SPECIATION STUDIES USING HPLC-ICP-MS and HPLC-ES-MS

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ABSTRACT

SPECIATION STUDIES USING HPLC-ICP-MS AND HPLC-ES-MS

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Knowledge about selenium content of foods containing selenium species is very important in terms of both nutrition and toxicity. Bioavailability of selenium species for human body is different from each other. Hence, speciation of selenium is more important than total selenium determination. In the selenium speciation study, chicken breast samples, selenium supplement tablets and egg samples were analyzed for their selenium contents. In chicken breast study, chickens were randomly categorized into three groups including the control group (25 chickens), inorganic selenium fed group (25 chickens) and organic selenium fed group (25 chickens). After the optimization of all the analytical parameters used throughout the study, selenomethionine, selenocystine, Se(IV) and Se(VI) were determined using Cation Exchange-HPLC-ICP-MS system. In selenium supplement tablet study, anion and cation exchange chromatographies were used to determine selenium species.

Arsenic is known as toxic element, and toxicity of inorganic arsenic species, As(III) and As(V), is much higher than organic arsenic species like arsenobetaine and arsenosugars. Hence, speciation of arsenic species in any matrix related with human health is very important. In the arsenic speciation study, Cation Exchange-HPLC-ICP-MS and Cation Exchange-HPLC-ES-MS systems were used to determine arsenobetaine content of DORM-2, DORM-3 and DOLT-4 as CRMs. All of the

parameters in extraction, separation and detection steps were optimized. Standard addition method was applied to samples to eliminate or minimize the matrix interference.

Thiols play an important role in metabolism and cellular homeostasis. Hence, determination of thiol compounds in biological matrices has been of interest by scientists. In the thiol study, Reverse Phase-HPLC-ICP-MS and Reverse Phase-HPLC-ES-MS systems were used for the separation and detection of thiols. For the thiol determination, thiols containing –S-S- bond were reduced using dithiothreitol (DTT). Reduction efficiencies for species of interest were found to be around 100%. Reduced and free thiols were derivatized before introduction on the column by *p*-hydroxymercuribenzoate (PHMB) and then separated from each other by using a C8 column. In the real sample measurement, yeast samples were analyzed using HPLC-ES-MS system.

Keywords: Selenium, arsenobetaine, thiol, speciation, HPLC-ICP-MS, HPLC-ES-MS.

ÖZ

HPLC-ICP-MS VE HPLC-ES-MS KULLANILARAK TÜRLEME ÇALIŞMALARI

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Selenyum türlerini içeren gıda örneklerindeki selenyum içeriği hakkındaki bilgiler, hem toksik hem de yararlı etkilerini açığa çıkarmak açısından çok önem taşımaktadır. İnsan sağlığı açısından her selenyum türünün biyoyararlılığı farklıdır. Bundan dolayı selenyum türlemesi toplam selenyum tayininden daha önemlidir. Selenyum türlendirme çalışmasında, tavuk göğüs eti, selenyum takviyeli tabletler ve yumurta örnekleri analiz edilmiştir. Tavuk göğüs eti çalışmasında, tavuklar gelişigüzel bir şekilde, kontrol grubu (25 tavuk), inorganik selenyumla beslenmiş grup (25 tavuk) ve organik selenyum ile beslenmiş grup (25 tavuk) olmak üzere 3 gruba ayrılmıştır. Deney boyunca kullanılacak tüm analitik parametrelerin optimizasyonu yapıldıktan sonra selenometiyonin, selenosistin, Se(IV) ve Se(VI) türleri, Katyon Değiştirici-HPLC-ICP-MS sistemi kullanılarak tayin edilmiştir. Selenyum takviyeli tablet çalışmasında, anyon ve katyon değiştirici kromatografik yöntemler kullanılmıştır.

Arsenik toksik bir element olarak bilinir ve inorganik arsenik türleri olan As(III) ve As(V)' in zehirlilik dereceleri, arsenobetain ve arsenoşeker gibi organik arsenik türlerinden çok daha fazladır. Bundan dolayı insan sağlığı ile ilgili matrikslerde arsenik türleme çalışmaları büyük önem taşımaktadır. Arsenik türleme çalışmasında, Katyon Değiştirici-HPLC-ICP-MS ve Katyon Değiştirici-HPLC-ES-MS teknikleri DORM-2, DORM-3 ve DOLT-4 CRM' lerinde bulunan arsenobetaini tayin etmek için kullanılmıştır.

Özütleme, ayırma ve tayin basamaklarındaki tüm parametreler optimize edilmiştir. Matriksten kaynaklanan girişimleri ortadan kaldırmak veya azaltmak için örneklere standart katma yöntemi uygulanmıştır.

Tiyoller insan metabolizması ve homeostaz (dengeleşim) alanlarında önemli role sahiptirler. Bundan dolayı biyolojik matrikslerde tiyol bileşiklerinin tayini bilim adamlarının ilgisini çekmektedir. Tiyol çalışmasında, Ters Faz-HPLC-ICP-MS ve Ters Faz-HPLC-ES-MS sistemleri tiyollerin birbirlerinden ayrılmaları ve tayinleri için kullanılmıştır. –S-S- grubu içeren tiyoller ditiyotretol (DTT) kullanılarak indirgenmiştir. Türlerin indirgenme verimleri %100 dolaylarında bulunmuştur. İndirgenmiş ve serbest tiyoller *p*-hidroksimerküribenzoat (PHMB) kullanılarak türevlendirilmiş ve C8 kolonu kullanılarak ayrılmışlardır. Gerçek örnek analizlerinde, maya örnekleri HPLC-ES-MS sistemi kullanılmıştır.

Anahtar Kelimeler: Selenyum, arsenobetain, tiyol, türlendirme, HPLC-ICP-MS, HPLC-ES-MS.

To my wife, Gülhan and my small princess, Bengisu Ece

No words can express my love.....

