonium phosphate and heptafluoro butyric acid, were able to separate inorganic species, i.e. Se(IV), Se(VI), As(III) and As(V), but unable to separate organic ones, i.e. selenomethionine, selenocystine and selenomethyl selenocysteine. On the contrary, small ion pairing reagents, like trifluoro acetic acid, were able to separate organic molecules, i.e. selenomethionine, selenocystine and selenomethyl selenocysteine, and failed to

baseline separate inorganic arsenic and selenium species, i.e. Se(IV), Se(VI), As(III) and As(V).

3.5.3 Anion exchange chromatography

Different mobile phase-anion exchange column compositions available in our laboratory were studied for the separation of five selenium and two arsenic species. A summary of column and mobile phase compositions are listed in Table3.15.

Table3.15 Analytical column and mobile phases studied for anion exchange chromatographic separation.

Column	**Flow rate, mL/min**	**Mobile phase**
Varian SAX	1.5	10 mM citric acid, pH 2.49, 5% methanol
Varian SAX	1.5	10 mM citric acid, pH 4.50, 5% methanol
Varian SAX	1.0	50 mM phosphate buffer (ammonium), pH 5.00, 5% methanol
Spherisorb S5 SAX	1.5	10 mM citric acid, pH 4.50, 5% methanol
Spherisorb S5 SAX	1.5	10 mM phosphate buffer (ammonium), pH 7.00, 5% methanol
Spherisorb S5 SAX	1.0	50 mM phosphate buffer (ammonium), pH 5.00, 5% methanol
Spherisorb S5 SAX	1.0	10 mM Citrate buffer, pH 5.00, 5% methanol
Dionex AS7	1.5	10 mM phosphate buffer (ammonium), pH 7.00, 5% methanol
Dionex AS7	1.0	50 mM phosphate buffer (ammonium), pH 5.00, 5% methanol
Dionex AS7	1.0	50 mM phosphate buffer (ammonium), pH 7.00, 5% methanol
Dionex AS4A	1.5	50 mM phosphate buffer (ammonium), pH 7.00, 5% methanol

Most of the above combinations were successful for the separation of ionic species, but organic species were not separated successfully. One of the most popular columns used for the speciation studies of arsenic and selenium in the literature is Hamilton PRP-X100 strong anion exchange column [164,205,206]. The column is filled with polystyrene-divinylbenzene (PSDVB) resin. The porosity of resin is high and is the base material. Active anion exchangers are trimethylammonium groups (Figure 3.23) that are covalently bonded to PSDVB matrix. Column can resist pH ranging from 1.00 to 13.00. This column was used for the further studies.

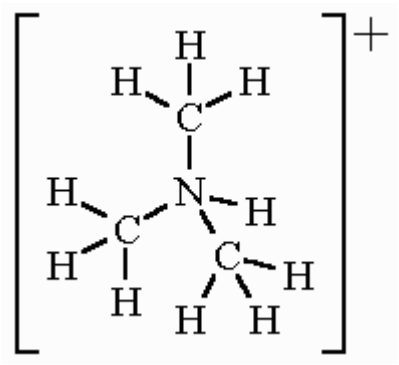


Figure 3.23 Structure of trimethyl ammonium group.

First mobile phase was ammonium phosphate buffer in 5% (v/v) methanol at acidic, neutral and basic pH regions (Table 3.16). At pH 9.00 As(III) and As(V) were successfully separated within 8 min as seen from Figure 3.24. However, selenocystine and selenomethionine were strongly retained in the column and did not elute within 40 min.

Table 3.16 Working conditions for first mobile phase.

Mobile phase	50 mM ammonium phosphate buffer, 5% methanol
Flow rate	1.0 mL/min
Column	Hamilton PRP-X100 strongly anion exchange column
pH values	9.00, 7.00, 5.00

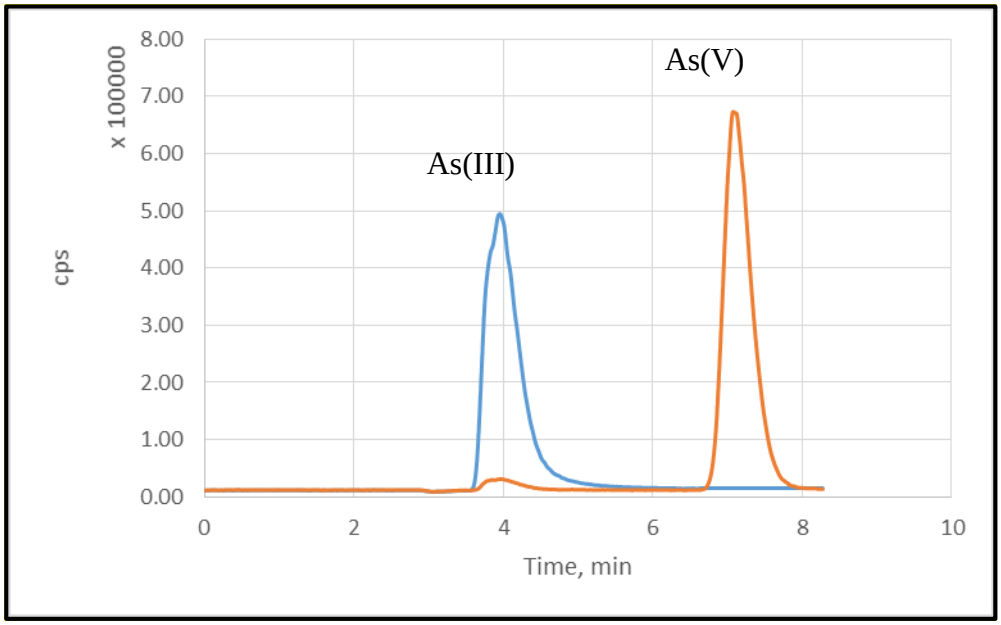


Figure 3.24 Chromatograms of 100 ng/mL As(III) and 100 ng/mL As(V) when 50 mmol/L ammonium phosphate buffer was used at pH value 9.00.

A lower pH value, ie pH 7.00, was studied and a better separation was achieved. As(III) and As(V) were properly separated. However, an overlapping of Se(IV), and selenomethyl selenocysteine was observed. Additionally, selenocystine peak was not properly seen in the chromatogram. Related chromatograms are given in Figure 3.25 and Figure 3.26.

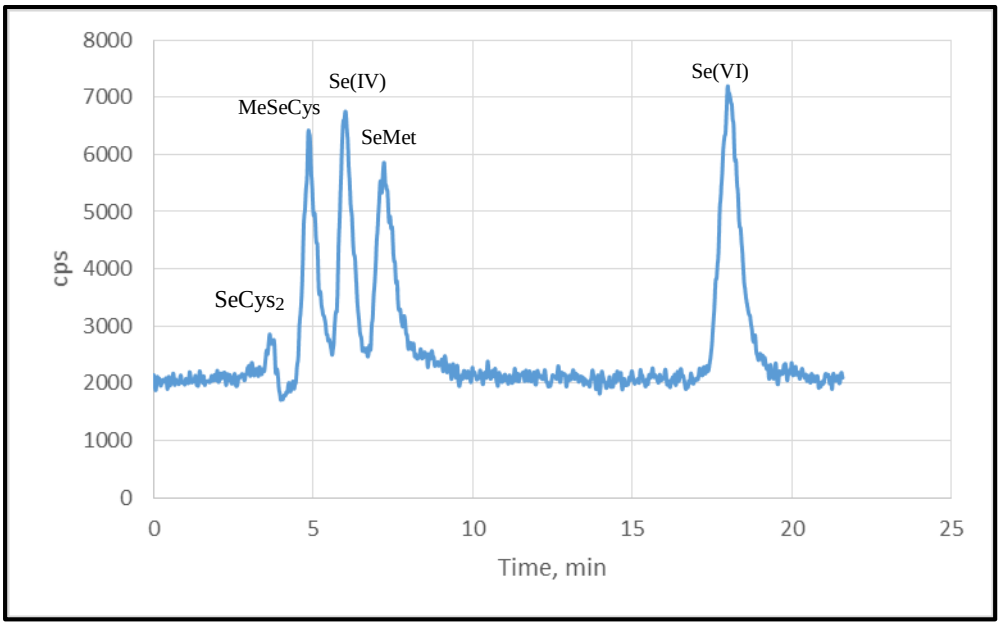


Figure 3.25 Chromatogram of 200 ng/mL selenocystine, selenomethyl selenocysteine, Se(IV), selenomethionine and Se(VI) mixed standards when 50 mM ammonium phosphate buffer was used at pH 7.00.

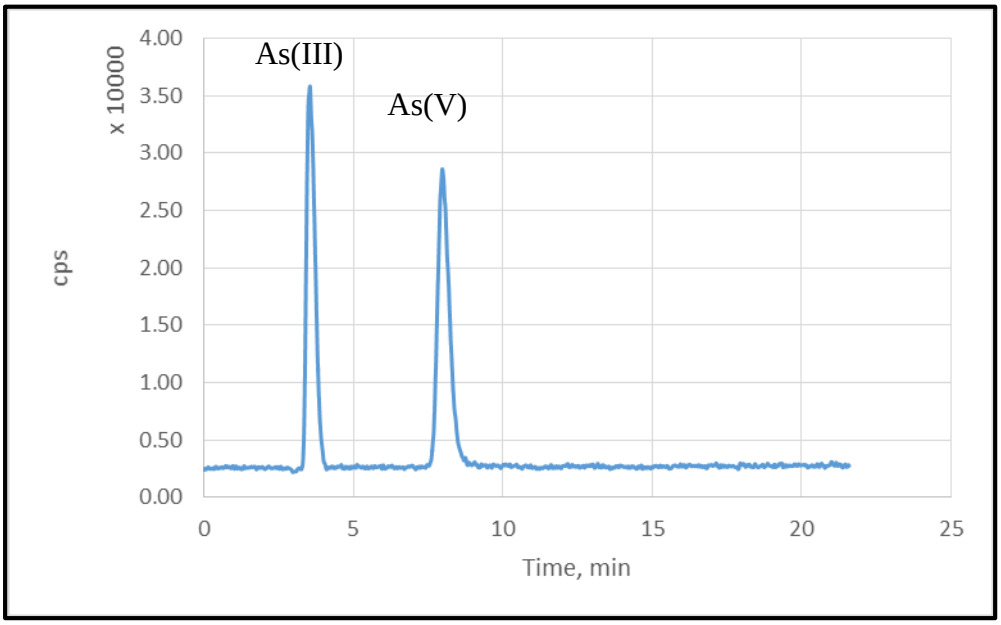


Figure 3.26 Chromatogram of 200 ng/mL As(III) and As(V) standard when 50 mM ammonium phosphate buffer was used at pH 7.00.

Using the lowest pH value, pH 5.00, resulted in full separation of arsenic and selenium species except Se(VI), which was not eluted within 40 min (Figure 3.27 and Figure 3.28).

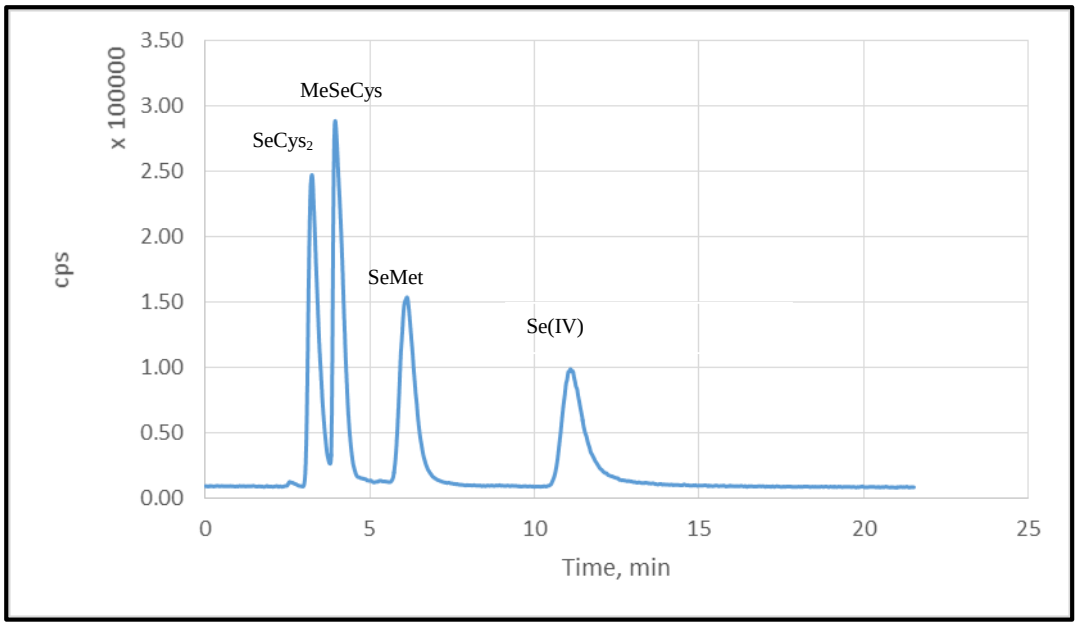


Figure 3.27 Chromatogram of 200 ng/mL selenocystine, selenomethyl selenocysteine, Se(IV), selenomethionine and Se(VI) mixed standards when 50 mM ammonium phosphate buffer was used at pH 5.00. Se(VI) did not elute within 30 min.

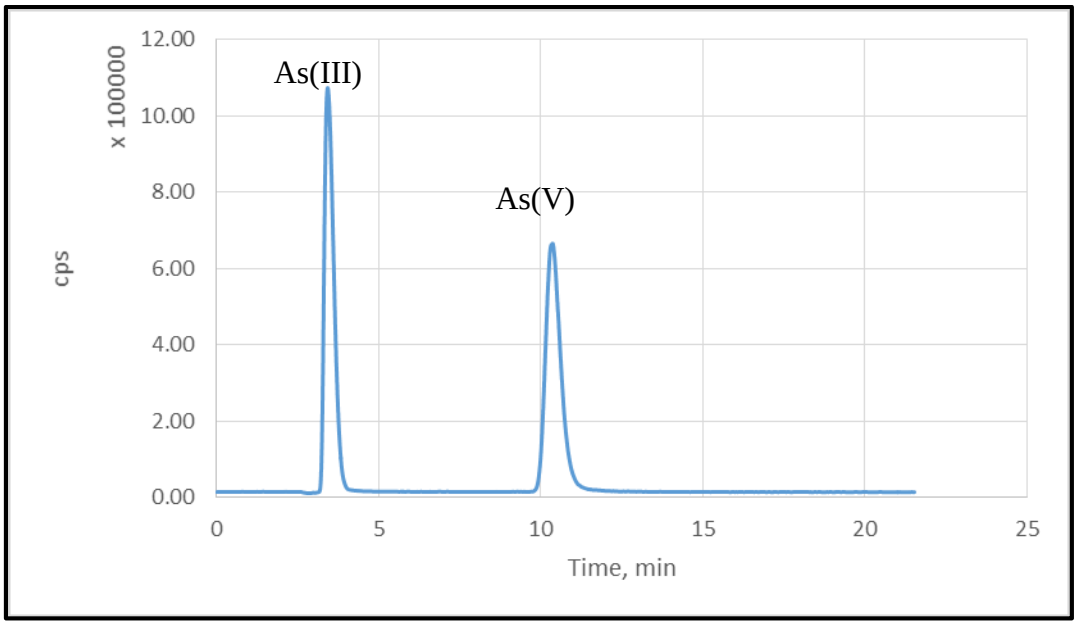


Figure 3.28 Chromatogram of 200 ng/mL As(III) and As(V) standard when 50 mM ammonium phosphate buffer was used at pH 5.00.

Second mobile phase system was ammonium citrate buffer at pH values 5.00, 5.50 and 6.50 (Table 3.17). At pH 6.50, all the analytes were separated except Se(IV) and selenomethyl selenocysteine. These two species were coeluted as seen from Figure 3.29.

Table 3.17 Working conditions for second mobile phase.

Mobile phase	5.0 mM ammonium citrate buffer, 2.0% methanol
Flow rate	1.0 mL/min
Column	Hamilton PRP-X100 strongly anion exchange column
pH values	6.50, 5.50, 5.00

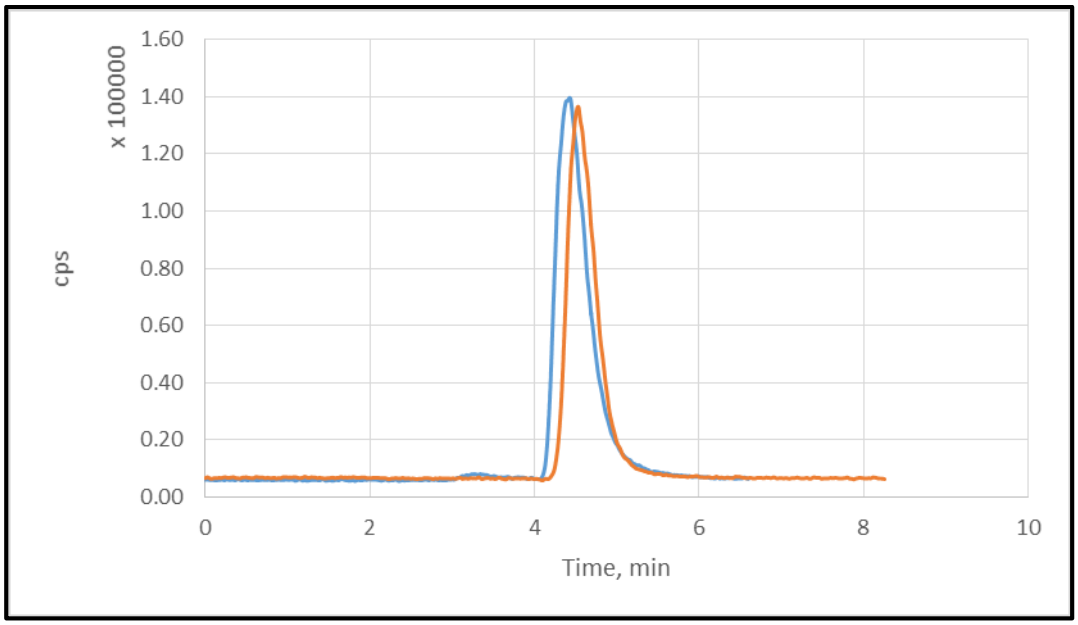


Figure 3.29 Chromatograms of 100.0 ng/mL Se(IV) and selenomethyl selenocysteine when ammonium citrate buffer at pH 6.50 was used.

At pH 5.50, separation was better, all species were separated but baseline resolution (as described in section 3.6) was not achieved. Related chromatogram is given in Figure 3.30.