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ABBREVIATIONS

AFS	Atomic fluorescence spectrometry
AI	Adequate Intake
a-Lac	a- lactalbumin
AsB	Arsenobetaine
AsB-D_3	Deuterated arsenobetaine
BSA	Butane sulfonic acid
CE	Capillary electrophoresis
CX	Cation exchange
CRM	Certified reference material
Cys	Cysteine
CysGly	Cysteinylglycine
DAD	Diode array detector
DF-ICP–MS	Double-focusing inductively coupled plasma mass spectrometer
DMDSe	Dimethyldiselenide
DMSe	Dimethylselenide
DTT	Dithiothreitol
EAR	Estimated average requirement
ES-MS	Electrospray mass spectrometry
ETAAS	Electrothermal atomic absorption spectrometry
FI	Flow injection
FPD	Flame photometric detector
FDNB	1-fluoro-2,4-dinitrobenzene
GC	Gas chromatography
GFAAS	Graphite furnace atomic absorption
GFC	Gel filtration chromatography
GPC	Gel permeation chromatography
GSH	Glutathione

GSSG	Glutathione disulfide
GPx4	Glutathione peroxidase
HAS	Human serum albumin
HCys	Homocysteine
HSA	Hexane sulfonic acid
HFBA	Heptafluorobutanoic acid
HGAAS	Hydride generation atomic absorption spectrometry
HPLC	High performance liquid chromatography
HPLC-MS/MS	High-performance liquid chromatography/tandem mass spectrometry
IAA	Iodoacetic acid
ICP-MS	Inductively coupled plasma-mass spectrometry
ICPS	Integrated count per second
IgG	Immunoglobulin G
LOD	Limit of detection
LOQ	Limit of quantitation
Lys	Lysozyme
Me_2Se	Dimethylselenide
Me_2Se_2	Dimethyldiselenide
Me_2SeO	Dimethylselenoxide
Me_2SeO_2	Dimethylselenone
Me_3Se^+	Trimethylselenonium cation
$MeHg^+$	Methylmercury
$MeSe(O)O^-$	Methylseleninic acid anion
MeSeH	Methylselenol
MeSSeMe	Dimethylselenosulfide
MIAC	N-(2-acridonyl)-maleimide
NEMI	N-ethylmaleimide
PHMB	P-hydroxymercuribenzoate
RDA	Recommended dietary allowance
RNI	Recommended nutrient intake
RPLC	Reversed phase liquid chromatography
RSD	Relative standard deviation

SAX	Strong anion exchange
SCX	Strong cation exchange
SDS	Sodiumdodecylsulfate
$Se=C(NH_2)_2$	Selenourea
SeCys	Selenocysteine
SEC	Size-exclusion chromatography
$SeCN^-$	Selenocyanate
SPME	Solid phase microextraction
SH/SS	Sulphydryl-disulphide
TBP	Tri*n*- butylphosphine
TCEP	Tris(2-carboxyethyl)phosphine
TFA	Trifluoroacetic acid
TMAH	Tetramethylammoniumhydroxide
TMPAB-o-M	1,3,5,7-tetramethyl-8-phenyl-(2-maleimide)-difluoroboradiaza-s-indacene
Tris-HCl	Tris(hydroxymethyl)aminomethanehydrochloride
UL	Upper tolerable nutrient intake level
USAED	Ultrasonic-Assisted Enzymatic Digestion
XRD	X-Ray diffraction
4-VP	4-vinylpyridine

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PART A

SPECIATION OF SELENIUM IN DIFFERENT MATRICES

CHAPTER 1

A. 1. INTRODUCTION

A.1.1. Selenium

A.1.1.1. General Information about Selenium

Selenium was discovered by the Swedish chemist, Jöns Jacob Berzelius, in 1817 after analyzing impurities that was contaminating the sulfuric acid being produced at a particular factory in Sweden. He initially suggested that it was tellurium but then he realized that it is an unknown element [1]. Selenium commonly found in rocks and soil is a naturally occurring element. In the elemental form, selenium is present in its pure form of metallic gray to black crystals. Elemental selenium is commercially produced, primarily as a by-product of copper refining. In the environment, selenium is not only found in its elemental form but also present in other forms where it is usually combined with other substances. In general, selenium is combined with sulfide minerals or with silver, copper, lead, and nickel minerals in rocks. In addition to these types of combinations, selenium is also found in some combinations with oxygen to form white or colorless crystals. In addition, it is known that some of the selenium compounds such as methylselenides (dimethylselenide (DMSe), dimethyldiselenide (DMDSe)) are in gases [2, 3, 4].

Selenium is released through both natural processes and human activities. It has many sources in environment such as agricultural drain water, sewage sludge, fly

ash released coal-fired power plants, oil refineries, and mining of phosphates and metal ores. Industrial and agricultural activities cause the releasing of selenium from geologic sources. Due to high amounts of released selenium by these sources, fish and wildlife in aquatic and terrestrial ecosystems around the globe are contaminated. Some conditions affect the amount of selenium in drain water. These conditions include: (1) a geologic source of selenium proximate to the project area, (2) low rainfall and high evaporation, and (3) topographically closed areas [5,6,7]. Biological activity of this element may be categorized in three main groups; a) Trace concentrations are sufficient for normal growth and development b) Moderate concentrations can be stored and homeostatic functions are maintained c) Elevated concentrations could result in toxic effects [7].

Selenium levels in soils and waters increase with time because of the fact that selenium settles from air and from waste. Mobility of selenium directly depends on oxygen. If selenium in soils does not react with oxygen it remains fairly immobile. Immobile selenium which is not dissolved in water is less of a risk for organisms. Higher oxygen levels and increased acidity of soils caused by human activities like industrial and agricultural processes increase mobile forms of selenium. All this processes affect not only the soil selenium levels but also selenium concentrations in surface water [8].

A.1.1.2. Selenium Species

Selenium species are mainly categorized in 3 groups; these are, i) inorganic selenium species, ii) organic selenium species and iii) amino acids and low molecular mass species. Some of the selenium species such as selenopeptides, selenoproteins are not included in any of these three groups. Hence, these types of selenium compounds form a 4^{th} group. Categorization of selenium species in environmental and biological systems can be seen in Table 1 [9]. In addition to selenium species given in Table 1 there are many other selenium species having different functions in living organisms and used in different applications.

Table 1. Selenium species in environmental and biological systems [9].