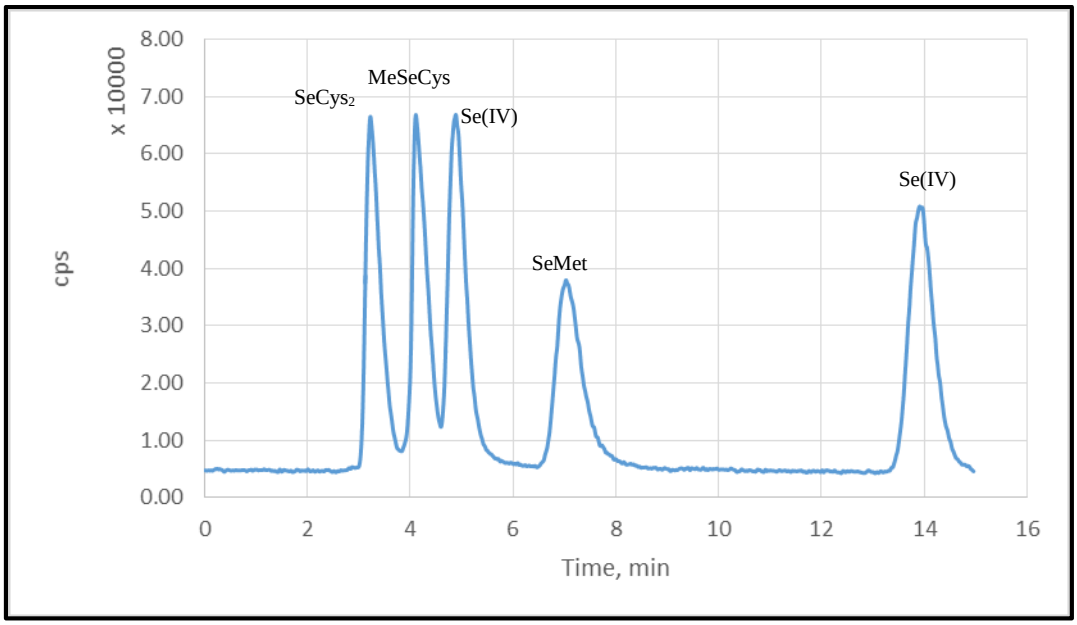


Figure 3.30 Chromatograms of 200 ng/mL selenocystine, selenomethyl selenocysteine, Se(IV), selenomethionine and Se(VI) mixed standards when 5 mM ammonium citrate buffer was used at pH 5.50.

Lowest pH value, i.e. 5.00, resulted in best separation of five selenium and two arsenic species (Figure 3.31 and Figure 3.32).

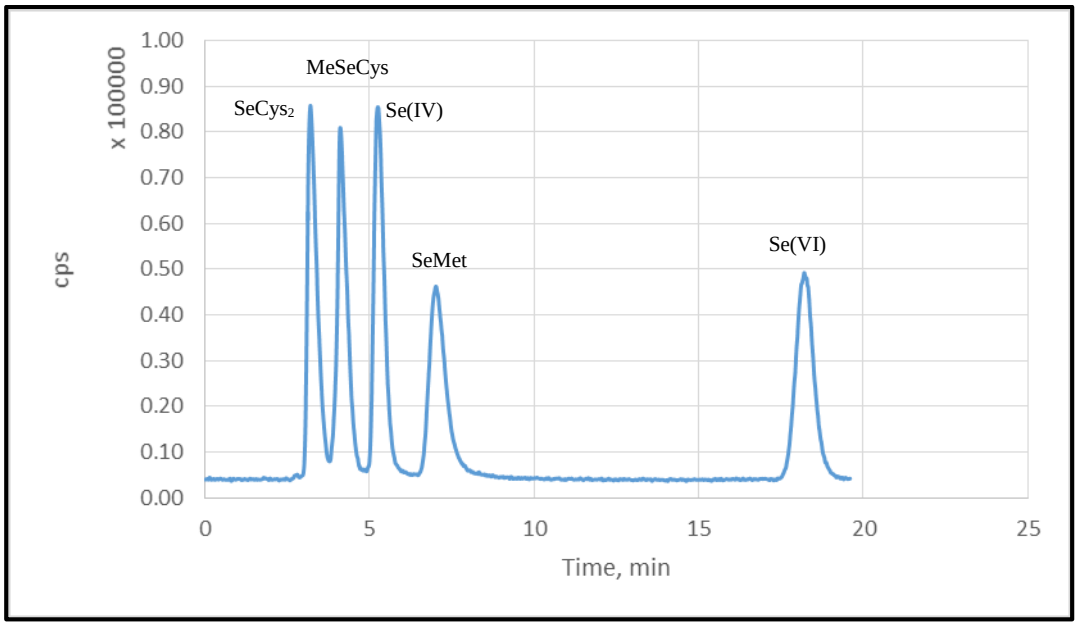


Figure 3.31 Chromatograms of 100 ng/mL mixed selenium standards when 5 mM ammonium citrate buffer was used at pH 5.00.

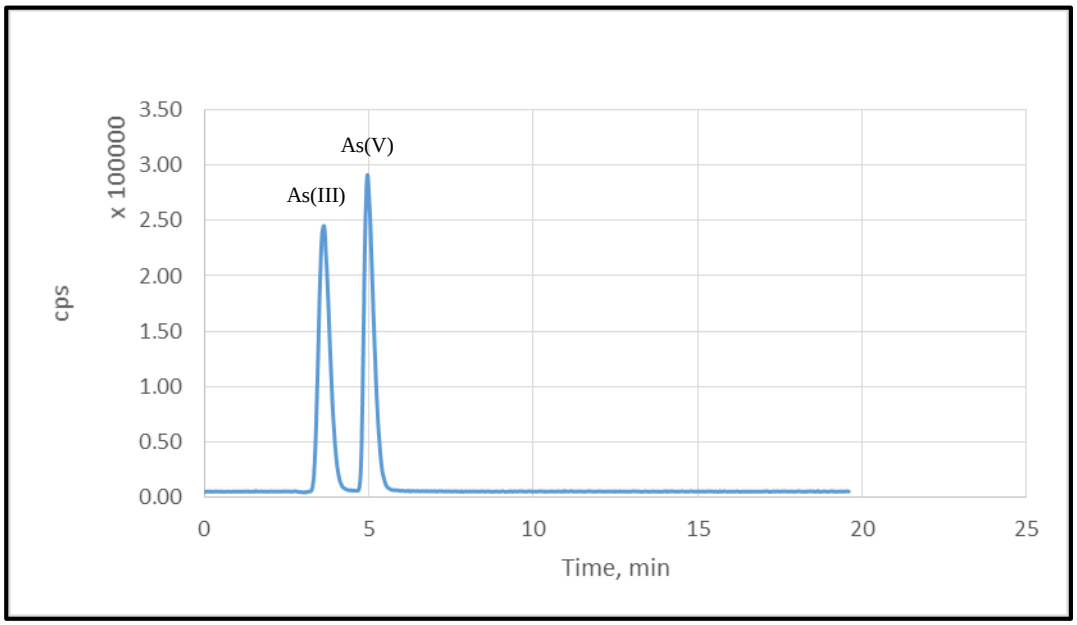


Figure 3.32 Chromatograms of 200 ng/mL As(III) and As(V) standards when 5 mM ammonium citrate buffer was used at pH 5.00.

Overall effect of pH on retention time of Se and As species are given in Figure 3.33 and Figure 3.34, respectively. As the pH increases, retention time of Se(IV) and Se(VI) decreases whereas retention time of selenomethyl selenocysteine and selenomethionine increase slightly. In case of As, pH does not have a drastic effect on the retention time of As(III) and As(V). Additionally, distribution of selenium species over the pH range is given in Figure 3.35. Increasing pH resulted in a decrease in retention time of Se(VI). In the pH range studied, Se(VI) is dominantly in the form of SeO_4^{2-}, as seen from Figure 3.35(a), and retention time was expected to increase as pH increases due to increasing anionic character. Additionally, concentration of citrate ion increases as pH increase as seen from Figure 3.35(e). Observed decrease in the retention time may be attributed to the competition between selenite and citrate ions for the available anion exchange sites of stationary phase. Similar discussion may be done with the selenite ions. In the pH range 5.00-6.50 selenite ion is mostly in form of $HSeO_3^-$ (Figure 3.35(b)) and as the pH increases, retention time was expected to increase, but a decrease in retention time was observed.

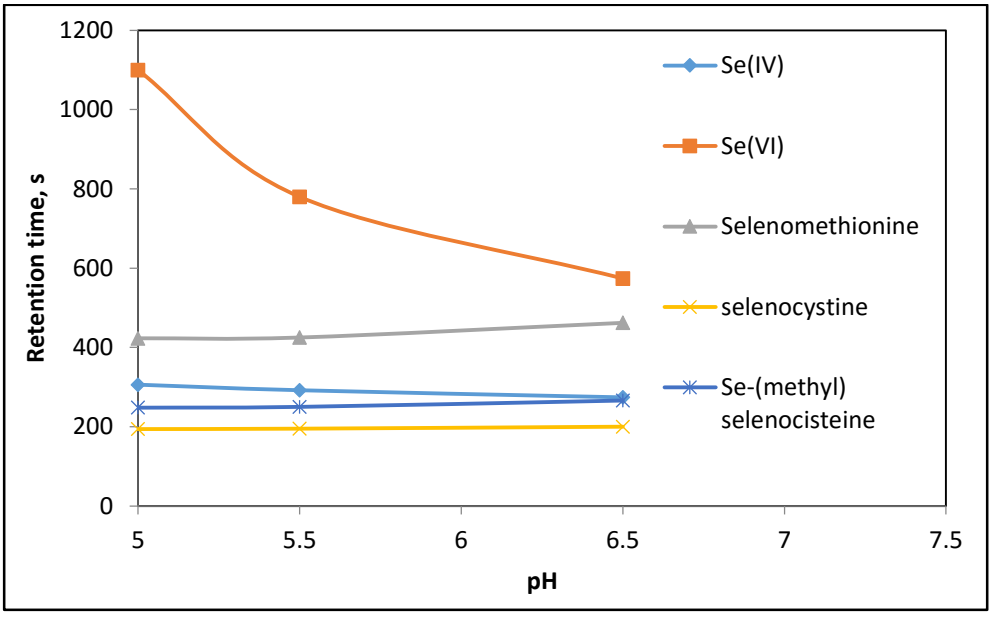


Figure 3.33 Effect of pH on retention time of selenium species. 5 mM ammonium citrate buffer was used with Hamilton PRP-X100 strongly anion exchange column.

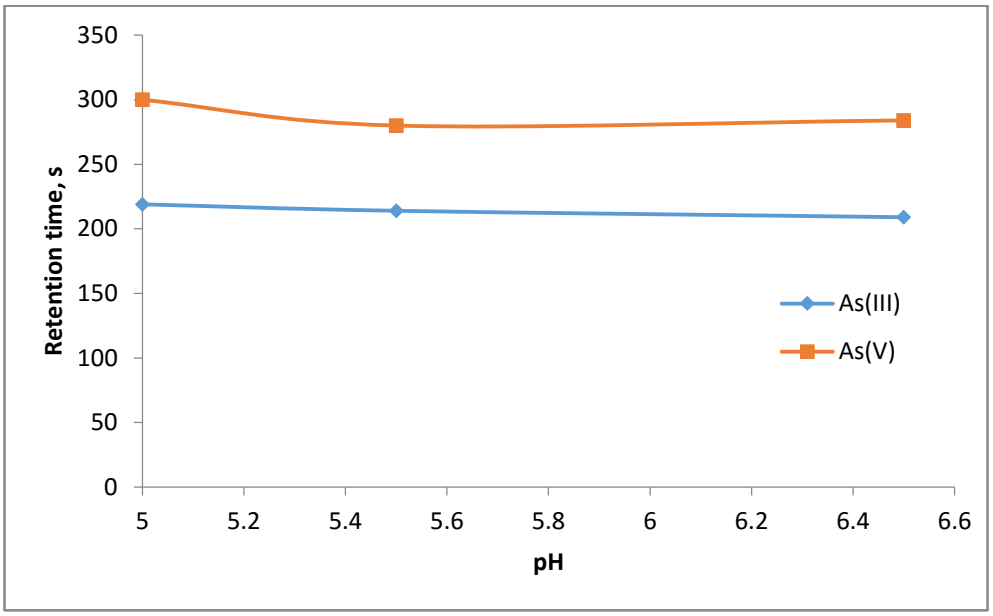


Figure 3.34 Effect of pH on the retention time of As(III) and As(V). 5 mM ammonium citrate buffer was used with Hamilton PRP-X100 strongly anion exchange column.

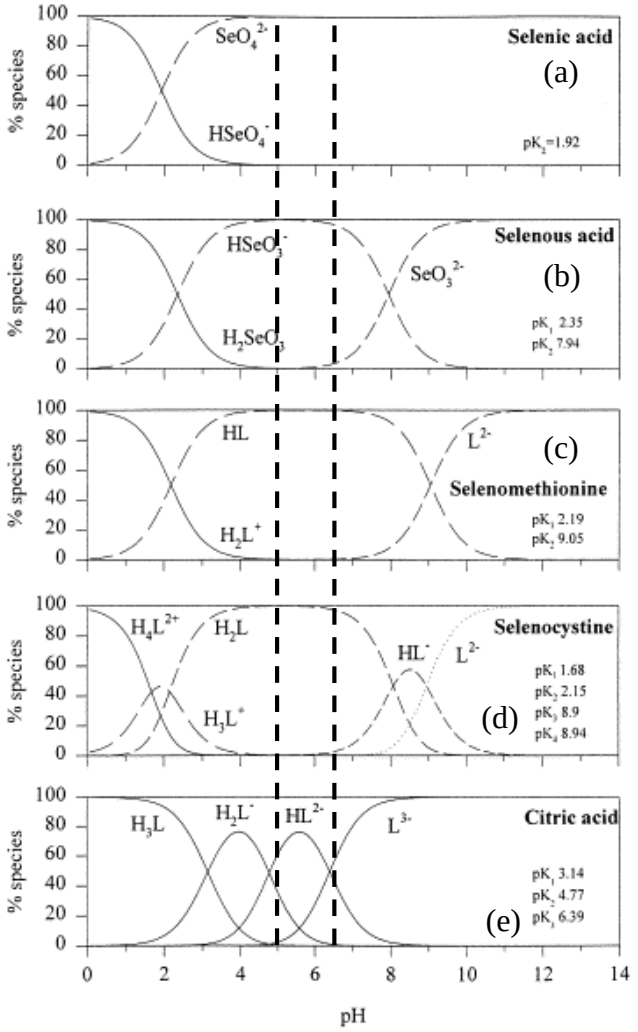


Figure 3.35 Ion distribution of Se species and citric acid with between pH 0.00 and 14.00. pH range used in this study is marked between dashed lines. Adopted from reference [205].

3.5.4 Concentration of citrate buffer

Concentration of citrate buffer was varied in order to see the effect of buffer concentration on retention time. As the buffer concentration increases, retention time of selenomethionine, selenocystine and selenomethyl selenocysteine did not changed significantly as seen in Figure 3.36. However, decrease in retention time of Se(IV) and Se(VI) were observed.

Increasing the buffer concentration decreased the analysis time but analyte peaks were not baseline resolved. 5.0 mmol/L citrate buffer was found as optimum for the elution.

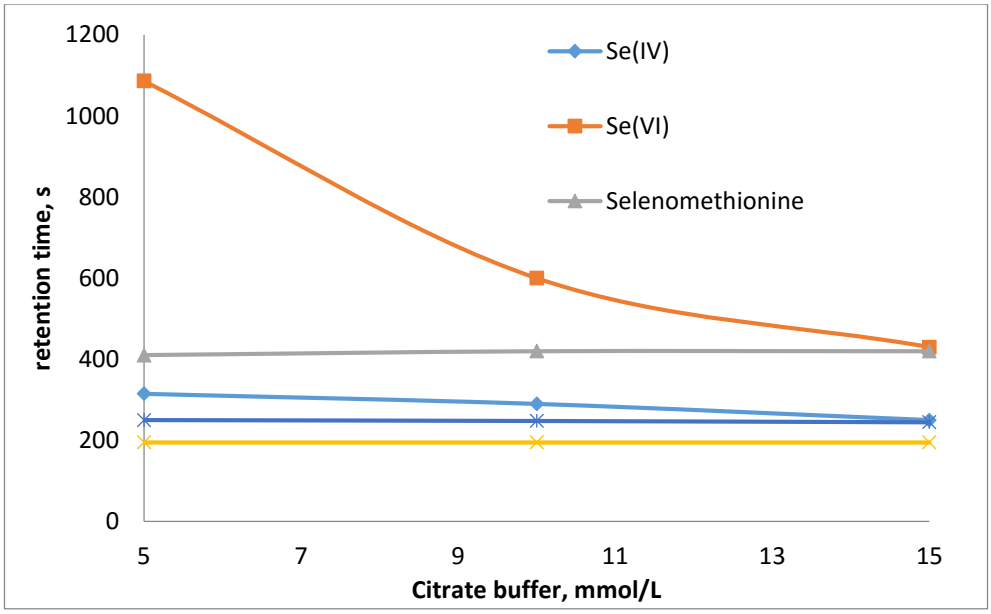


Figure 3.36 Effect of buffer concentration on the retention time of selenium species at pH 5.00.

3.5.5 MMA and DMA

Inorganic arsenic species are dominating in plant studies as explained in the previous sections and organoarsenicals are rarely observed. Consequently, MMA and DMA are not the target species in this study hence full separation of these species was not aimed. However, presence of these species was also monitored. Chromatogram of 200 ng/mL MMA and DMA mixture is given in Figure 3.37.

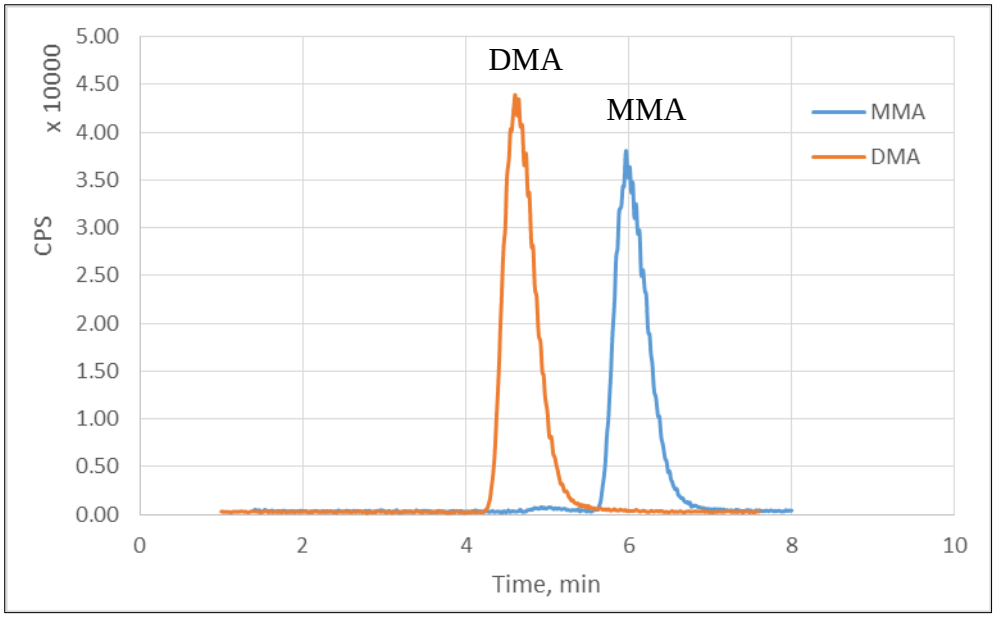


Figure 3.37 Chromatograms of 200 ng/mL MMA and 200 ng/mL DMA eluted with 5.0 mmol/L citrate buffer in 5% (v/v) methanol mobile phase over PRP-X100 strong anion Exchange column at pH 5.00.