Selenium Groups	Species
Inorganic Selenium Species	Se [element], Selenide [Se(–II)] Selenite [Se(IV), (SeO_3^{2-})], Selenate [Se(VI), (SeO_4^{2-})]
Organic Selenium Species	Methylselenol (MeSeH) Dimethylselenide (Me_2Se) Dimethyldiselenide (Me_2Se_2) Trimethylselenonium Cation (Me_3Se^+) Dimethylselenone (Me_2SeO_2) Dimethylselenoxide (Me_2SeO) Methylseleninic Acid Anion ($MeSe(O)O^-$ Dimethylselenosulfide (MeSSeMe) Selenocyanate ($SeCN^-$) Selenourea ($Se=C(NH_2)_2$
Amino Acids and Low Molecular Mass Selenium Species	Selenomethionine, Selenocysteine Selenocystine, Se-methylselenocysteine Selenocysteic acid Se-methylselenomethionine *S*-(methylseleno)cysteine, Selenoxide hydrate, Selenohomocysteine Gama-glutamyl-Se-methylselenocysteine Se-adenosylselenohomocysteine Selenocholine Selenobetaine, Selenoglutathione
Other Types of Selenium Species	Selenopeptides, Selenoproteins Selenoenzymes, Selenosugars Se-metal metallothionines

A.1.1.3. Industrial Applications

Selenium has many applications in different fields. It is most commonly used in electronics due to its semiconductor and photoelectric properties. Semiconductor properties make selenium useful in different fields such as electric eyes, coating the metal cylinders and photographic exposure meters. In addition, selenium is widely used in the glass industry in the production of both red and black glasses. Some of colored pigments contain selenium and these are used in the production of ceramics, glass,

photoelectric cells, pigments, rectifiers, plastics, paints, enamels, inks, and rubber vulcanizing [10, 11]. Selenium can find applications also in photocopying [8]. Selenium has catalytic activity in some reactions. Hence, it is used as a catalyst in the production of pharmaceuticals like antidandruff shampoos where selenium sulfide is used [10]. Some of the selenium compounds have radioactive properties. Hence, it is used in diagnostic medicine [12].

A.1.1.4. Selenium and Health

Selenium has not only beneficial but also harmful effects on human body. It is mentioned that selenium as a trace mineral is an essential nutrient to human biology. Each selenium species has a different function in human body. Selenium takes roles in activities of different enzymes like glutathione peroxidases, Iodothyronine deiodinases and thioredoxin reductases. In Table 2, main functions of selenium species are given [13].

Table 2. Functions of selenium species [13]

Glutathione peroxidases	Antioxidant enzymes: remove hydrogen peroxide, lipid and phospholipid hydroperoxides (thereby maintaining membrane (GPx1, GPx2, GPx3, GPx4) integrity, modulating eicosanoid synthesis, modifying inflammation and likelihood of propagation of further oxidative damage to biomolecules such as lipids, lipoproteins, and DNA)
(Sperm) mitochondrial capsule selenoprotein	Form of glutathione peroxidase (GPx4): shields developing sperm cells from oxidative damage and later polymerises into structural protein required for stability/motility of mature sperm
Iodothyronine deiodinases (three isoforms)	Production and regulation of level of active thyroid hormone, T3, from thyroxine, T4
Thioredoxin reductases (probably three isoforms)	Reduction of nucleotides in DNA synthesis; regeneration of antioxidant systems; maintenance of intracellular redox state, critical for cell viability and proliferation; regulation of gene expression by redox control of binding of transcription factors to DNA.
Selenophosphate synthetase, SPS2	Required for biosynthesis of selenophosphate, the precursor of selenocysteine
Prostate epithelial selenoprotein (15kDa)	Found in epithelial cells of ventral prostate. Seems to have redox function (resembles GPx4), perhaps protecting secretory cells against development of carcinoma
DNA-bound spermatid selenoprotein (34 kDa)	Glutathione peroxidase-like activity. Found in stomach and in nuclei of spermatozoa. May protect developing sperm
18 kDa selenoprotein	Important selenoprotein, found in kidney and large number of other tissues. Preserved in selenium deficiency
Selenoprotein P	Found in plasma and associated with endothelial cells. Appears to protect endothelial cells against damage from peroxynitrite
Selenoprotein W	Needed for muscle function

Some deficiency problems occur in the case of low doses of selenium while the toxic effect of this element appears at high doses. It is known that in the case of short-term exposure to high concentrations of selenium in oral way, some problems including nausea, vomiting, and diarrhea may appear. In high exposure doses of selenium, a disease called *selenosis* is induced. By the effect of *selenosis*, hair losses, nail brittleness, and neurological abnormalities may appear [14]. The main function of selenium is to function as antioxidant that protects the human body from oxidative effects of oxidants such as hydrogen peroxide, other lipid hydroperoxides and derivatives [15]. Selenium has a role to prevent or improve the health conditions including acne, multiplesclerosis, ovarian cysts, cervical dysplasia, Parkinson disease, colorectal cancer, psoriasis, esophageal cancer, stomach cancer [16].

It is stated that selenium is a very important element for human body because it is a component of the enzyme glutathione peroxidase that is used to remove hydrogen peroxide, and lipid and phospholipid hydroperoxides from the cell [17]. There are many studies about the health effects of different species of selenium. In a study, turnover of noradrenalin and many of its metabolites were studied in the Se deficient animals [18].

A.1.1.4.1. Selenium Deficiency and Toxicity

Low amount of selenium is crucial for human body, but high amounts can cause serious health effects. When the amount of selenium taken daily is lower than the essential value, some health problems including protein energy malnutrition, haemolytic anaemia, cardiomyopathy (Keshan disease), hypertension, ischemic heart disease, alcoholic cirrhosis, infertility, cancer, arthritis, muscular dystrophy and multiple sclerosis may appear [19]. Studies have showen that if the intake of selenium for men and women is higher than 19.1 and 13.3 µg/day for an adult, respectively, deaths caused by Keshan disease were not observed. It was found that in the case of intaking of approximately 27 µg/day of selenium by a 65 kg-male, activity of GSHPx improves. Average normative requirements of selenium, $Se_R^{normative}$ and Recommended Nutrient Intake might be criteria for selenium intake [20].

Upper tolerable nutrient intake level (UL) of selenium for adults is 400 µg/day. Hence, the maximum concentration of selenium in dry diet should be lower than 2 mg/kg in order to balance the selenium concentration. In that case, UL of 400 µg/day of

selenium for humans provides an adequate margin of safety. If the consumption of selenium exceeds 900 µg/day, a health problem named nail dystrophy was observed [20].

Estimated Average Requirement (EAR), Recommended Dietary Allowance (RDA), Adequate Intake (AI), Tolerable Upper Intake Levels (UL) and Recommended Nutrient Intakes (RNI) values of selenium (µg/day) are shown in Table 3 [20, 21, 22, 23].

Table 3. Recommended Nutrient Intakes (RNI), Estimated Average Requirement (EAR), Recommended Dietary Allowance (RDA), Tolerable Upper Intake Levels (UL) and Adequate Intake (AI) values of selenium (µg/day) [20, 21, 22, 23].

Group	EAR, µg/day	RDA, µg/day	UL, µg/day	RNI, µg/day
Infants and children				
0-6 months		No RDA, 15 (AI)	45	6
7-12 months		No RDA, 20 (AI)	60	10
1-3 years	17	20	90	17
4-8 years	23	30	150	
4-6 years				22
7-9 years				21
Adolescents				
Female,10-18 years				26
9-13 years	35 (Female-male)	40	280	
Male, 10-18 years				32
14-18 years	45 (Female-male)	55 (14 years or older)	400	

Table 3. Continued.

Adults				
19-65 years			400	26
65+ years			400	25
Pregnancy		55		
2nd trimester				28
3rd trimester				30
Lactation		70		
0-6 months post-partum				35
7-12 months post-partum				42

A.1.1.4.2. Selenium Pathway in Plants and Animals

The concentration of selenium in plants is directly related with concentration of selenium in soil where the plants grow up. When the plant species grow up in seleniferrous soils they accumulate very high concentrations of selenium; such plants are called as Se accumulators [24]. In general, plants take selenite and selenate from the soil. Selenate is firstly converted to selenite and then selenide. After production of selenide, this species is transformed to Se–Cys. In plant metabolism, Se–Cys is converted to selenomethionine (Se-Met). Se-Met is also metabolized to Se–adenosyl–Se–Met, Se–MeSeMet [25, 26]. The metabolism of Se in plants can be seen in Figure 1 [26];

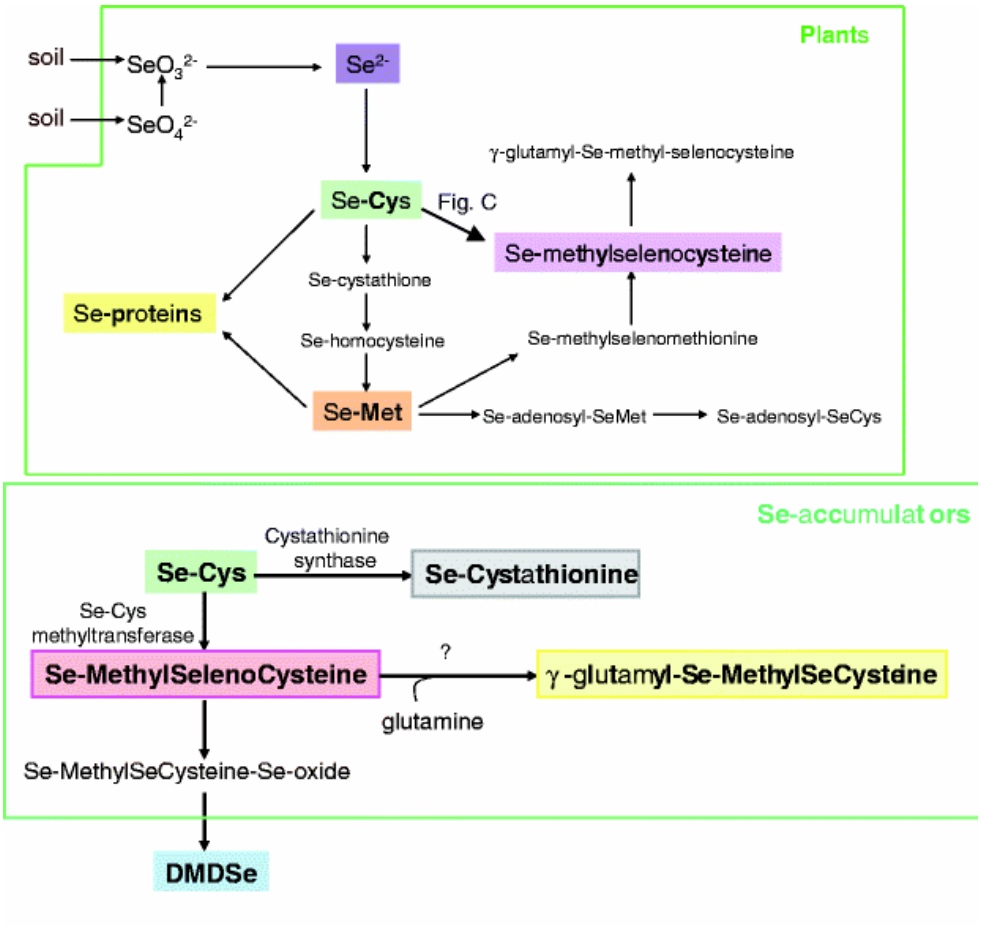

Figure 1. The metabolism of Se in plants [26].

Humans take selenium from food, drinks, air and other environmental sources. Metabolic patway of selenium species are different from each other. The general metabolism of selenium in animals is shown in Figure 2 [25].

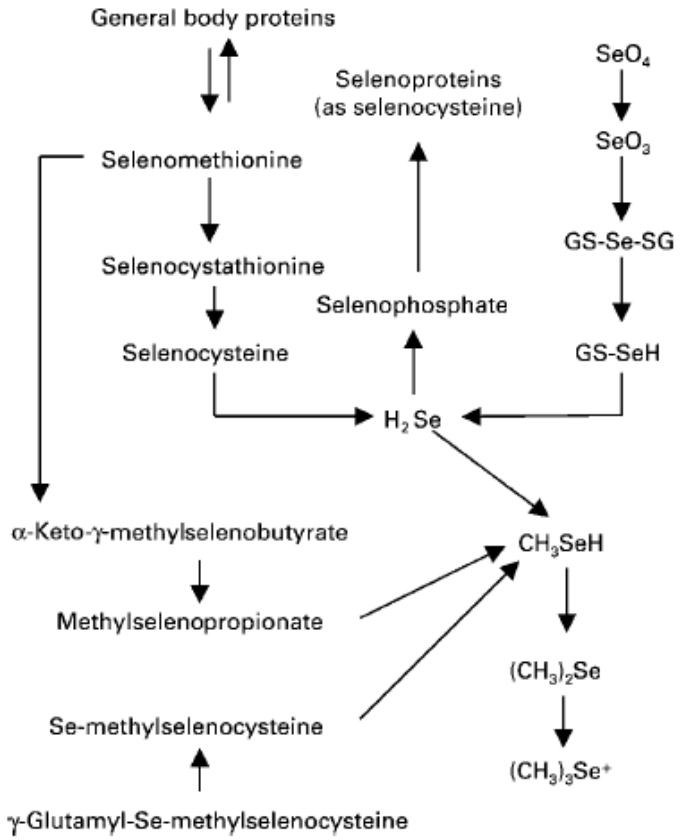

Figure 2. The metabolism of Se in animals [25].