If this chromatogram is overlaid with As(III) and As(V), it can be seen that MMA is almost entirely resolved from other peaks while DMA is partially overlapped with As(V) (Figure 3.38).

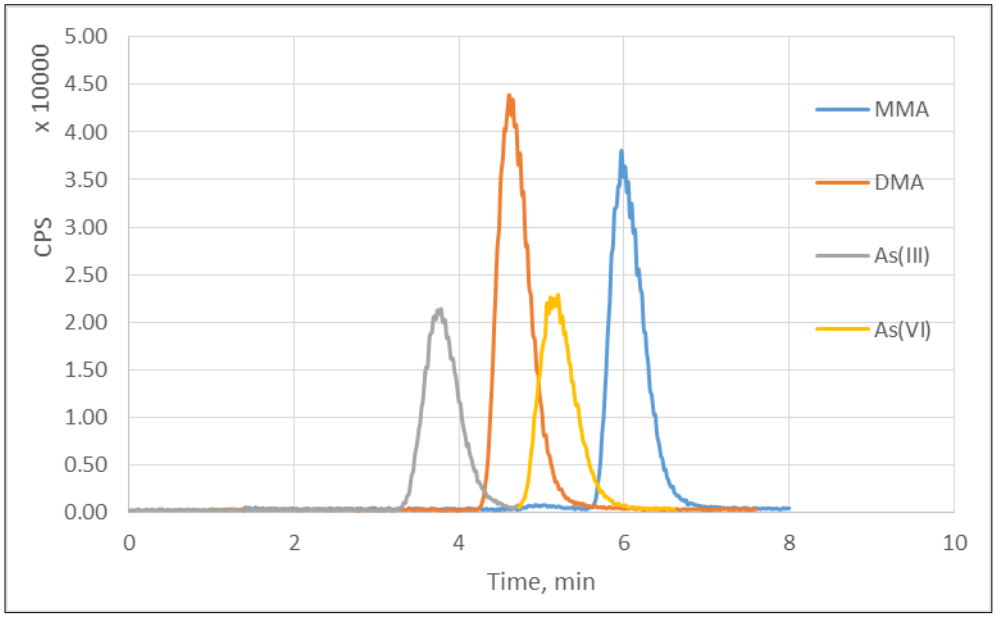


Figure 3.38 Overlay chromatogram of 200 ng/mL MMA, 200 ng/mL DMA, 100 ng/mL As(III) and 100 ng/mL As(V). Species were eluted over PRP-X100 strong anion Exchange column using 5.0 mmol/L citrate buffer and 5% (v/v) methanol.

Trying to resolve these peaks by changing experimental parameters distort the separation of selenium species. Thus, no further effort was made and traces of these species were carefully monitored in sample chromatograms. It will be seen in the later sections that amount of As(V) will be very low and consequently it is easy to track for MMA and DMA.

3.5.6 Summary of the optimum HPLC conditions

As a summary, 5.0 mM ammonium citrate buffer in 2% (v/v) methanol at pH 5.00 was used as a mobile phase in combination with strong anion exchange column and target molecular and ionic species were successfully resolved from each other.

3.5.7 Column recovery

Recoveries of selenium and arsenic species were studied so as to see whether analyte species were permanently bonded to the column filling material. For this purpose, a mixed standard of Se(IV), Se(VI), selenomethionine, selenocystine, selenomethyl selenocysteine, As(III) and As(V), 1.0 mg/L each, was prepared. This mixture was first injected to the column with a 100 µL injection loop. Outlet of the column was disconnected from ICP-MS instrument and the eluent was collected in a 50 mL test tube for 1300 s, which is the elution time for all of the arsenic and selenium species. Same experiment was repeated one more time however, this time without analytical column.

Both of the collected samples were injected directly to ICP-MS through conventional nebulization system in flow injection mode using 1.0 mL sample loop. Signals with and without analytical column for As and Se are given in Figure 3.39 and Figure 3.40, respectively.

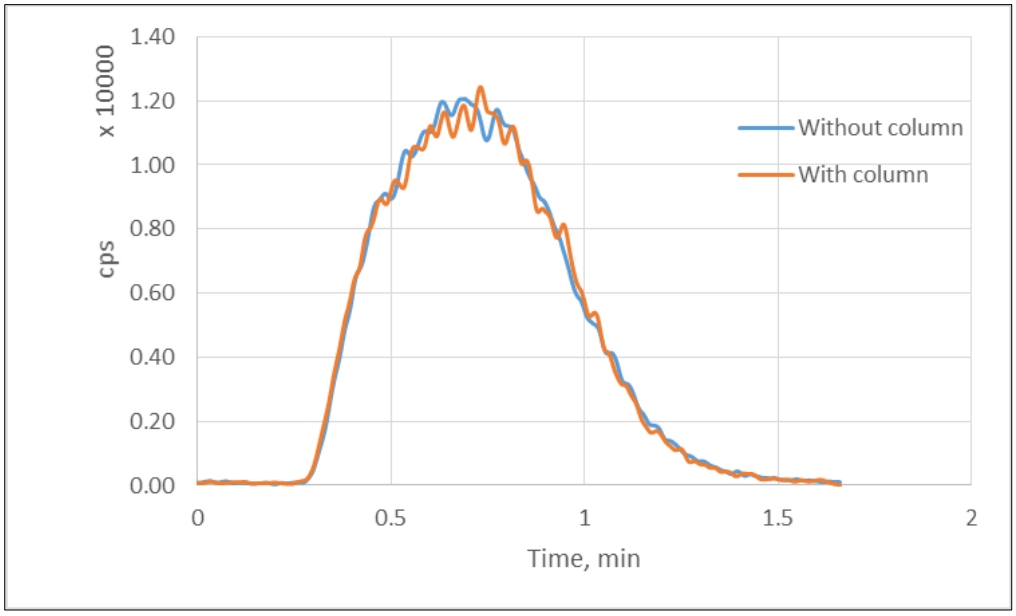


Figure 3.39 Signals for 100 µL of 1.0 mg/L mixed arsenic standard, As(III) and As(V), with and without analytical column, following $^{75}As^{+}$.

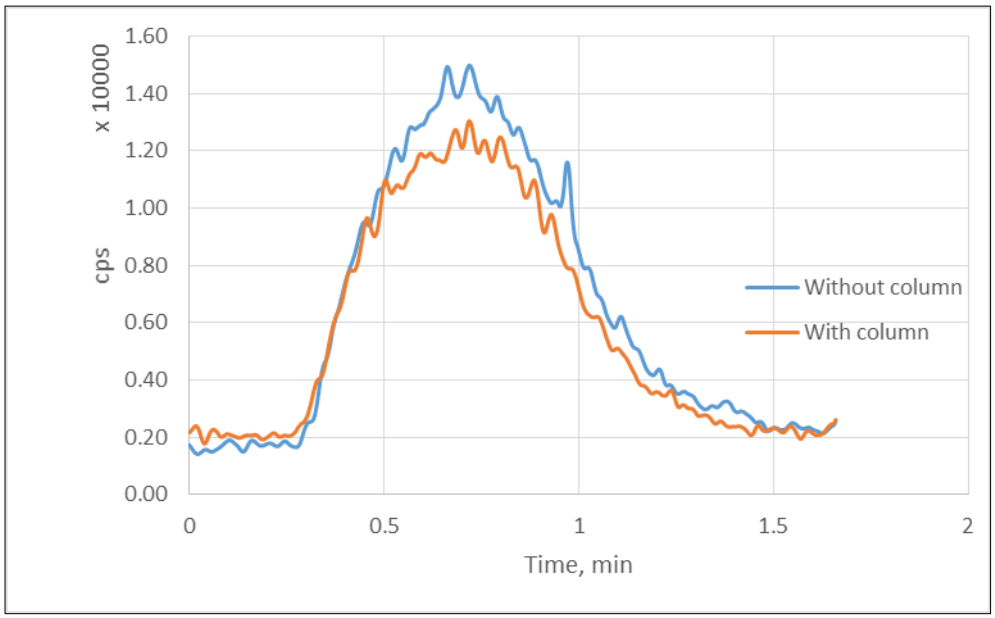


Figure 3.40 Signals for 100 µL of 1.0 mg/L mixed selenium standard, Se(IV), Se(VI), selenomethionine, selenomethyl selenocysteine, selenocystine, with and without analytical column, following $^{78}Se^{+}$.

Ratio of peak area values of As with and without analytical column is 0.995 while peak area ratio of Se with and without analytical column is 0.905. These results indicate that most of the As species were reversibly bonded to the stationary phase and recovery values are high enough for quantitative measurements. About 10% of selenium is irreversibly bonded to column leading in decrease in recovery values.

Same experiment was performed by injecting 100 µL of root extracts with and without the presence of analytical column. Root extract was selected since highest amount of arsenic was found only in roots. Resulting flow injection signals for arsenic and selenium are given in Figure 3.41 and Figure 3.42, respectively.

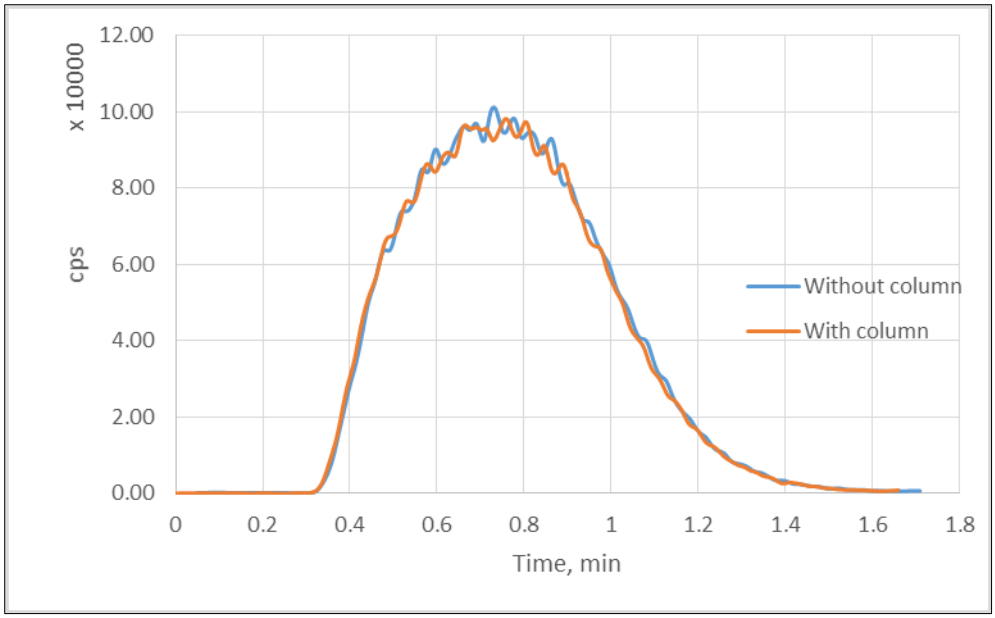


Figure 3.41 $^{75}As^{+}$ signals for 100 µL of root extract with and without analytical column.

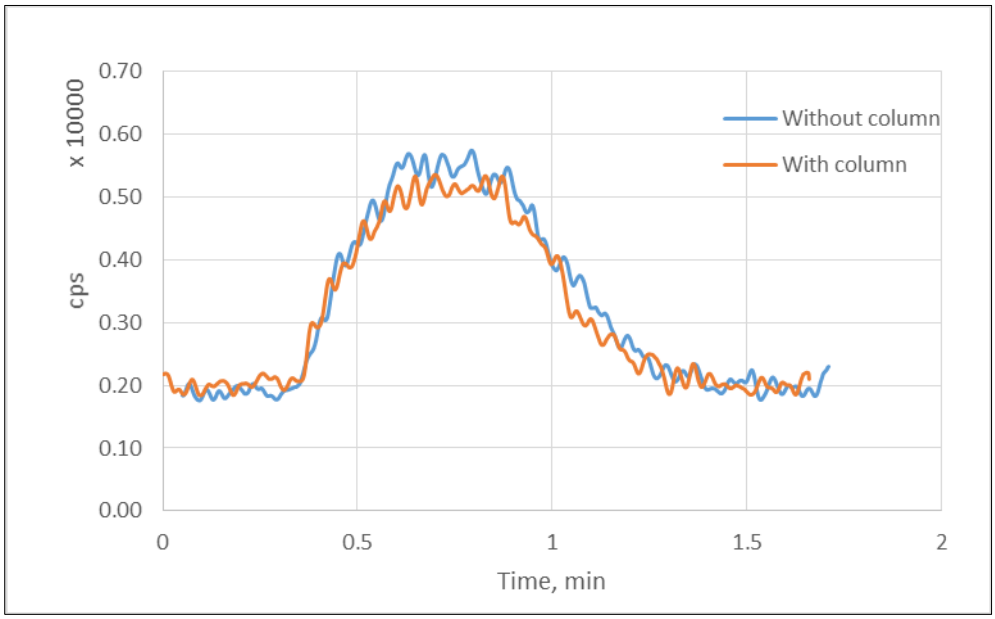


Figure 3.42 $^{78}Se^{+}$ signals for 100 µL of root extract with and without analytical column.

Ratio of peak area values of As and Se with and without analytical column were 0.998 and 0.961, respectively. Extend of irreversible bonding for selenium was decreased. Nonetheless recovery values are high enough for quantitative measurements. The difference may be attributed to the matrix effect caused by the sample.

3.6 Analytical figures of merit

Analytical performance of the system was evaluated by injecting different concentrations of mixed selenium and arsenic standards. Quantification of each species was performed by calculating the peak area values of corresponding peaks. Calibration plots were constructed for individual species based on peak area values. Overlay of chromatograms obtained from different concentrations of mixed selenium and arsenic standards are given in Figure 3.43 and Figure 3.44, respectively.

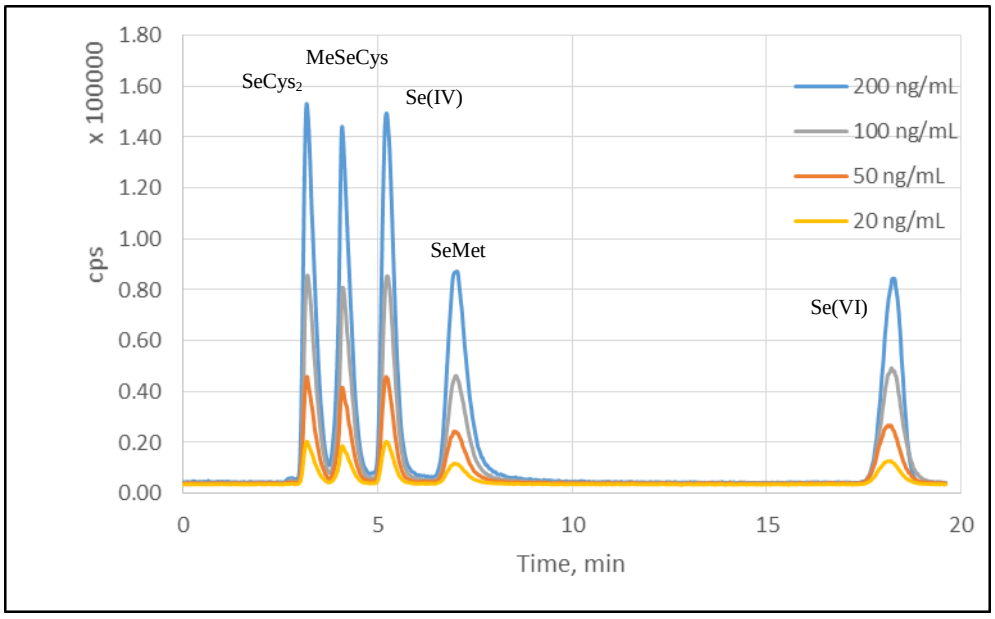


Figure 3.43 AE-HPLC-ICP-MS chromatograms of 200 µg/L, 100 µg/L, 50 µg/L and 20 µg/L selenium mixed standard solutions.

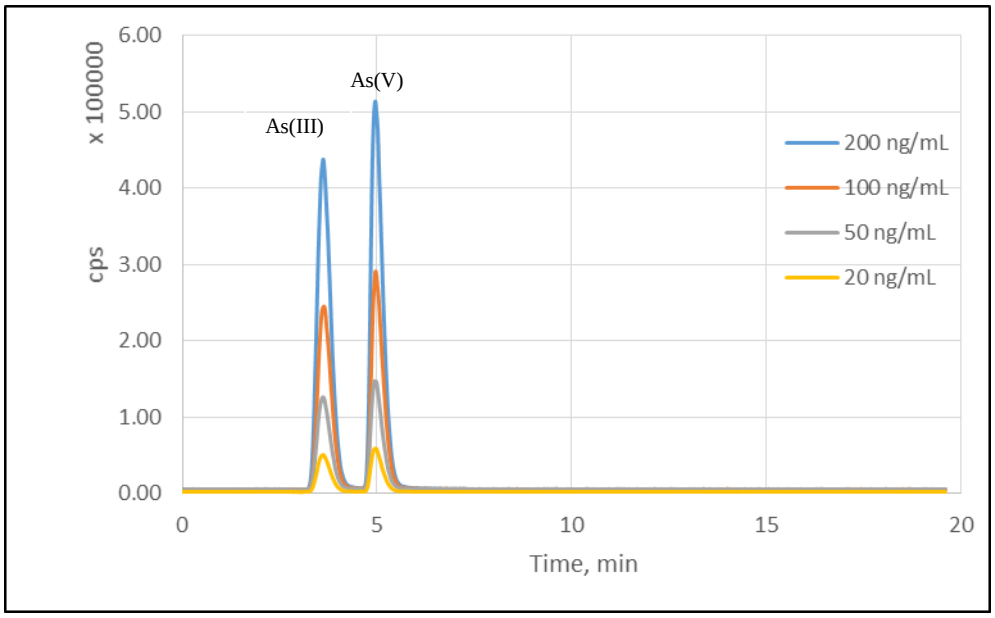


Figure 3.44 AE-HPLC-ICP-MS chromatograms of 200 µg/L, 100 µg/L, 50 µg/L and 20 µg/L arsenic mixed standard.

Using the data given in Figure 3.44 calibration graphs, best line equations and R^2 values of Se(IV), Se(VI), selenomethionine, selenocystine, selenomethyl selenocysteine were calculated and reported in Figure 3.45, Figure 3.46, Figure 3.47, Figure 3.48 and Figure 3.49, respectively.