As it is seen in Figure 2, in the animal body, most of the selenium species are converted to hydrogen selenide regarded as the precursor for supplying selenium for the synthesis of selenoproteins as selenocysteine. In literature there are many studies and reviews about selenium pathways in animal and plants [26, 27, 28].

The intake of selenium in the form of SeMet was significantly higher than selenium absorption as inorganic selenium species, selenate or selenite. It is reviewed that in the case of the injection of selenite to rats, the majority of selenium is metabolized to selenocysteine [25].

A.1.1.5. Determination of Selenium

Knowledge about the selenium content of foods containing selenium species is very important in terms of both nutrition and toxicity. Consequently, adequate analytical methods should be developed for the control of Se in trace levels. Atomic spectrometric techniques such as hydride generation atomic absorption spectrometry (HGAAS) [29, 30, 31, 32, 33] and graphite furnace atomic absorption spectrometry (GFAAS) [34, 35, 36, 37] are most widely used for selenium determination in different matrices.

HGAAS is one of the most commonly used techniques for Se determination at trace levels. Sufficiently low cost, low detection limits, high accuracy and precision and reasonably high speed of analysis are some of the advantages for HGAAS technique. In the organic matrices, firstly the organic matrix should be completely decomposed and all selenium species present should be oxidized into inorganic form of Se(VI). In the digestion procedure, in general, closed-vessel microwave technique is applied due to its ability to control the reaction parameters such as temperature and pressure, and permission to minimal reagent volumes ensuring low blanks for low detection limits. In digestion process, possible contaminations coming from laboratory environment can be minimized by isolating the process from surrounding as much as possible. In addition, possible volatilization losses in GFAAS can be eliminated or minimized by using hydride generation method [29]. Sample must be acid digested and Se is reduced to Se(IV) before measurement [31]. In general, after total digestion procedure all selenium species oxidized to Se(VI) are reduced easily to Se(IV) by heating at proper temperature after addition of HCl. In this final form, hydride species are efficiently formed.

Although some spectral interferences are observed, GFAAS is also widely used for the determination of selenium in different media. Low LOD, selectivity, sensitivity, and minimum sample quantity requirement are some of the advantages of this technique. In addition, by applying appropriate temperature program, organic matrix of sample can easily be eliminated before measurement step. Using this method, some spectral interference problems coming from the matrices containing high amounts of iron and phosphate compounds may appear. Possible volatilization losses of selenium during ashing can be minimized by using chemical modifiers such as palladium, nickel or magnesium [37]. In GFAAS, LOD for Se is at the level of low µg/L or lower [34].

In addition to AAS techniques, atomic fluorescence spectrometry (AFS) has also been used for the determination of this element [38, 39, 40, 41, 42]. When AFS is coupled to hydride system, it offers a simple and sensitive alternative. This method can provide detection limits that are lower than μg L^{-1}. By applying this method, Se has been determined in different matrices such as in Ni metal, shampoos, urine and food supplements [43, 44, 45, 46].

Stripping voltammetry is another technique for the determination of selenium. Piech used a new cyclic renewable mercury film silver based electrode for the determination of Se(IV) traces in the presence of copper ions using differential pulse cathodic stripping voltammetry [47]. Nascimento et al. determined Se(IV) and Se(VI) in high saline media (seawater, hydrothermal and hemodialysis fluids) by using cathodic stripping voltammetry [48]. Ashournia and Aliakbar also used CSV for the determination of selenium in natural waters. In this study, albumin was used as a medium for adsorptive accumulation of selenium on thin mercury film electrode [49].

Electrospray mass spectrometry (ES-MS) is used to make quantitative measurement of selenium species. ES-MS is also used in the characterization of selenium species. In literature, there are many studies published where ES-MS was used for determination and identification of selenium species [50,51,52,53].

Inductively-coupled plasma-mass spectrometry (ICP-MS) is a technique that has been widely used for selenium determination. ICP-MS has been used both in total element determination and isotope analysis for Se. In general, detection limit for Se is at ng/L level using ICP-MS. ICP-MS is also an effective technique for speciation analysis when used with chromatography or electrophoresis. Due to the very low detection limits ICP-MS has been mostly used in literature [54, 55, 56, 57, 58, 59]. In Table 4, studies where ICP-MS was used can be seen. ICP-MS can be combined with other instrument for the determination of different element.

Table 4. Some studies where ICP-MS was used in Se determination.

Time	Matrix	Analyte	Instrumentation	Limit of Detection	Ref.
2007	Otoliths	Se	LA-ICP-MS)	2.27 ppm	60
2007	Soil and sludge	As, Se	Slurry sampling electrothermal vaporization (ETV) ICP-MS	0.03 and 0.02 mg kg^{-1} for As and Se, respectively	61
2006	Foodstuffs of animal origin	Cr, Fe, Se	A hexapole collision cell with ICP-MS	0.025, 0.086 and 0.041 mg kg^{-1} for Cr, Fe and Se, respectively (LOQ)	62
2006	Epithelial cell homogenates	Selenotrisulfides	LC–ICP–MS and LC–ES-MS	Not given	63
2005	Human breath	Dimethylselenide	GC–ICP–MS	Se as DMSe is 10 pg Se, or 0.02 ng L^{-1}	64
2004	Phosphoric acid extracts of sediment samples	As, Se	HPLC-ICP-MS	2 and 40 ng g^{-1}	65
2003	Shark Tissue	Hg, Se	High resolution inductively coupled plasma mass spectrometry	Hg and Se with detection limits (3σ blank signal, *n*=10) of 26 and 4 ppt, respectively.	66

A.1.2. Speciation

A.1.2.1. General Information

Speciation is a technique that is used for identification and determination of the different chemical forms of element in any matrix. In addition, process that is used to find the quantities of different species of an element is called as *speciation analysis*. It is know that total element determination in environmental matrices is important in some fields like the water quality guidelines and maximum permissible levels of element. It is also well know that determination of species of element is more important than total amount because toxicity of many elements depends on their chemical forms. For example, Cr(VI) is more toxic than Cr(III) and As(III) is also more toxic than As(V) that is retained more easily in human body. In order to obtain more information about necessity or toxicity of element in environment we need to determine not only the total concentration of element but also its different chemical forms [67, 68].