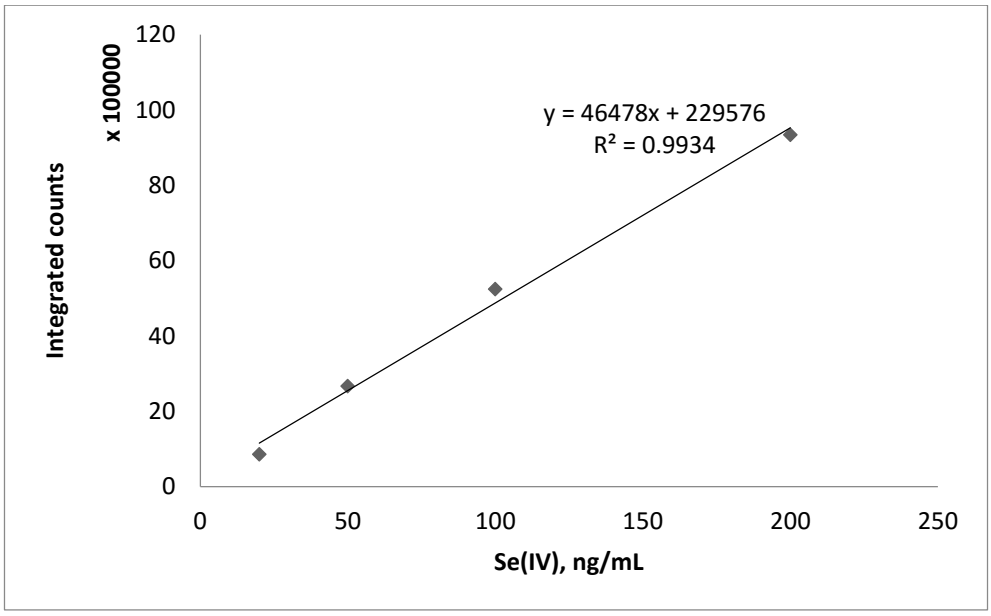


Figure 3.45 Calibration graph and best line equation for Se(IV). Integration time is 100 ms.

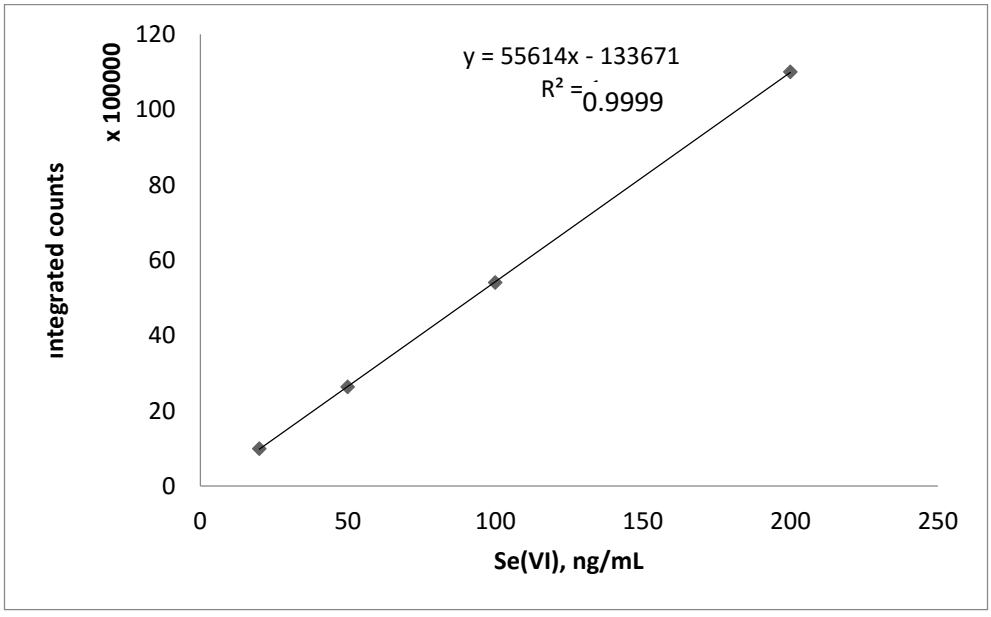


Figure 3.46 Calibration graph and best line equation for Se(VI). Integration time is 100 ms.

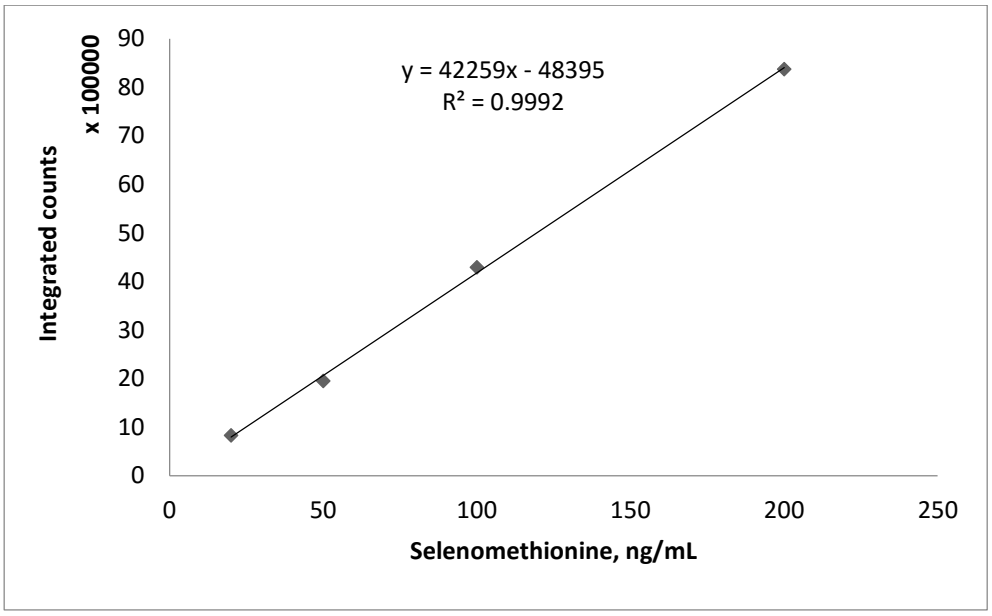


Figure 3.47 Calibration graph and best line equation for selenomethionine.

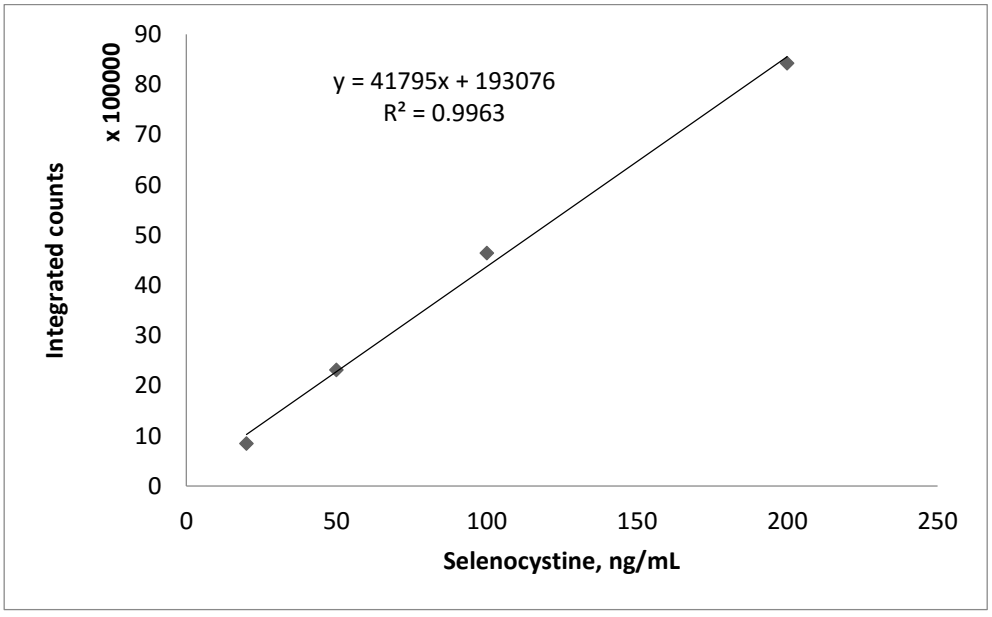


Figure 3.48 Calibration graph and best line equation for selenocystine.

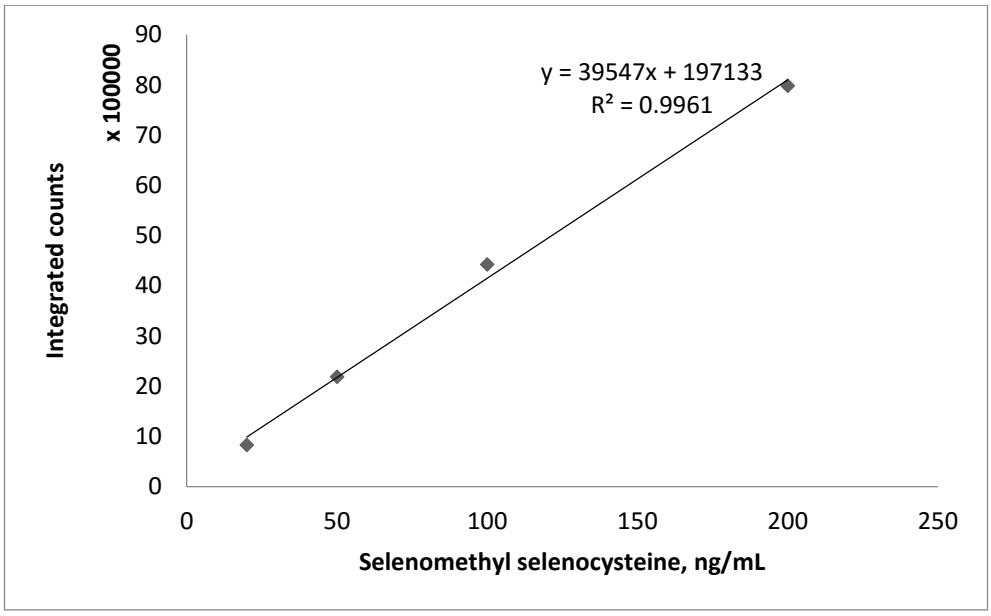


Figure 3.49 Calibration graph and best line equation for selenomethyl selenocysteine.

Sensitivity of inorganic selenium species were relatively higher compared to organic ones. Differences may be coming from the different nebulisation efficiencies of each species and the differences between retention behaviour in HPLC column.

Analytical performances of As(III) and As(V) with the developed AE-HPLC-ICP-MS system were evaluated by constructing the corresponding calibration graphs. Best line equations, were calculated based on least square method, and the R^2 values are given in Figure 3.50 and Figure 3.51 for As(III) and As(V), respectively. Sensitivity of both species were almost the same indicating a similar, retention, elution and nebulisation profile in HPLC-ICP-MS system.

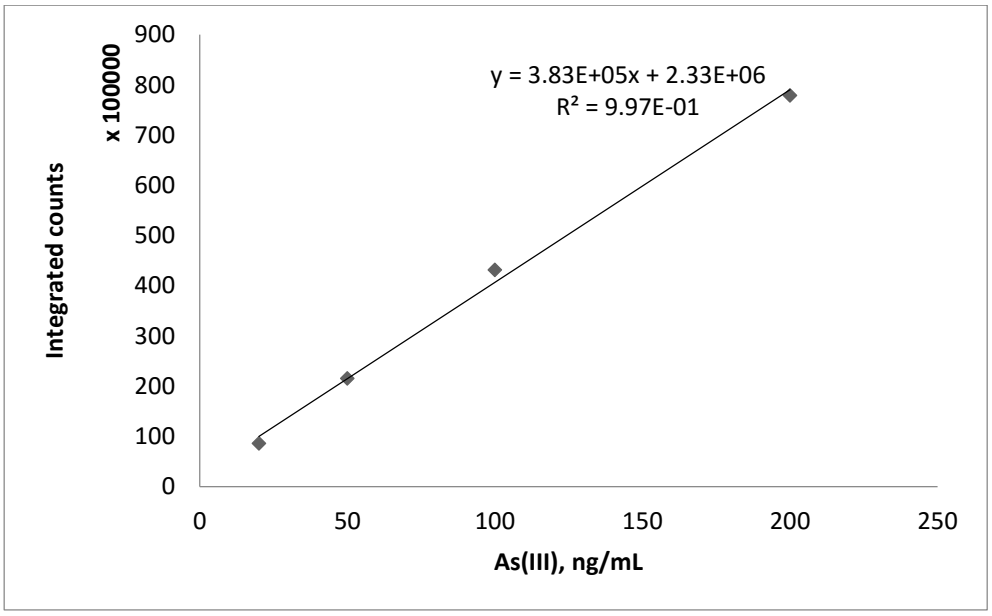


Figure 3.50 Calibration graph and best line equation for As(III) under optimum HPLC-ICP-MS conditions.

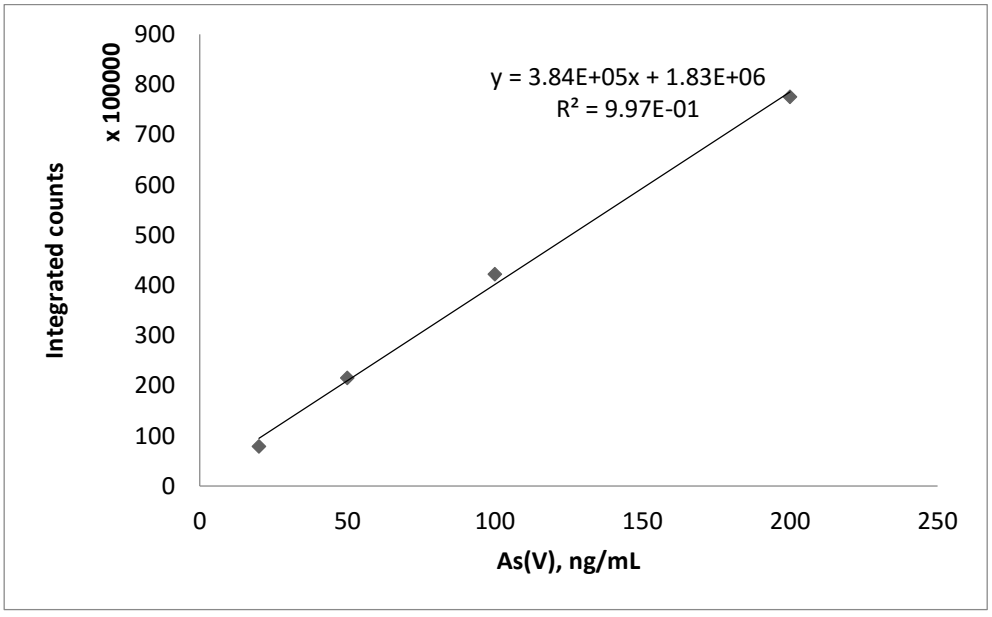


Figure 3.51 Calibration graph and best line equation for As(V) under optimum HPLC-ICP-MS conditions.

Limit of detection and limit of quantification values of each species were calculated by injection of lowest standard, i.e. 5.0 ng/mL, for 5 times. Results are given in Table 3.18. Peak area values were used in high smoothing mode. In high smoothing mode, moving box approach was used with a window width of 15 points and three orders of iteration. Time resolution of the unsmoothed data was between 100-150 ms. LOD values of arsenic species were in sub ppb level, whereas LOD levels of selenium species were at ppb level.

Table 3.18 LOD and LOQ values of arsenic and selenium species with HPLC-ICP-MS system.

Species	**LOD, 3s/m, ng/mL**	**LOQ, 10s/m, ng/mL**	**LOD, 3s/m, mg/kg**	**LOQ, 10s/m, mg/kg**	**Working range, ng/mL**
As(III)	0.52	1.73	0.042	0.12	20-200
As(V)	0.61	2.03	0.045	0.15	20-200
Se(IV)	0.92	3.07	0.064	0.21	20-200
Se(VI)	0.88	2.93	0.063	0.21	20-200
Selenomethionine	1.00	3.34	0.071	0.23	20-200
Selenocystine	0.91	3.03	0.064	0.21	20-200
Selenomethyl selenocysteine	1.49	4.98	0.10	0.35	20-200

LOD: Limit of detection, LOQ: Limit of quantification

Performance of the HPLC system was evaluated by calculating number of theoretical plates, plate height and resolution between adjacent peaks. Number of theoretical plates and the corresponding peak height values were calculated based on *half peak height method* since the peaks are not Gaussian. Calculations were conducted using Equation 4 and Equation 5 where N designates number of theoretical plates, t_R is the retention time of a species, $W_{0.5}$ is the peak width at half maximum and H is the plate height.

Peaks of 100 ng/mL standard selenium and arsenic solutions and ^{75}As and ^{78}Se signals were used.

Equation 4 $N=5.54 \times (t_R/W_{0.5})^2$

Equation 5 $H=L/N$

Summary of number of theoretical plates and plate height values for arsenic and selenium species are given in Table 3.19. Highest number of theoretical plates was calculated for Se(VI) which has the longest retention time. This result indicates that corresponding peak is not broadened significantly.

Table 3.19 Number of theoretical plates and plate height values of arsenic and selenium species with optimum conditions.

Species	**Number of theoretical plates, N**	**Plate height, H, mm**
As(III)	501	0.499
As(V)	1123	0.223
Se(IV)	1257	0.199
Se(VI)	4642	0.054
Selenomethionine	854	0.293
Selenocystine	515	0.485
Selenomethyl selenocysteine	941	0.266

Resolution of the adjacent peaks were calculated by using Equation 6, where R_s is the resolution, t_R is the retention time and W is the peak width at baseline. W was calculated by drawing tangent lines on sides of the peaks.

Equation 6 $Rs= 2x[(t_R)_B-(t_R)_A]/(W_A+W_B)$

Resolution values of adjacent peaks were calculated and reported in Table 3.20. All of the values are greater than the critical value, i.e. 1.5, [207] implying full resolution of the adjacent peaks.

Table 3.20 Resolution values of the adjacent peaks with optimum conditions.

Species	**Resolution, R_s**
As(III)-As(V)	2.04
Selenocystine-selenomethyl selenocysteine	1.63
Selenomethyl selenocysteine – Se(IV)	2.01
Se(IV)- selenomethionine	2.88
Selenomethionine-Se(VI)	17.4

3.7 Speciation of onion samples

Concentrations of arsenic and selenium species in nine different samples grown under controlled hydroponic conditions were calculated using the developed HPLC-ICP-MS system. Enzymatic extraction was performed since this method yields the highest extraction efficiency. All concentrations given are based on dry weight of the sample. Elution of a single sample last about 1300 s. Column was washed for 5 min with mobile phase between each consecutive injection. Regeneration of the column was performed regularly in order to keep the column at highest performance. Regeneration aims to desorb any species that are adsorbed on the column active material and remove impurities.

Regeneration procedure involves elution of 0.5 mol/L HNO_3 in methanol at a flow rate of 0.5 mL/min for 2 h. During the regeneration procedure, analytical column was connected in reverse orientation to remove impurities stacked on the entrance of column.

3.7.1 Arsenic supplementation

Root, bulb and leave extract of plant supplemented with 1.0 mg/L As(III) was analysed. Concentration of arsenic species and percent distribution of arsenic in different parts of the plant are given in Figure 3.52 and Figure 3.53, respectively. Results revealed that arsenic is almost entirely found in the form of As(III). Conversion of As(III) to As(V) is less than 10% in the root part. Highest arsenic concentration was observed in root part. These results indicate that plants prefer As(III) and a possible As(III)-As(V) conversion is not desirable.

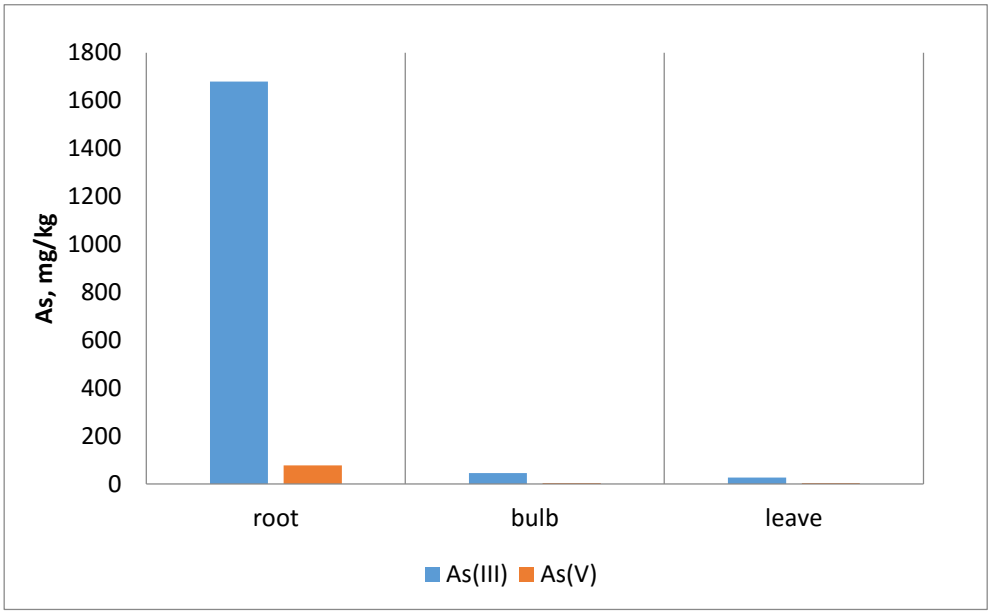


Figure 3.52 Concentration of As(III) and As(V) in root, bulb and leave of plants supplemented with 1.0 mg/L As(III) solution.

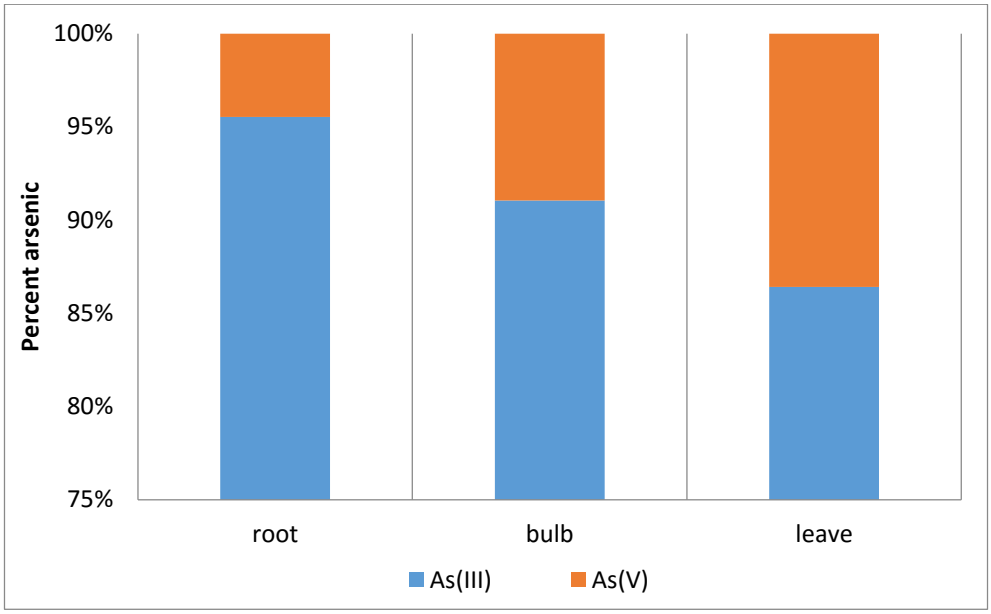


Figure 3.53 Percent mass distribution of As(III) and As(V) in root, bulb and leaves of plants supplemented with 1.0 mg/L As(III).

Speciation of arsenic in plants supplemented with 1.0 mg/L As(V) was performed. Concentration of As(III) and As(V) in different parts of plants and percent distribution of species are given in Figure 3.54 and Figure 3.55, respectively. In this case, the dominating species was not As(V), as the supplemented form, but As(III).

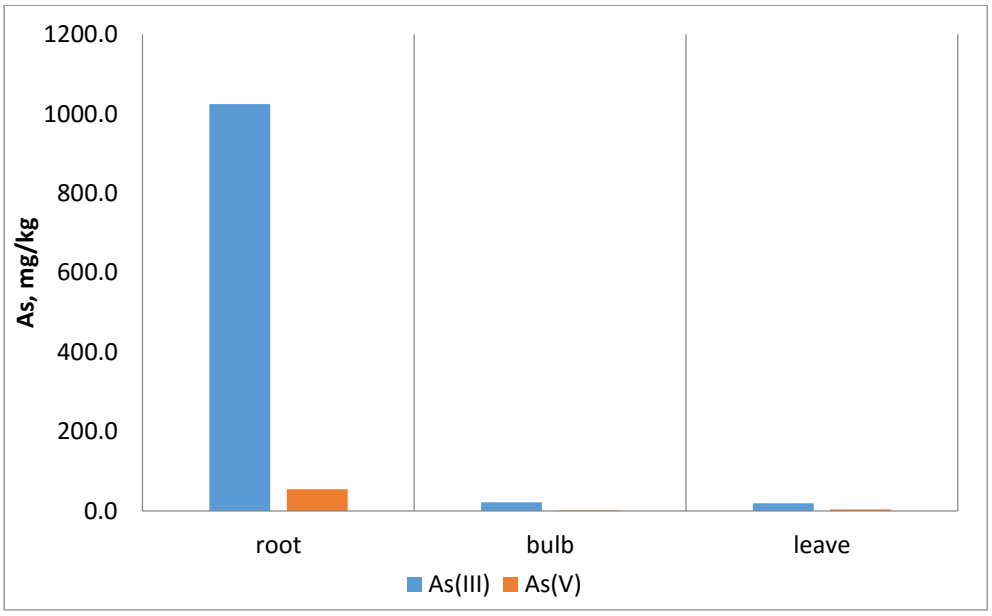


Figure 3.54 Concentration of As(III) and As(V) in root, bulb and leave of plants supplemented with 1.0 mg/L As(V) solution.

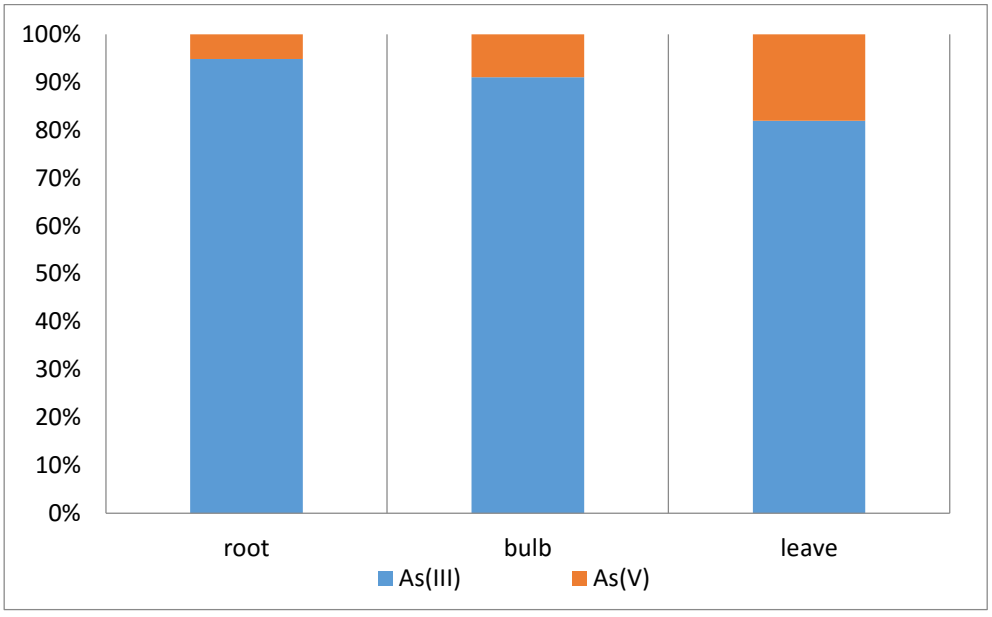


Figure 3.55 Percent mass distribution of As(III) and As(V) in root, bulb and leaves of plants supplemented with 1.0 mg/L As(V).

Conversion of As(V) to As(III) occurs either in the feed solution or within the plant. In order to test the feasibility of conversion in the feed solution, pH of the feed solutions at the time of harvesting were measured and given in Table 3.21.

Table 3.21 pH of feed solutions at the time of harvesting.

Feed Solution	pH
Control	7.77
As (III)	8.07
As (V)	8.01
Se (IV)	8.13
Se (VI)	7.38
As (III) + Se (IV)	8.76
As (III) + Se (VI)	7.75
As (V) + Se (IV)	7.15
As (V) + Se (VI)	7.72

In this pH range, arsenic is thermodynamically expected to be in the form of As(V) [208]. According to this result, conversion of As(V) to As(III) took place within the plant.

3.7.2 Selenium supplementation

Speciation of plants supplemented with 1.0 mg/L Se(IV) was performed and plotted in Figure 3.56 and Figure 3.57. Selenium was mainly deposited as selenomethionine in root and leaves of the plant. SeMet was below the detection limit in bulb. Selenomethyl selenocysteine was the second most abundant selenium species. Additionally, very small concentration of Se(IV) was quantified in root part only. This result indicate that Se(IV) was converted to organoselenium species mainly in

the root part. Amount of free Se(IV) was very low indicating a high conversion efficiency of Se(IV) to organic selenium species.

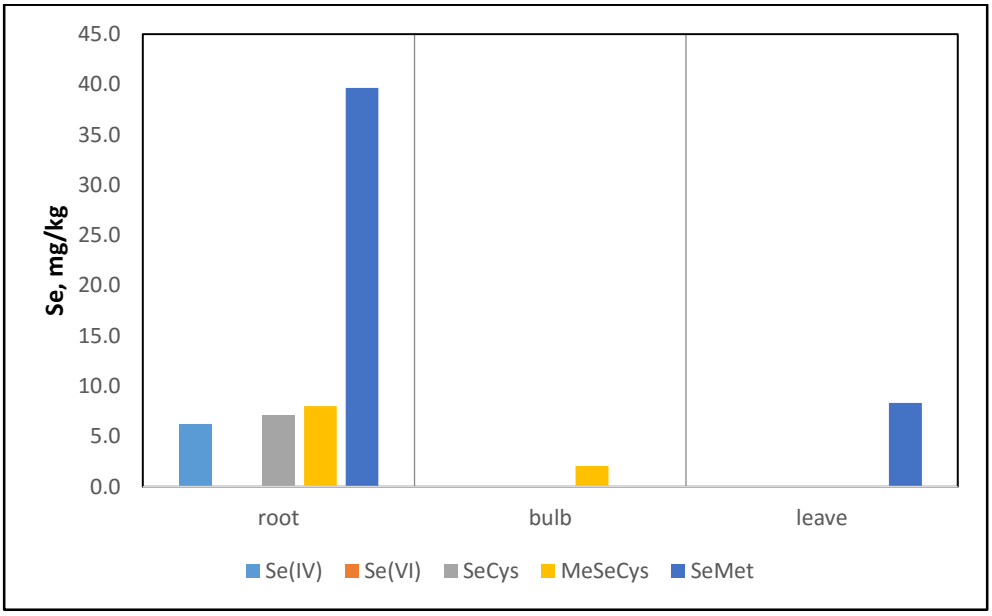


Figure 3.56 Concentration of selenium species in root, bulb and leaves of plants supplemented with 1.0 mg/L Se(IV).

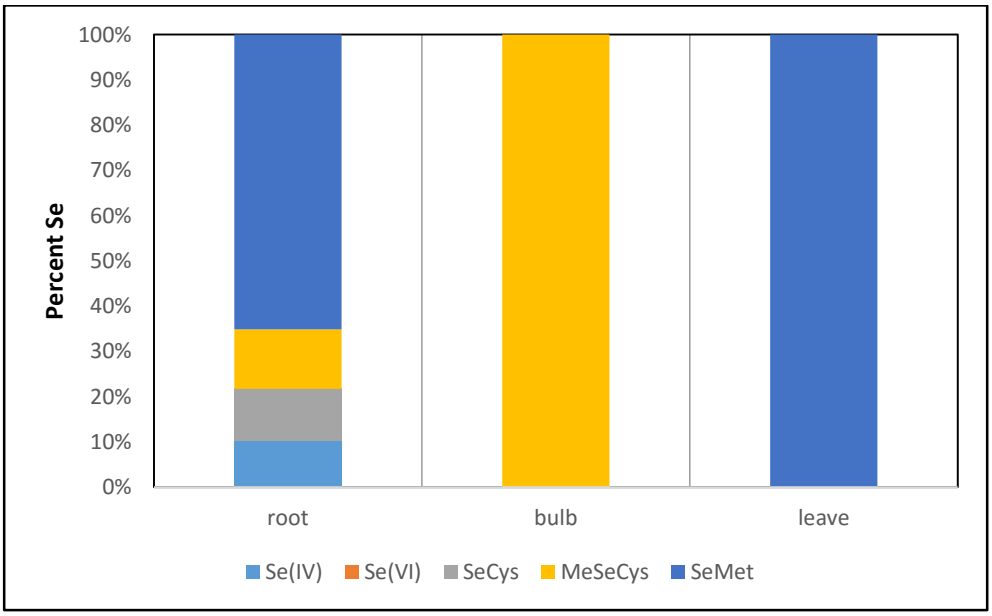


Figure 3.57 Percent distribution of selenium species in root, bulb and leaves of plants supplemented with 1.0 mg/L Se(IV).

Concentration of different selenium species and percent selenium distribution for plants supplemented with 1.0 mg/L Se(VI) are given in Figure 3.58 and Figure 3.59, respectively. In this case, selenium was deposited mainly in the form of Se(VI) and conversion to organoselenium species was limited as compared to Se(IV) supplementation. Highest Se(VI) concentrations were measured in leaves. These results indicate that bioavailability of Se(VI) is very low and conversion to organoselenium species is limited. This is because selenate must be reduced to selenite in order to be used by plant metabolism as explained in the introduction section. Additionally, selenomethionine was the only organoselenium species with relatively high concentration. Highest concentration of selenomethionine was reported in leave part implying that conversion of Se(VI) to selenomethionine took place in the leaves more efficiently. This is because selenate must be converted to selenite in order to metabolise to organoselenium species which is taking place in leave chloroplast. In both Se(IV) and Se(VI) supplementation, amount of selenium deposited in bulb was lowest. It may be concluded that main function of bulb is not the conversion of selenium species, rather it behaves as a transport medium between roots and leaves.

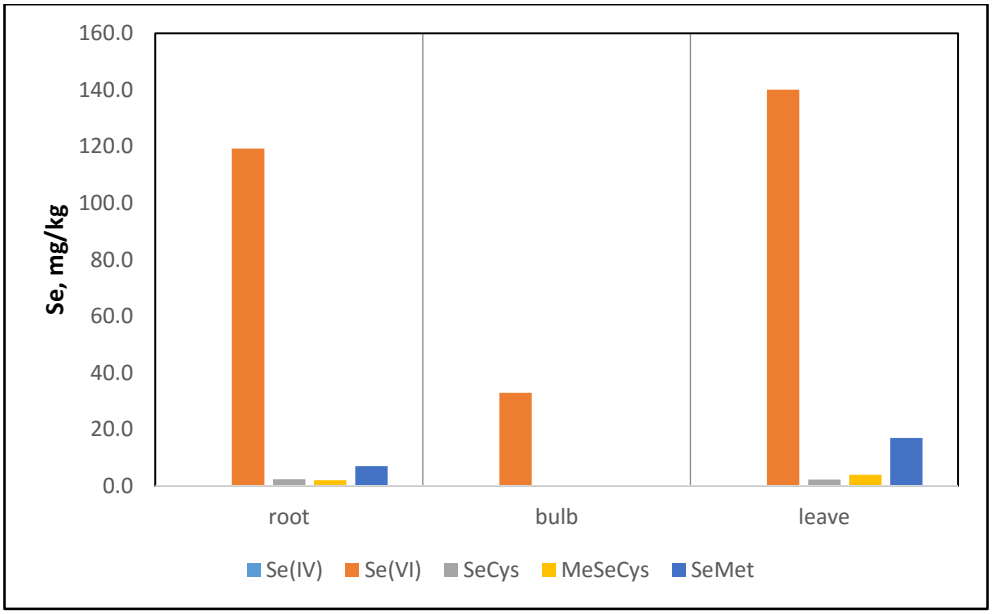


Figure 3.58 Concentration of selenium species in root, bulb and leaves of plants supplemented with 1.0 mg/L Se(VI).