Speciation analysis have been used in different fields such as studies of biochemical cycles of elements, toxicity of elements, quality control of vegetation or foods, technological improvement in environment, clinical studies and the control of medicinal and pharmaceutical products [68, 69].

In literature, there are many speciation studies for different element in different matrices such as water, sediments, foods and wastes. In Table 5, some examples of speciation studies for As, Hg and Cr are shown [70, 71, 72, 73, 74, 75, 76]. These studies are very important for human health because it is known that inorganic types of arsenic, namely arsenite and arsenate, are the most toxic arsenic species. Some of the arsenic species such as monomethylarsonic acid and dimethylarsinic acid are less toxic than the inorganic forms; arsenocholine and arsenobetaine are known as non-toxic arsenic species [70].

The similar situation is also seen for chromium. It is well known that Cr(VI) is about 1000 times more toxic than Cr(III). While Cr(III) is essential for human health in maintenance of glucose, lipid, and protein metabolism, Cr(VI) damages the renal system and mucous membranes [72].

Table 5. Some examples of speciation studies for As, Hg and Cr.

Year	Matrix	Analytical Method	Instrumentation and System	Ref.
1998	Environmental samples of contaminated soil	Arsenate, arsenite, monomethylarsonic acid, dimethylarsinic acid, arsenocholine and arsenobetaine	HPLC-ICP-MS	70
2001	Gas condensates	Triphenylarsine and triethylarsine	GC-ICP-MS	71
1997	Aqueous samples	Chromium(VI) and (III)	Adsorptive stripping voltammetry with pyrocatechol violet	72
1995	Aerosols	Chromium(VI) and (III)	Rapid single-particle mass spectrometry	73
2005	Mineral Waters	Chromium(VI) and (III)	GFAAS	74
2005	Water samples	Inorganic mercury (Hg^{2+}) and methyl mercury (CH_3Hg^+)	Polyaniline and gold trap-CVAAS	75
2007	Sediments at a municipal sewage	Total and Methyl Hg (MeHg)	Cold vapor atomic fluorescence spectrometry	76

Speciation studies are very important for mercury. It is well known that among the mercury species, methylmercury is the most toxic one and it causes many health risks for human. In general, humans take the most toxic mercury species through the consumption of polluted fish samples [76].

A.1.2.2. Selenium Speciation

Simply determining the total amount of selenium in body fluids or tissues without distinguishing the various chemical forms is not sufficient to understand whether the intake will function as toxic or beneficial. It is investigated that LD_{50} value for oral exposure for selenite and selenate are 4.8–7.0 mg Se/(kg body weight) and 2.5 mg Se/(kg body weight), respectively, while this value is 6700 mg Se/(kg body weight) for elemental selenium according to the US National Institute of Occupational Safety and Health [77]. Hence, speciation of selenium has become popular in recent years and much effort has been devoted to the development of analytical methods in order to make selective determination of selenium compounds in environment, body fluids and tissues.

In general, selenium species related to human health have been determined in literature. Chemical formulas of species of selenium that are of interest have been analyzed in speciation studies are given in Table 6 [78].

Table 6. Chemical formulas of Se species analyzed in some speciation studies.

Selenium Species	Formula	References
Selenite	SeO_3^{2-}	[79, 80, 81, 82]
Selenate	SeO_4^{2-}	[80, 81, 82]
Trimethylselenonium ion	Me_3Se^+	[82]
Dimethylselenide	Me_2Se	[79, 82]
Selenocystine	$H_3N^+–CH(COO^-)–CH_2–Se–Se–CH_2–CH(COO^-)–NH_3^+$	[80, 83]
Selenomethionine	$H_3N^+–CH(COO^-)–CH_2–CH_2–Se–Me$	[80, 82, 84, 83]
Se–Methylselenocysteine	$H_3N^+–CH(COO^-)–CH_2–Se–Me$	[85]
Gama-Glutamyl-*Se*-methylselenocysteine	$H_3N^+–CH(COO^-)–CH_2–CH_2–CO–NH–CH(COO^-)–CH_2–Se–Me$	[86]
Selenocystathionine	$H_3N^+–CH(COO^-)–CH_2–CH_2–Se–CH_2–CH(COO^-)–NH_3^+$	[87]
Selenohomocysteine	$H_3N^+–CH(COO^-)–CH_2–CH_2–SeH$	[88]
Selenocystamine	$H_2N–CH_2–CH_2–Se–Se–CH_2–CH_2–NH_2$	[82]

Chemical species or forms of selenium can be categorized to three parts [89];

1) Nuclear level (Isotopic distributions of Se),

2) Electronic level (Different redox states of Se)

3) Organometallic level (Forms of organic Se).

If the nuclear levels of selenium are taken into consideration, it is well known that selenium has 6 different isotopes, ^{74}Se, ^{76}Se, ^{77}Se, ^{78}Se, ^{80}Se and ^{82}Se. Most abundant isotope among them is ^{80}Se with 49.6 %, but this isotope has not been mostly used in literature when ICP-MS is used; because of Ar-Ar interferences that has a nominal mass of 80 amu. Hence, in general, ^{78}Se and ^{82}Se isotopes have been selected in selenium monitoring due to relatively high abundances and low interferences. While the abundance of ^{78}Se is 23.7%, this value is 8.7% for ^{82}Se. The abundances of ^{74}Se, ^{76}Se, ^{77}Se are 0.8%, 9.3% and 7.6%, respectively [90].

In the selenium speciation studies, 3 steps are crucial; these will be handled separately in the following sections.

a) Extraction
b) Separation
c) Detection

A.1.2.3. Extraction Step

The most crucial step in the speciation of selenium is the extraction procedure. In this step, all selenium species of interest should be taken from matrix to the solution without any alteration in the original form. Although some of the selenium species can be dissolved in water, many of them attached to the proteins can be only taken with hydrolysis. It is known that some selenium species can decompose during the hydrolysis step in the case of high pH and temperature. Hence, in order to eliminate any decomposition caused by pH or temperature, different hydrolysis procedures such as enzymatic solutions should be used instead of employing acid aqueous solution. Different analytical approaches could be used to find the effective extraction procedure for elements like selenium. Efficiency of extraction procedures depends on the matrix of the sample. The following extractions have been most commonly used in literature [89];

a) Solvent extraction
b) Basic hydrolysis
c) Enzymatic hydrolysis
d) Supercritical fluid extraction
e) Accelerated solvent extraction
f) Solid phase extraction
g) Derivatization
h) Fast extraction methods.