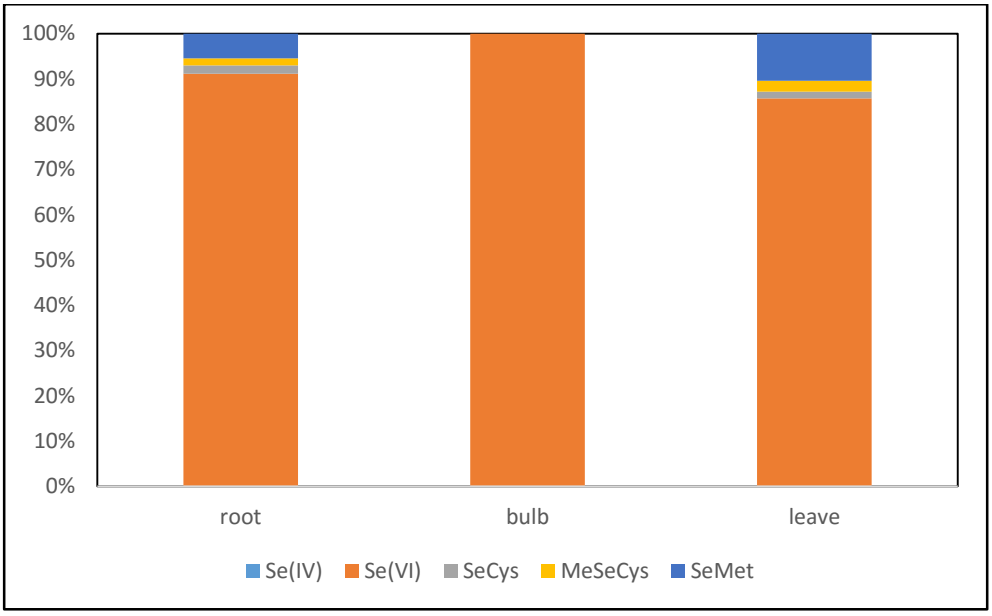


Figure 3.59 Percent mass distribution of selenium species in root, bulb and leaves of plants supplemented with 1.0 mg/L Se(VI).

It is well known from the literature that organoselenium species are more bioavailable than inorganic ones since selenomethionine directly takes part in protein structure simply by replacing the methionine and selenomethyl selenocysteine are converted to methylselenol (CH_3SeH) that is used in the selenium metabolism [209–214]. If selenium enriched onion growth is the main purpose, selenium should be introduced in the form of Se(IV) rather than Se(VI) since conversion efficiency of Se(IV) to organoselenium forms is higher. Another conclusion is that leave parts of the plants are rich in terms of organoselenium species, hence green parts, instead of bulb, should be consumed if a selenium enriched diet is aimed.

3.7.3 Simultaneous supplementation of arsenic and selenium

Interactive effect of selenium and arsenic was investigated by simultaneous addition of species of selenium and arsenic to green onion plants. Concentration of each species was calculated and differences were discussed in the following sections.

3.7.3.1 Application of Se(IV) and As(III)

Speciation of arsenic and selenium in plants supplemented with 1.0 mg/L Se(IV) and 1.0 mg/L As(III) was conducted. Concentration of arsenic species in root, bulb and leave of the plants are plotted in Figure 3.60 and percent distribution of species were given in Figure 3.61. If the distribution of arsenic was compared with application of As(III) alone, concentration of As(III) in roots was about 25% higher and ratio of As(III) to As(V) in root, bulb and leaves were about the same. Amount of As(V) was not changing significantly. We may conclude that the presence of Se(IV) increased the As(III) concentration in the root hence Se(IV) increase the uptake of As.

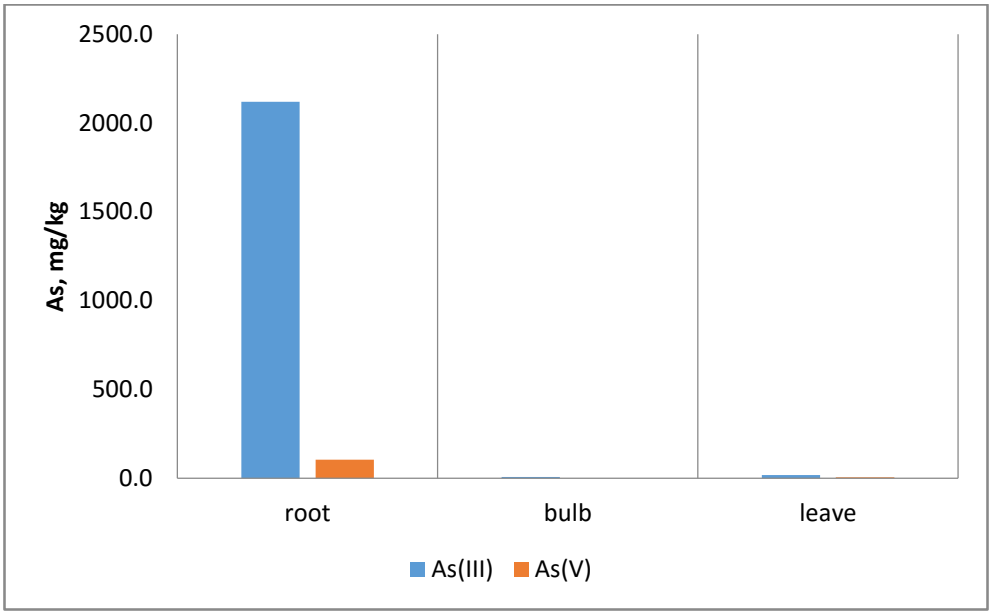


Figure 3.60 Concentration of arsenic species in root, bulb and leaves of plants supplemented with mixture of 1.0 mg/L Se(IV) and 1.0 mg/L As(III).

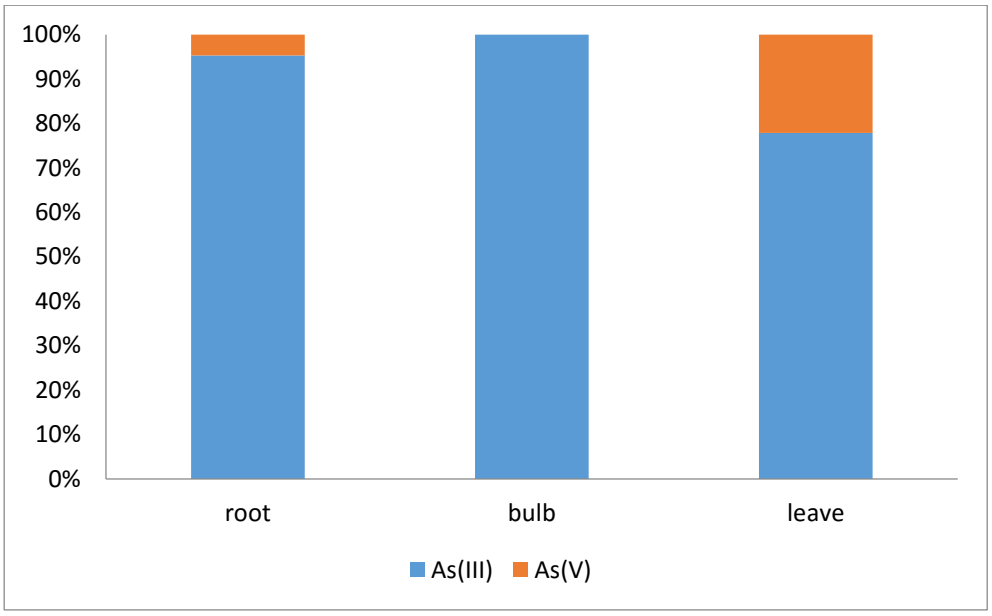


Figure 3.61 Percent mass distribution of selenium species in root, bulb and leave part of plants supplemented with mixture of 1.0 mg/L Se(IV) and 1.0 mg/L As(III).

Selenomethionine was the dominant selenium species in root and leaves as seen from Figure 3.62 and Figure 3.63, root having the highest concentration. If the selenomethionine deposition was compared with and without the presence of As(III), selenomethionine concentration decreased about 36% in roots and increased about 3 times in leaves. Additionally, concentrations of other organoselenium species in leave part increase as compared to Se(IV) supplementation only. Traces of Se(VI) were observed in root and leaves if As(III) was present. Presence of As(III) cause transformation of Se(IV) to Se(VI) to some extent. Since most of the As was deposited in the root part, it may be concluded that presence of As(III) inhibits the conversion of Se to organoselenium species in root part. Later these forms were transported to bulb and leaves where they were converted to organoselenium species. So another conclusion is that presence of As(III) drives the selenium species from roots to leaves.

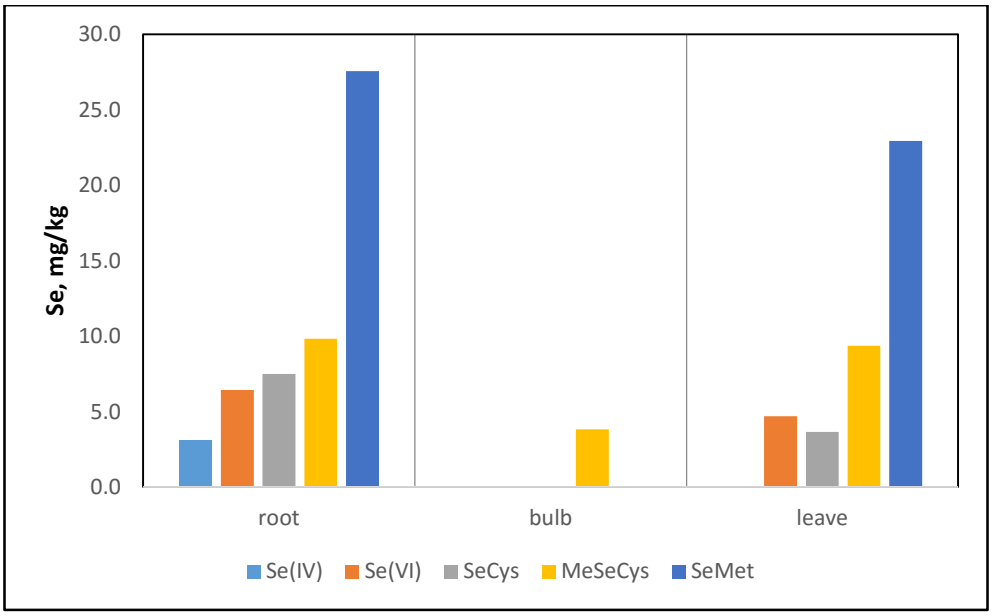


Figure 3.62 Concentration of selenium species in root, bulb and leaves of plants supplemented with mixture of 1.0 mg/L Se(IV) and 1.0 mg/L As(III).

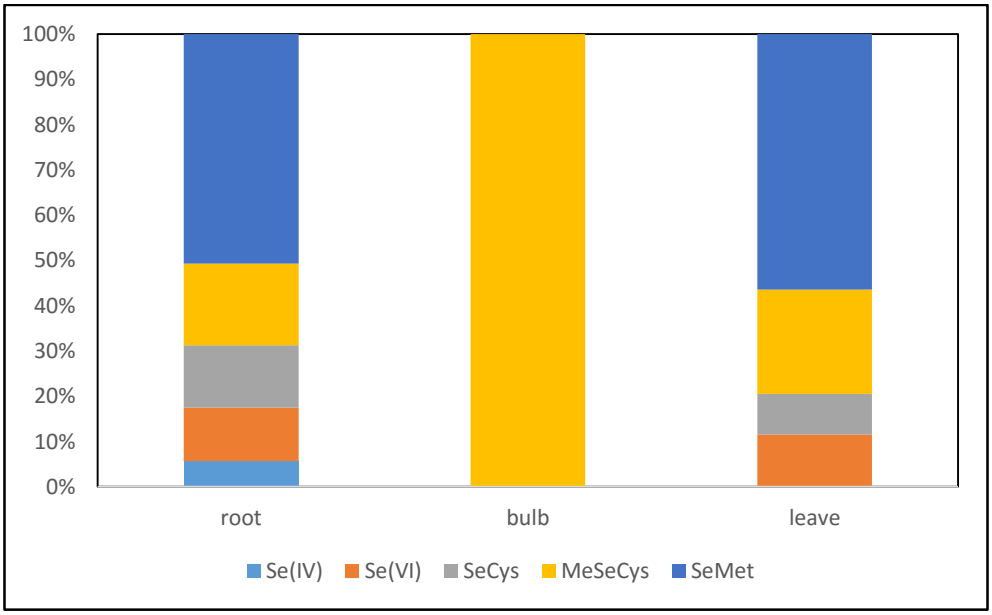


Figure 3.63 Percent mass distribution of selenium species in root, bulb and leave part of plants supplemented with mixture of 1.0 mg/L Se(IV) and 1.0 mg/L As(III).

3.7.3.2 Application of Se(IV) and As(V)

Dominating arsenic species in this combination was again As(III) although As was administered as As(V) as seen from Figure 3.64 and Figure 3.65. Concentration of As(III) was highest in roots and it was 96% higher if compared with As(V) alone supplementation. Additionally, concentration of As(III) in bulb and leave were two times higher when compared with onion samples supplemented with As(V) alone. These finding shows that presence of Se(IV) increased the concentration of As(III) in root, bulb and leave significantly while As(III) to As(V) ratio remains fairly constant except in leave part where ratio decreases.

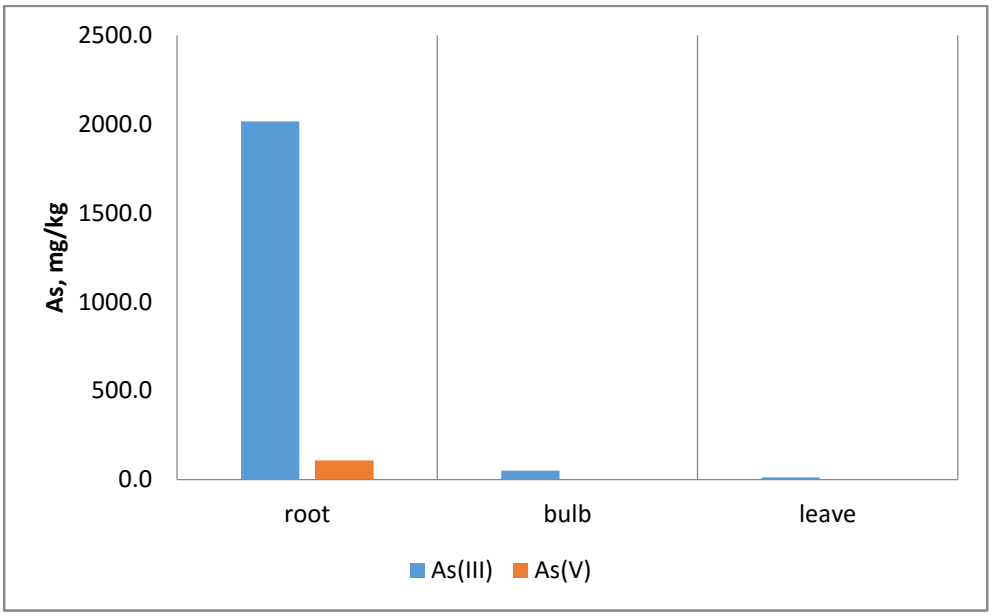


Figure 3.64 Concentration of arsenic species in root, bulb and leaves of plants supplemented with mixture of 1.0 mg/L Se(IV) and 1.0 mg/L As(V).

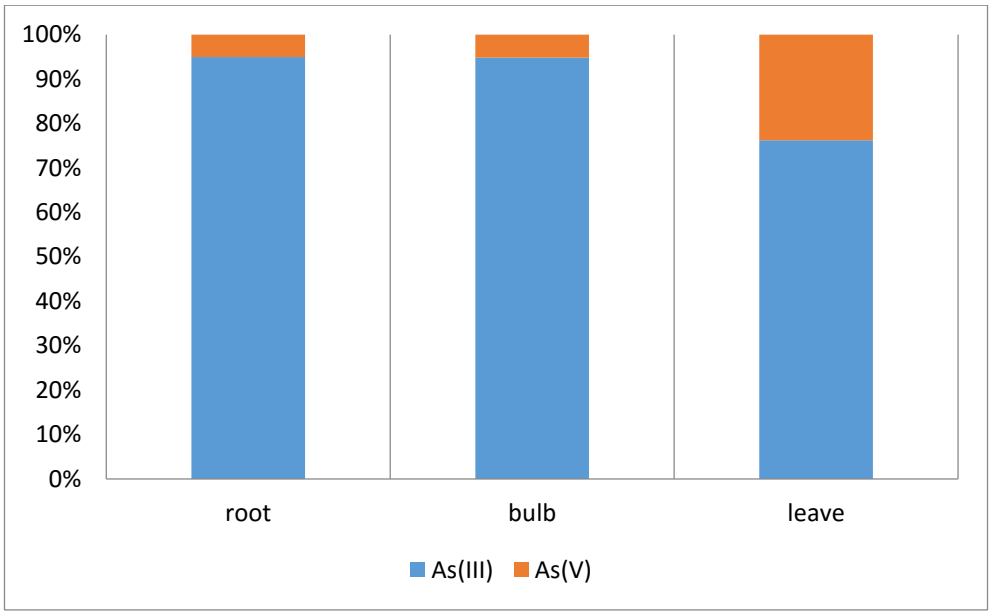


Figure 3.65 Percent mass distribution of arsenic species in root, bulb and leave part of plants supplemented with mixture of 1.0 mg/L Se(IV) and 1.0 mg/L As(V).

Distribution of selenium species were given in Figure 3.66 and Figure 3.67. While selenomethionine concentration decreased about 10% in root, its concentration was doubled in leaves if compared with Se(IV) supplementation only. Concentration of selenomethyl selenocysteine and selenocystine did not change significantly. These results indicate that there is a synergic relation between As(III) and Se(IV) species.

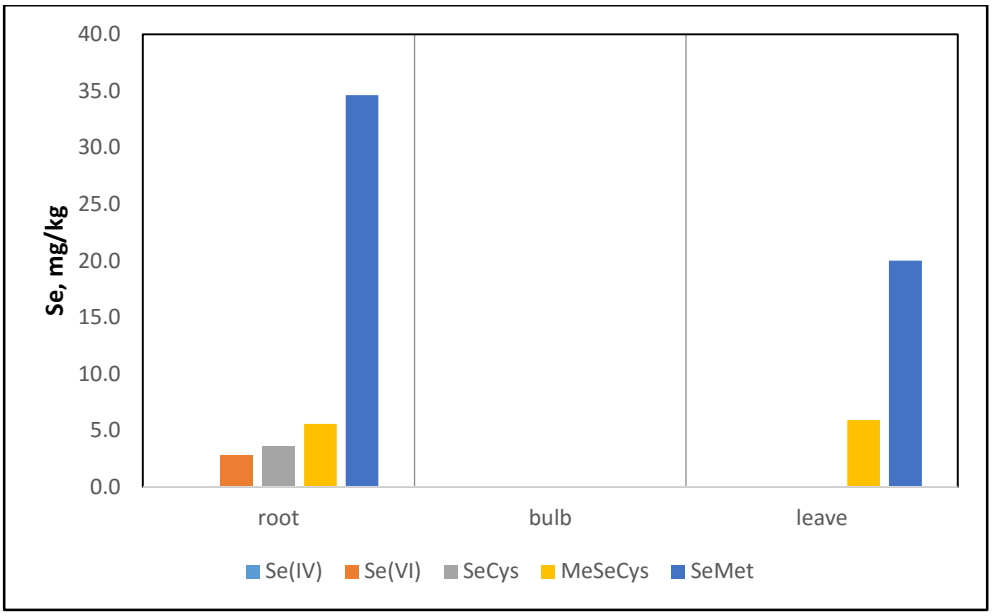


Figure 3.66 Concentration of selenium species in root, bulb and leaves of plants supplemented with 1.0 mg/L Se(IV) and As(V).