In the extraction procedure, the main purpose is to obtain a high efficiency. While liberating the selenium species that are bounded to protein chains by breaking down the peptide bonds, Se-containing amino acids or their derivatives should be intact [91]. In brief, extraction conditions should be chemically mild but yet efficient to release the selenium species from the matrix [89].

The acidity of the extraction solution is very important regarding the protection of selenium species from conversion to each other by the effect of pH changes. Hence, selenium species have been generally liberated from medium using different buffers like Tris HCl [92, 93], phosphate [94, 95] and acetate [96]. Due to the deposition of different phosphate compounds on sampler and skimmer cones, phosphate buffer has not been mostly used in literature in order to protect the ICP-MS system.

In general, water soluble selenium species are mostly taken from matrices by adding different reagents. In a recent study, proteins containing selenium were extracted using 30 mM Tris–HCl (pH 7.5) buffer containing 1% Sodiumdodecylsulfate (SDS) and protease inhibitor (2.0 mM Phenylmethanesulfonyl fluoride, PMSF) from Se-enriched chives (*Allium schoenoprasum*). By using Tris–HCl buffer water soluble proteins can be effectively taken from the sample. In addition, SDS known to be a good anionic detergent was used for solubilizing water insoluble proteins by the effect of ion-pair formation [97].

Microwave extraction method can also be applied for extraction of selenium. Caruso et al. used a 10.0 mL of water or 0.5 M HCl for selenium extraction from the ground nut sample after lipid removal. In the lipid removal step, powdered ground nuts were sonicated with a 1:2 mixture of CH_3OH-$CHCl_3$ and then solvent was taken from system. After this step, samples were located into microwave system for the extraction [98]. Sequential extraction procedure is another method to improve extraction efficiency. Mounicou et al. developed a sequential extraction procedure for the fractionation of different classes of selenium species in garlic sample [99]. Casiot et al. used 8 different extraction procedures using different chemicals; extraction procedures used in this paper can be listed as follows [100].

a) Hot water (85–90 °C)

b) 10% MeOH in 0.2 M HCl

c) 30 mM TRIS-HCl buffer (pH 7) and 0.1 mM PMSF

d) Driselase in 30 mM TRIS-HCl buffer (pH 7) in the presence of 1 mM PMSF

e) 30 mM TRIS-HCl buffer (pH 7) containing 4% SDS

f) 20 mM ammonium phosphate buffer (pH 7.4), 0.15 M NaCl, 0.1 mM PMSF, 1 mM EDTA, 5% SDS

g) Phosphate buffer (pH 7.5) containing 20 mg of pronase and 10 mg of lipase

h) 25% TMAH solution in hot (60 °C) water

In order to improve the extraction efficiency, different enzymes have been used for the extraction of selenium species bonded to large molecules (proteins, peptides) from different matrices. Protease XIV [101,102], Subtilisin (a non-specific protease) [103], Pronase E [104], Proteinase K [89, 93, 105] and pepsin [106] have been used for the extraction of selenium. In the enzymatic extractions, peptide bonds of molecule are broken and amino acid containing selenium can be easily liberated. In the enzyme studies, some of the parameters like temperature, enzyme amount, pH of the system are very important. The activities of many enzymes are temperature dependent. Hence, in order to obtain high activity, temperature of system should be constant at proper temperature. In addition, pH of the system is very important for the stability and activity of enzymes. Enzymes have "working pH ranges". If the pH is outside of this range, enzyme denaturation and inactivation may occur. In order to keep the pH of the system constant (generally around pH 7) to maintain maximum activity, in general, extraction procedures containing enzymes are performed in different buffer media as mentioned above. In the extraction procedure, all the parameters should be optimized to obtain high extraction efficiency. The most important optimization parameters are amount of enzymes used and extraction period [89]. Amount of enzyme to be added depends on the matrix. In literature, the ratio of sample and enzyme has been mostly used as 10:1 (w/w) while the different ratios have also been used. In addition, optimization of extraction period is very crucial. In literature, extraction periods ranging between 1 h and 48 h have been used. Duration of periods mostly used are categorized into 3 groups [89];

a) Short incubation period, 4-6 hours
b) Medium incubation period, 10 hours
c) Long incubation period, 24 hours

In general, 24 hours of extraction period is employed in literature.

Uden et al. used protease XIV as an enzyme for the extraction of selenium from phytoremediation plants (*Astragalus praleongus* and *Brassica juncea*), yeast, ramp (*Allium tricoccum*), onion (*Allium cepa*) and garlic (*Allium sativum*). In the enzymatic extraction, proper amount of de-ionized water and 0.02 g of protease XIV enzyme were added to 0.2 g of samples and then the mixture was shaken for 24 h at room temperature [101].

Gergely et al. used proteinase K enzyme as an enzyme for the extraction of selenium from *Agaricus bisporus* and *Lentinula edodes* mushroom samples. For this purpose, 5 mL of 30 mM Tris–HCl buffer solution (pH 7.5) containing 1 mM $CaCl_2$ was added to 0.2 g of sample and 0.03 g of proteinase K enzyme and then 0.03 g of protease XIV enzyme was also added to system. The mixture was stirred for 12 hours; the temperature of system was kept at 50 ^{0}C [93].

Ultrasonic-Assisted Enzymatic Digestion (USAED) method is another efficient extraction method. Capelo et al. firstly used ultrasounds in order to speed up enzymatic reactions for selenium speciation in yeast samples; simplicity, robustness and rapidity are some of the advantages of this method [107]. USAED has been widely used in literature for the extraction of selenium species; other elements were also studied using this approach [108,109,110]. Vale et al. described for total elemental determination and elemental speciation under the most recent achievements using USAED [111].

Extraction efficiencies depend on not only chemicals or enzymes used but also matrix. Hence, extraction efficiencies obtained in literature show variations. Extraction procedures and corresponding extraction efficiencies obtained thereby are given in Table 7.

Table 7. Extraction and speciation studies for Se.