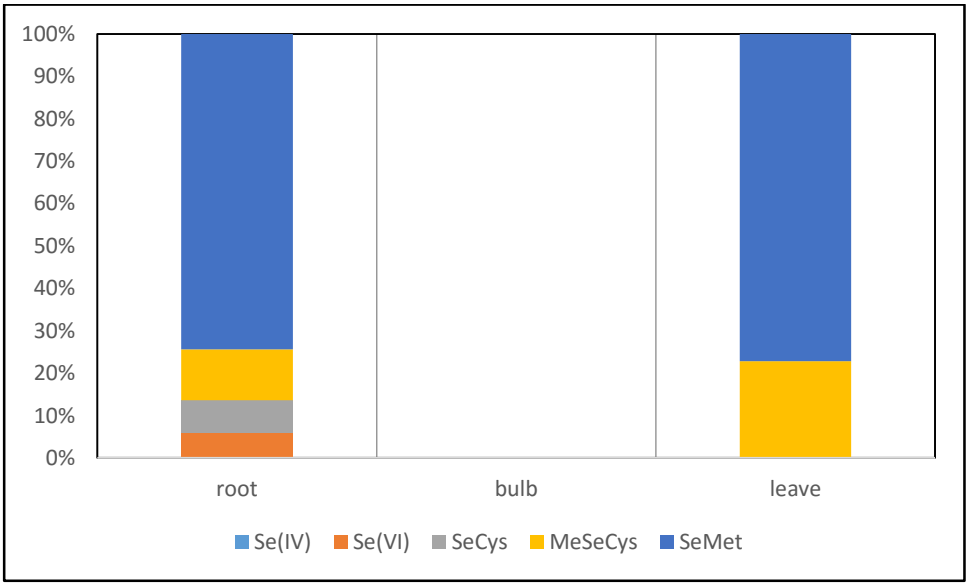


Figure 3.67 Percent mass distribution of selenium species in root, bulb and leave part of plants supplemented with 1.0 mg/L Se(IV) and As(V).

3.7.3.3 Application of Se(VI) and As(III)

When As(III) was introduced together with Se(VI), As(III) ion concentration in root part decreased 53% compared to As(III) alone while As(III) concentration in bulb and leave part did not change significantly as seen from Figure 3.68. As(V) concentration, on the other hand, decreased about 50% for root part. As(III) to As(V) ratio decreased significantly in leave part (Figure 3.69). It can be concluded that in the presence of Se(VI), arsenic uptake decreased about 50%. Amount of arsenic left in feed solution and total As determination supports this conclusion (Figure 3.11).

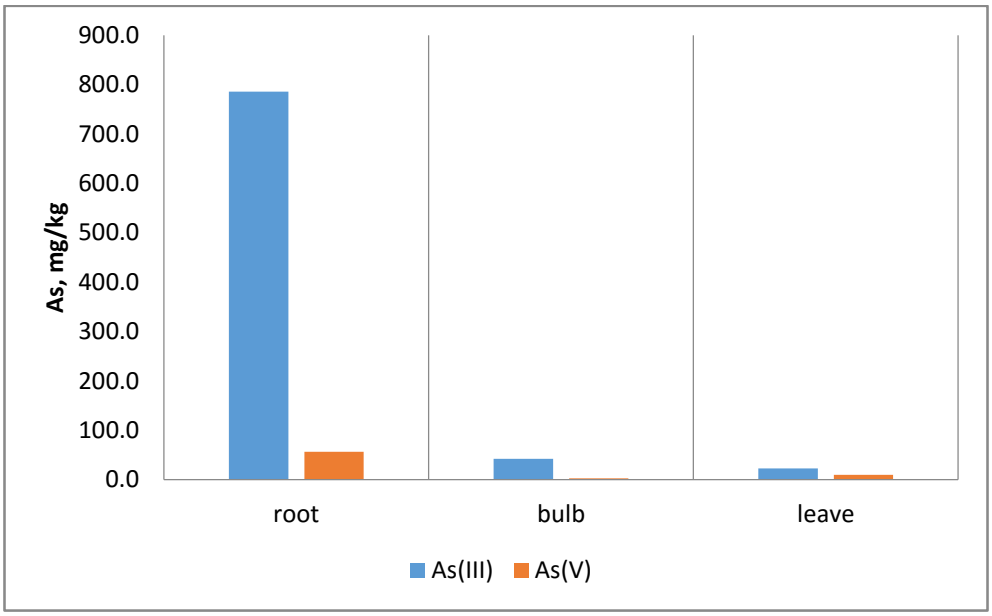


Figure 3.68 Concentration of arsenic species in root, bulb and leaves of plants supplemented with mixture of 1.0 mg/L Se(VI) and 1.0 mg/L As(III).

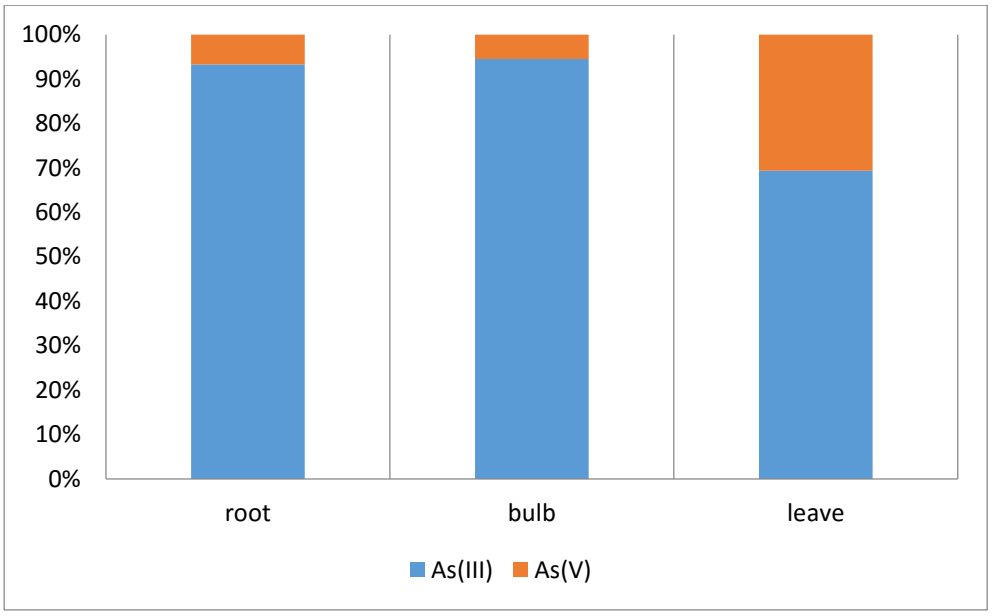


Figure 3.69 Percent mass distribution of arsenic species in root, bulb and leave part of plants supplemented with mixture of 1.0 mg/L Se(VI) and 1.0 mg/L As(III).

If we compare the Se distribution, dominating species is again Se(VI) with and without As(III) (Figure 3.70 and Figure 3.71). However, Se(VI) concentration in root decreased by 50%, while concentration in leave was doubled. This shows that As(III) drives the Se(VI) to leave part as suggested in selenium distribution section (Section 3.3.2). Concentration of selenomethionine, which is the dominating organic form, did not change significantly in root and leave parts indicating that presence of As(III) did not affect the formation of selenomethionine. Percent distribution of selenium species did not change significantly for root and bulb. It may be concluded that there is an antagonistic relation between As(III) and Se(VI) in root part of the plant.

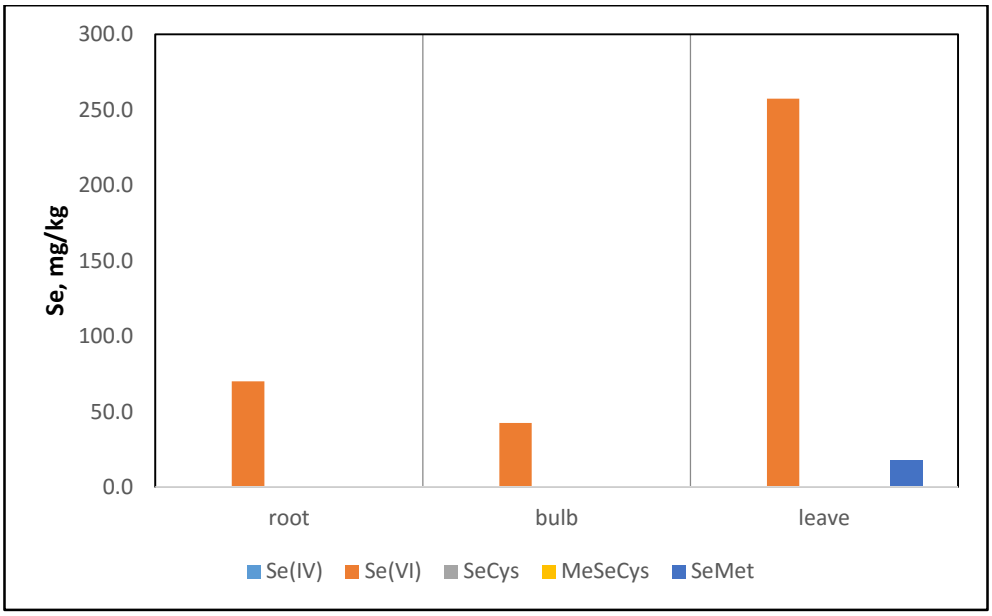


Figure 3.70 Concentration of selenium species in root, bulb and leaves of plants supplemented with mixture of 1.0 mg/L Se(VI) and 1.0 mg/L As(III).

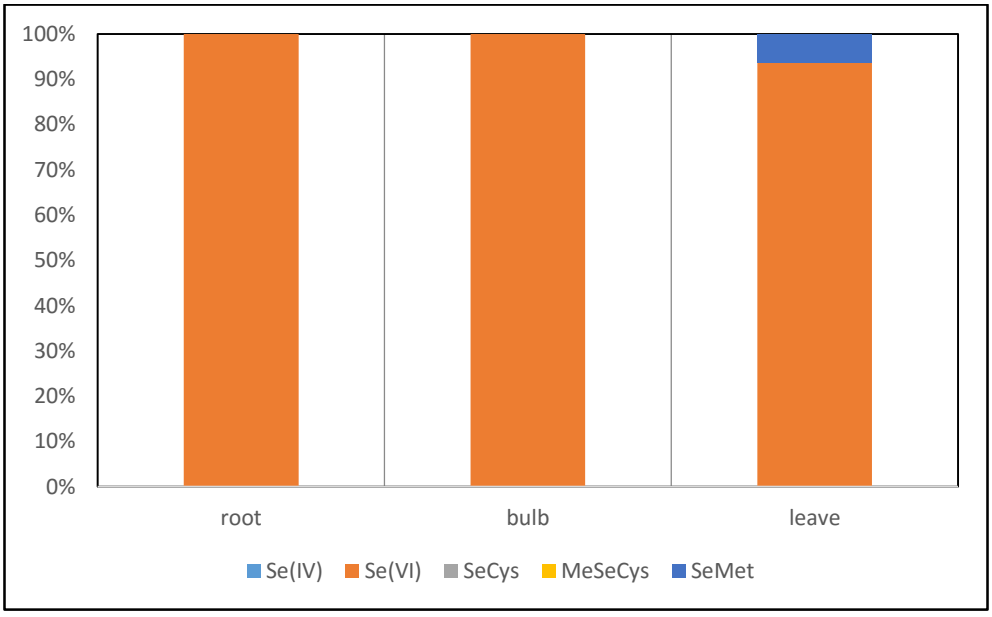


Figure 3.71 Percent mass distribution of selenium species in root, bulb and leave part of plants supplemented with mixture of 1.0 mg/L Se(VI) and 1.0 mg/L As(III).

3.7.3.4 Application of Se(VI) and As(V)

Concentration of As(III) decreased 23% in root if As(V) was administered together with Se(VI). Concentration of As(V), on the other hand, increased about 40% in root decreasing the As(III) to As(V) ratio (Figure 3.72 and Figure 3.73). Concentration of As(III) in bulb and leave did not change significantly. Presence of Se(VI) increased the conversion efficiency of As(V) to As(III).

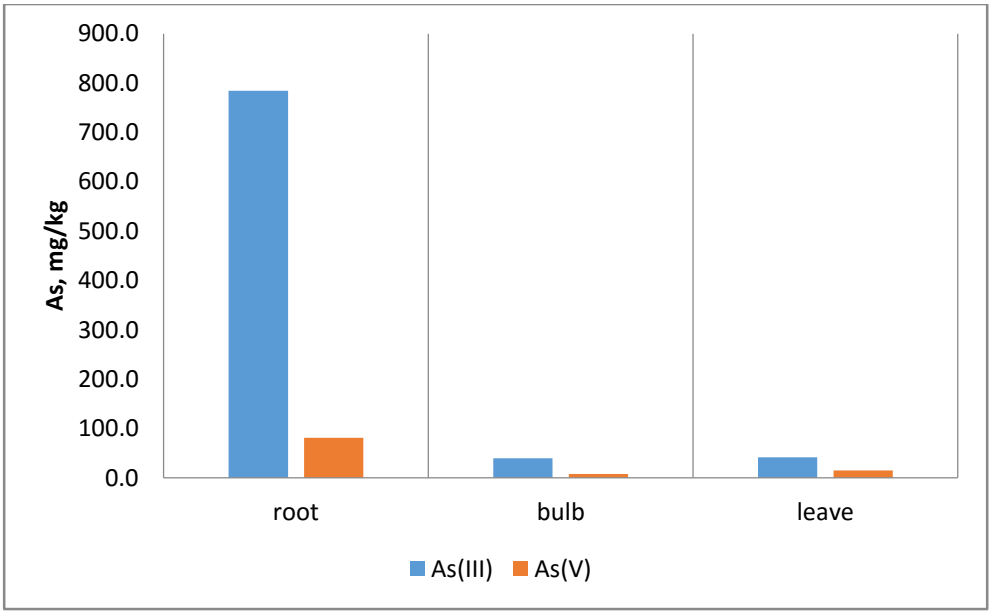


Figure 3.72 Concentration of arsenic species in root, bulb and leaves of plants supplemented with mixture of 1.0 mg/L Se(VI) and 1.0 mg/L As(V).

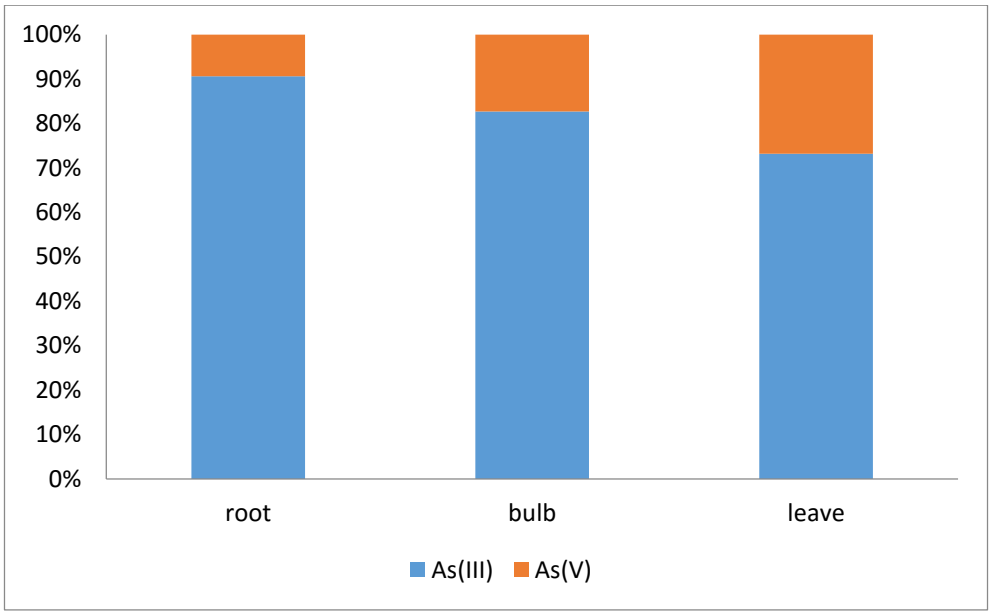


Figure 3.73 Percent mass distribution of arsenic species in root, bulb and leave part of plants supplemented with mixture of 1.0 mg/L Se(VI) and 1.0 mg/L As(V).

If we study the distribution scheme of selenium species, the dominant species was Se(VI) as seen from Figure 3.74 and Figure 3.75. Concentration of Se(VI) and selenomethionine were not changing in the presence of As(V) for root and bulb but Se(VI) concentration increased by about 30% in leaves. Similarly, concentration of selenomethionine was increased about 50% in the leaves indicating that As(V) derives Se to the leaves.

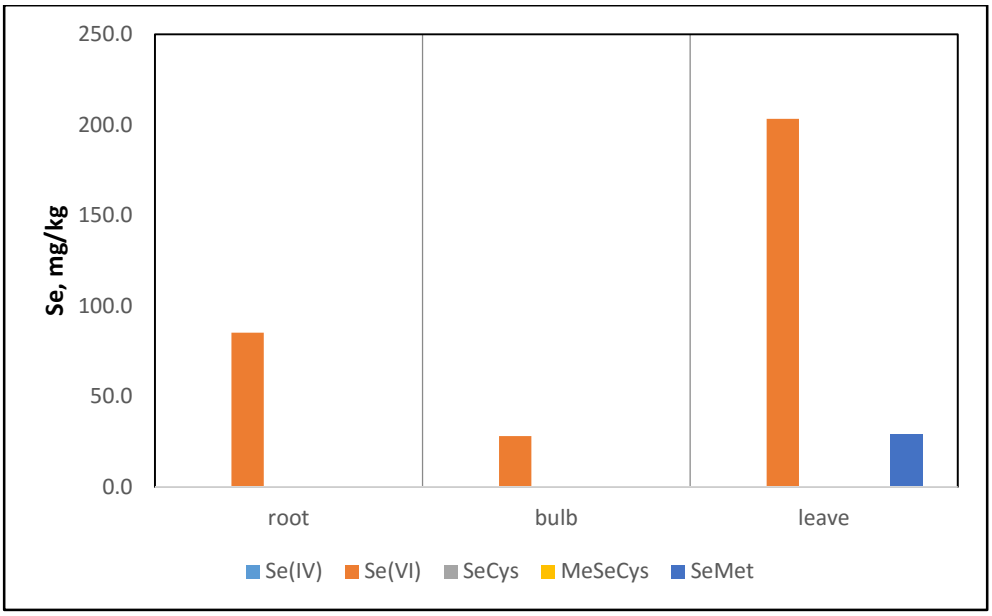


Figure 3.74 Concentration of selenium species in root, bulb and leaves of plants supplemented with mixture of 1.0 mg/L Se(VI) and 1.0 mg/L As(V).

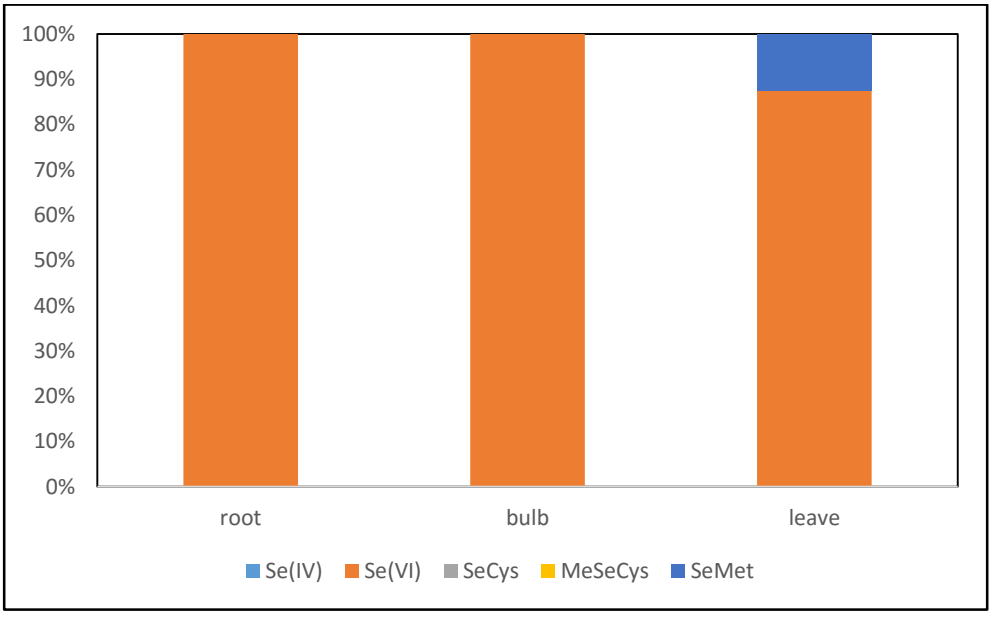


Figure 3.75 Percent mass distribution of selenium species in root, bulb and leave part of plants supplemented with mixture of 1.0 mg/L Se(VI) and 1.0 mg/L As(V).

3.7.4 Recovery values of As and Se

Percent recovery values of Se and As were calculated by dividing the amount of As or Se found by summing up the absolute masses of individual species by total As or Se found by digesting the samples. Results for As and Se are reported in Table 3.22 and Table 3.23, respectively.

Recovery values for bulb samples are not included since amount of Se and As quantified are generally low or below quantification limit and for some samples recovery values can not be calculated. Recovery of As from root samples are about 70% if As present alone, about 100% if present with Se(IV) and about 30% if present together with Se(VI). These results indicate that presence of Se species affect the recovery of As species either positively or negatively depending on the chemical form. On the other hand, recovery values of As from leave samples are relatively constant, about 60%.

Table 3.22 Percent recovery values of As

	Percent As recovery	
	Root	Leave
As(III)	71.2	60.1
Se(IV)-As(III)	109.7	64.4
Se(VI)-As(III)	25.1	68.2
As(V)	71.8	53.0
Se(IV)-As(V)	93.5	52.7
Se(VI)-As(V)	36.9	67.9

Recovery values calculated for Se are tabulated in Table 3.23. Recovery of Se from samples supplemented with Se(IV) are fairly low, about 18%, regardless of As species. This result indicates that Se(IV) is incorporated to plant matrix very strong and extraction is difficult. Similarly, low Se recovery values were calculated, about 30%, by other researchers if Se was administered as Se(IV) [159]. On the other hand, recovery of Se from plants supplemented with Se(VI) are comparably higher. Presence of As decreases the recovery values from root part whereas increases the recovery values for leave part.

These results indicate that presence of Se or As affect the recovery values and possibly the chemical form of elements considerably.

Table 3.23 Percent recovery values of Se

	Percent Se recovery	
	Root	Leave
Se(IV)	16.8	15.7
Se(IV)-As(III)	18.1	19.7
Se(IV)-As(V)	16.1	19.6
Se(VI)	71.7	48.9
Se(VI)-As(III)	27.9	76.1
Se(VI)-As(V)	37.5	61.8

CHAPTER 4

CONCLUSIONS

In this study a sensitive HPLC-ICP-MS method was developed for the simultaneous speciation of five selenium, namely Se(IV), Se(VI), selenomethionine, selenocystine and selenomethyl selenocysteine; and two arsenic, As(III) and As(V), species. Additionally, two arsenic species, monomethyl arsenic and dimethyl arsenic, were also monitored. Developed methodology was successfully applied to investigate antagonistic and synergic relation between As and Se in green onion samples supplemented with different inorganic forms of arsenic and selenium under hydroponic conditions.

Firstly, a chromatographic method capable of separating the target species was developed. For this purpose, ion pairing chromatography was studied but full separation was not achieved. Use of small ion pairing reagent resulted in separation of organic forms but failed to separate inorganic ones. On the other hand, inorganic species were successfully separated but not organic ones if bulky ion pairing reagents were used. As a result, anion exchange chromatography approach was studies. Different column-mobile phase combinations were studied and use of PRP-X100 anion exchange column with 5.0 mmol/L ammonium citrate buffer and 5.0% (v/v) methanol mobile phase at pH 5.00 was found to be the optimum elution mixture for the separation of target molecules and ions.

Green onion samples were grown under hydroponic conditions using halve strength Hoagland nutrient solution in the presence of As and/or Se species. Use of 10.0 and 5.0 mg/L for As and/or Se spiked nutrient solution resulted in poor root and leave development of plants compared to control plant. It was concluded that at this concentration level As and/or Se shows toxic effect. For instance, root and leave

mass of plants supplemented with 5.0 mg/L As and Se together was 85% and 50% lower compared with control pant. If 1.0 mg/L As and/or Se was used this ration is lower than 50% for all plants. According to these results, 1.0 mg/L As and/or Se was used for throughout of this thesis.

Total As and Se in root, bulb and leave parts of plants were determined by digestion of samples using 3.0 mL of concentrated nitric acid and 3.0 mL of 30% (v/v) hydrogen peroxide mixture with the help of microwave assisted digestion unit. Diluted samples were quantified by ICP-MS. Additionally, As and Se left in nutrient solutions were also determined.

If As distribution was investigated, it was seen that arsenic is almost entirely absorbed by the plant and 90% of it was deposited in the root part. This behaviour is similar for As(III) and As(V) supplementation. It may be considered as a defence mechanism developed by the plant against the toxic behaviour of As.

Se distribution showed a different trend. Highest Se concentration was found in roots if Se was administered as Se(IV) and highest Se concentration was found in leaves if Se was supplanted as Se(VI). This behaviour may be explained by the difference in bioavailabilities of selenium species. Se(IV) was converted to organoselenium species mostly in the root part, while conversion efficiency of Se(VI) was lower. It was transported to leaves easier.

Se concentration in root decreased and Se concentration in leaves increased if Se(IV) only supplementation was compared with Se(IV) supplementation together with As(III) or As(V). These observations shows that presence of As decreased the conversion efficiency of Se(IV) to organoselenium species; more Se(IV) was transported to leaves and converted to organoselenium in the leaves where As concentration was much lower.

Extraction procedure of Se and As species from plant matrix were optimised. Different extraction solvents were studied and enzymatic extraction was yield the

highest extraction efficiency. Without use of enzyme, protein bonded amino acids, such as selenomethionine, was not be extracted. Protease type XIV was used in phosphate buffer for the extraction of selenium and arsenic species.

Speciation of plants supplemented with As(III) and As(V) revealed that dominating species in root, bulb and leave was As(III) and concentration of As(V) was much lower. Speciation of As in nutrient solution was conducted and no detectible change in forms of species were detected. It is understood that As(V) was converted to As(III) after absorption by the plant.

Speciation of Se in plants supplemented with Se(IV) was also studied. Dominating selenium species in these plants were found as selenomethionine and selenomethyl selenocysteine and highest concentration was measured in root part. Conversion of Se(IV) to organoselenium species were 90% for roots and 100% for leaves. These results revealed the high bioavailability of Se(IV).

On the other hand, conversion efficiency of Se(VI) to organoselenium species was comparably low, 8% for roots, 0% for bulb and 14% for leaves. Most of the Se was found as Se(VI). It was concluded that bioavailability of Se(VI) is much lower compared to Se(IV) and conversion to other selenium forms was limited.

Above results were supplementation of single element only. From this point on results of supplementation of As and Se together will be given. If As(III) and Se(IV) were added to nutrient solution together, concentration of As(III) in root part was increased 25% compared to As(III) supplementation. Arsenic species in bulb and leave did not changed significantly. This results point a synergic relation between As(III) and Se(IV) in roots.

Selenomethionine concentration, on the other hand, decreased 30% in root and increased 2.5 times in leave if Se(IV) supplementation was compared with Se(IV) and As(III) supplementation.

Plants supplemented with As(V) and Se(IV) were studied. As(III) concentration in root part increased two times if compared with only As(V) supplementation. On the other hand, concentration of organoselenium species did not change significantly in root part and increased about 2.5 times in leaves. This is an indication of a synergic relation between As(V) ans Se(IV).

Results of supplementation with As(III) and Se(VI) were investigated. As(III) concentration was decreased two times in root and did not changed significantly in bulb and leaves if As(III) supplementation was compared with the mixture. Results revealed an antagonistic relation between Se(VI) and As(III) and As(V) species.

Plants supplemented with As(III) and Se(VI) indicates both antagonistic and synergic relations. Se(VI) concentration in leave was 1.8 times higher if compared with only Se(VI) supplementation and Se(VI) concentration was 1.7 times lower in root compared to Se(VI) only supplementation.

In case of As(V) and Se(VI) supplementation, interaction level between arsenic and selenium is not significant. As(V) was converted to As(III) species within the plant as discussed earlier. As(III) level in root part decreased 20% in case of mixture supplementation.

Both antagonistic and synergic interactions were observed if Se(VI) level in plants supplemented with As(V) and Se(VI) were compared with Se(VI) supplementation only. Se(VI) level decreased 1.4 times in root and increased 1.5 times in leave. Se(VI) conversion to organoselenium species was very low in all parts of the plant and the dominating selenium species was Se(VI) in all parts of the plant.

Results given above are valid only for onion plant grown under conditions given in experimental part and cannot be generalised to all plants or plants grown under different experimental conditions.

Simultaneous speciation studies are not common in literature. In some studies plants were grown under uncontrolled conditions in green houses using soil [159] or in water media using tap water [215]. As a result, some parameters such as possible conversions of the elements in growing media or distribution of elements between plant and growing media, cannot be studied. However, in the present study distribution of element in root, bulb and leave of the plant as well as feed solution was studied. It was proven that As(III)-As(V) conversion was took place after absorption by the plant.

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WORK EXPERIENCE

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AWARDS AND FELLOWSHIPS

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PUBLICATIONS

Books and Book Chapters

1. **Yıldırım, E.,** Yıldırım, L. S., Aspects of Speciation, In S. Bakırdere (ed.), Speciation Studies in Soil, Sediment and Environmental Samples, p.p. 1-18 (2013) CRC Press, London.

Peer Reviewed International Journals

1. **Yıldırım, E.**, Akay, P., Arslan, Y., Bakirdere, S., Ataman, O. Y., Tellurium speciation analysis using hydride generation *in situ* trapping electrothermal atomic absorption spectrometry and ruthenium or palladium modified graphite tubes, **Talanta**, 102 (2012) 59-67.
2. Bakırdere S., Bora S., Bakırdere E. G., Aydın F., Arslan Y., Komesli O. T., Aydın I., **Yıldırım E.**, Aflatoxin species: their health effects and determination methods in different foodstuffs, **Central European Journal of Chemistry**, 10 (2012) 675-685
3. Arslan Y., **Yıldırım E.**, Gholami M., Bakirdere S., Lower limits of detection in speciation analysis by coupling high-performance liquid chromatography

and chemical-vapor generation, **Trends in Analytical Chemistry**,30 (2011) 569-585

4. Bakirdere S., Aydin F., Bakirdere E. G., Titretir S., Akdeniz İ., Aydin I., **Yıldırım E.**, Arslan Y., From mg/kg to pg/kg Levels: A Story of Trace Element Determination: A Review, **Applied Spectroscopy Reviews**,46 (2011) 38–66

International Conference Papers

1. **Yıldırım, E**., Ertaş, G., Ataman, O. Y., Arsenic and selenium uptake by rocket plant (Eruca Sativa), **European Winter Conference on Plasma Spectrochemistry 2005**, 22-26 February 2015, Münster, Germany.
2. **Yıldırım, E**., Dügencili, B., Ertaş, G., Ataman, O. Y., Simultaneous speciation of arsenic and selenium in green onion (Allium Cepa) using HPLC-ICP-MS, **9th Aegean Analytical Chemistry Days**, 29 September-3 October 2014, Chios, Greece.
3. Ibraheim, M. A., **Yıldırım, E.,** Bora, S., Abdellalah M. H., Ataman, O. Y., A new phenomenon of cd vapor trapping inside burner head using vapor generation flame atomic absorption spectrometry, **9th Aegean Analytical Chemistry Days**, 29 September-3 October 2014, Chios, Greece.
4. **Yıldırım, E.,** Ertaş, G., Ataman, O. Y., Simultaneous separation of arsenic and selenium species by anion exchange chromatography and ICP-MS detection, **6th Black Sea Basin Conference on Analytical Chemistry**, 10-14 September 2013, Trabzon, Turkey.
5. **Yıldırım, E.** and Ataman, O. Y., Simultaneous extraction of Arsenic and Selenium species from plant matrix and Anion Exchange Chromatography and ICP-MS Detection, **The XVII European Conference on Analytical Chemistry,** 25-29 August 2013, Warsaw, Poland.
6. Dügencili, B., **Yıldırım, E**., Akay, P., Ataman, O.Y., Interactive Effects of Arsenic and Selenium in Green Onion (Allium cepa L.), **8th Aegean Analytical Chemistry Days**, 16-20 September 2012, İzmir, Turkey.

7. **Yıldırım, E.**, Akay, P., Arslan, Y., Bakırdere, S., Ataman, O.Y., Tellurium speciation using hydride generation atomic absorption Spectrometry and in-situ graphite cuvette trapping, **7th Aegean Analytical Chemistry Days**, 29 September-3 October 2010, Lesvos, Greece.
8. Akay, P., Arslan, Y., Bakirdere, S., **Yıldırım, E.**, Ataman, O.Y., Inorganic antimony speciation using tungsten coil atom trap and hydride generation atomic absorption spectrometry, **6th International Conference Instrumental Methods of Analysis Modern Trends and Applications**, 4-8 October 2009, Athens, Greece.
9. **Yıldırım, E.**, Arslan, Y., Bakirdere, S., Akay, P., Ataman, O.Y., Determination of Zinc by vapor generation atomic absorption spectrometry, **6th International Conference Instrumental Methods of Analysis Modern Trends and Applications**, 4-8 October 2009, Athens, Greece.
10. **Yıldırım, E.**, Akay, P., Bakirdere, S., Ataman, O.Y., Determination of lead by in-situ graphite cuvette trapping hydride generation atomic absorption spectrometry, **5th Black Sea Basin Conference on Analytical Chemistry**, 23-26 September 2009, Fatsa-Ordu, Turkey.

National Conference Papers

1. **Yıldırım, E.,** Dügencili, B., Ertaş, G., Ataman, O.Y, Yeşil Soğan (Allium Cepa L) Bitkisinde Arsenik ve Selenyum Etkileşimi , **7. Ulusal Analitik Kimya Kongresi**, 1-5 Eylül 2014, Kahramanmaraş, Türkiye
2. Akay, P., **Yıldırım, E.**, Arslan, Y., Bakırdere, S., Ataman, O.Y., Inorganic Antimony Speciation Using Tungsten Coil Atom Trap and Hydride Generation Atomic Absorption Spectrometry, **Uluslararası Katılımlı XII. Ulusal Spektroskopi Kongresi**, 18-22 Mayıs 2011, Side-Antalya, Türkiye
3. Şimşek, N.E., Arslan, Y., Yıldırım, E., Ataman, O.Y., Elektrotermal Atomik Absorpsiyon Spektrometri ile Bor Tayininde Kimyasal Değiştiriciler Kullanılması, **Uluslararası Katılımlı XII. Ulusal Spektroskopi Kongresi,** 18-22 Mayıs 2011, Side-Antalya, Türkiye

4. Ibraheim, M.A., Arslan, Y., **Yıldırım, E.**, Ataman, O.Y., Determination of Antimony (III) by Hydride Generation Slotted Quartz Tube-Atom Trapping-Flame Atomic Absorption Spectrometry, **Uluslararası Katılımlı XII. Ulusal Spektroskopi Kongresi**, 18-22 Mayıs 2011, Side-Antalya, Türkiye
5. **Yıldırım, E.**, Bora, S., Arslan, Y., Bakırdere, S., Duydu, Y., Ataman, O.Y., Hava Filtresi Örneklerinde ICP-OES ile Bor Tayini, **5. Ulusal Analitik Kimya Kongresi**, 21-25 Haziran 2010, Erzurum, Türkiye.
6. Karaman, G., Atakol, A., Bora, S., Njie, N., **Yıldırım, E.**, Bakırdere, S., Ataman, O.Y., Gıda Örneklerinde ICPMS ile Bor Tayini, **XI. Ulusal Spektroskopi Kongresi**, 23-26 Haziran 2009, Ankara, Türkiye.

Other Publications

1. **Yıldırım, E.**, Yıldırım, N., Turkish National Report about Volunteering and Lifelong Learning, October 2009.

PROFESSIONAL SERVICE

- Referee for Arabian Journal of Chemistry
- Practical exam team leader at the 43rd International Chemistry Olympiads, 9-18 June 2011, Ankara, Turkey, icho43.metu.edu.tr
- Theoretical and practical exam instructor for Turkish Chemistry Olympiad team

PROJECTS

1. Development of Analytical Technology for Simultaneous Speciation of Arsenic and Selenium and Its Application to Arsenic-Selenium Interactions in Plants, 2013-2015, TUBİTAK grant number 112T790.
2. Development of visual educational materials for high school chemistry students, Ministry of Education, TUBİTAK and METU joined project, 2013-2014.

3. Volunteering and Lifelong Learning in Universities in Europe (VALUE), European Commission's Lifelong Learning Programme Grundtvig Strand, 2008-2011, www.valuenetwork.org.uk

CERTIFICATES AND SEMINARS

1. Preparation of TÜBİTAK 1001 research project education, 2013, Antalya
2. First aid certificate, Turkish Red Crescent, 2009, 2013, Ankara
3. ISO 17025 Laboratory Accreditation education, 2010, Continuing Education Center, METU, Ankara

OTHER INFORMATION

Foreign Languages	Advance reading and writing in English Introductory German
Computer skills	Advance Windows, MS Office Good Macromedia Flash, Dreamweaver Good CambridgeSoft ChemDraw Average Adobe Photoshop
Memberships	METU İLKYAR (an organization that aims to help primary school students) student society administrative-board member Member of METU Chemistry Society