Matrix	Analytes	Extraction procedure	Extraction efficiency, %	Ref.
Dill (*Anethum graveolens L.*)	Se-methyl-selenocysteine (MeSeCys),Se-methyl-selenomethionine (MeSeMet)	0.25 g of sample was extracted with 0.1 M HCl by magnetic stirrer for 24 h at room temperature.	31 (Root) 35 (Stem) 33 (Leaf)	112
Enriched green onions (*Allium fistulosum*)	Se(IV) and Se(VI), organic selenomethionine (SeMet), selenocysteine (SeCys) and Se-(methyl) selenocysteine (SeMeCys)	0.050 g of freeze-dried onion sample was weighed into a plastic centrifuge tube and 3 mL of sodium hydroxide solution (0.1 mol/L) were added. This mixture was shaken on a Vortex device for 15 min.	55	91
		Deionized water (5 mL) was added to 0.05 g of the sample and Pronase E (0.005 g) was applied. This mixture was shaken at room temperature for 24 h.	80	
Edible mushroom (*Agaricus bisporus*)	Seleno-DL-methionine, Seleno-DL-ethionine, Seleno-DL-cystine and Se(IV) and Se(VI)	*Step 1)* 3.6 ml of deionised water (pH 5.7); the sample was stirred at 37 °C at 200 rpm for 3 h. *Step 2)* 45 mg of pepsin was added in 3.6 ml of 0.05 mol/L Tris–HCl buffer (pH 2.1); the sample was stirred for 20 h. *Step 3)* 45 mg of trypsin was added in 3.6 ml of 0.1 mol/L phosphate buffer (pH 7.6)	75	113

Table 7. Continued.

Yeast	DL-Selenocysteine, DL-selenomethionine, Se(IV) and Se(VI) and selenoethionine	Hot water (85–90 °C); the sample was stirred for 1 h	10	100
		10% MeOH in 0.2 M HCl; the sample was stirred for 1 h	13	
		30 mM TRIS-HCl buffer (pH 7) containing 4% SDS; the sample was stirred for 1 h	42	
		Phosphate buffer (pH 7.5) containing 20 mg of pronase and 10 mg of lipase; sample was incubated for 16 h at 37 ^{0}C	88	
Mushroom *(L. edodes)*	D,L-Selenomethionine (SeMet), Selenocystine (SeCys) Methylselenocysteine (MSC) Se(IV) and Se(VI)	Sodium hydroxide (0.1 M)	65.2	93
		5 ml 30 mM Tris–HCl buffer (pH 7.5) containing 1 mM $CaCl_2$ was added to 0.2 g of sample and 0.03 g of proteinase K enzyme. The solution was kept at constant temperature of 50 °C and constantly stirred for 18 h. Then, 0.03 g of protease XIV enzyme was added and stirred for 12 h.	60.6	
		5 ml 30 mM Tris–HCl buffer (pH 7.5) containing 1 mM $CaCl_2$ was added to 0.2 g of sample and 0.03 g of driselase enzyme. The solution was kept at constant temperature of 50 °C and constantly stirred for 18 h. After that, 0.03 g of proteinase K enzyme was added to the above mixture and kept at 50 °C with constantly stirring for 18 h.	57.5	

A.1.2.4. Separation Step

Separation of selenium species from each other before quantification is an important step. Capillary Electrophoresis (CE), Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC) have been used in literature for the separation of different selenium species from each other. All these methods can be utilized in different matrices. In addition to these methods, some nonchromatographic methods were used for the separation of species from each others. Huang et al. developed a novel, fast, and cheap nonchromatographic method for direct speciation of dissolved inorganic and organic selenium species in environmental and biological samples [114]. In addition, Gonzalvez et al. recently reviewed the non-chromatographic methods for the speciation analysis; in this review, advantages and drawbacks of non-chromatographic methods based on published papers were discussed [115].

A.1.2.4.1. Capillary Electrophoresis (CE)

Capillary electrophoresis has been used for speciation analysis for many elements in addition to selenium. It can be used for a small amount of samples, and for species such as cations, anions, organometallic molecules, metal–organic ligand complexes. CE depends on the mobility of analytes in an electric field. In this method, chemical interaction of species with stationary phase does not take place. When this method is compared with chromatographic methods, it is clear that distribution of the elemental species of selenium is disturbed less severely [116]. In the CE process, species are separated from each other according to their mass-to-charge ratios [117]. In the speciation analysis, CE has some disadvantages. In order to increase the resolution, complexing electrolytes or different pH conditions can be utilized in this method. This may affect the species and alter their distribution in the sample [116].

In literature, there are many studies for selenium speciation using CE [117, 118, 119, 120, 121]. In a study, capillary electrophoresis that was coupled with ICP-MS was used as a powerful separation and detection tool for six selenium species, Se(IV), Se(VI), selenium carrying glutathione (GSSeSG), selenomethionine, selenocystine, and selenocystamine. The Se species appeared at 7s (selenocystamine), 16s (Se(VI)), 22s

(Selenocystine), 27s (Se(IV)), 35 s (Selenomethionine) and 56s (GSSeSG) during the detection step [122].

Deng et al. used CE for the separation of inorganic selenium species [118]. In addition, they used hydride generation system in order to increase the analyte transport efficiency. ICP-OES has also been used to detect selenium species. Se(IV) and Se(VI) were first separated by CE and then converted into the volatile hydrides of selenium. Hydride species were determined using ICP-OES. Using 50 ng/mL of Se standards the relative standard deviations based on peak area and six measurements for Se(IV) and Se(VI) were found as 1.5% and 1.8%, respectively. The limit of detections for Se(IV) and Se(VI) were 2.1 ng/mL and 2.3 ng/mL, respectively [118]. It is seen that the detection limits for selenium species obtained were higher than those in HPLC due to low sample volume used in CE. Morales et al. reviewed the recent progress in CE with special focus on separation conditions, detection systems, interfaces and its relevant applications [123].

CE combined with different detectors has been used to make selenium speciation in many matrices such as human urine [124], selenized yeast samples [125,126] and milk [127].

A.1.2.4.2. Gas Chromatography (GC)

Separation of selenium species in speciation studies can be performed by using gas chromatography. GC is one of the most sensitive method among others in the selenium speciation studies. It is known that volatile selenium compounds have been mostly analyzed using GC. Some of the volatile organic species of selenium, namely dimethylselenide, dimethyldiselenide and dimethylseleniumdioxide, are mostly present in environmental and biological samples [117]. Hence, there are many studies about speciation of dimethylselenide [128,129], dimethyldiselenide [129] and diethylselenide [129] in any matrix related with human health. Volatile forms of selenium play an important role for its global cycling because these volatile species are easily released to atmosphere [129]. Hence, selective determination of these species is very crucial. In addition, some of the organoselenium compounds such as SeMet can be analyzed using GC-ICP-MS after some derivatization methods. For instance, Yang et al. used cyanogen bromide for the derivatization of SeMet into the volatile species. After

derivatization, determination was made using GC-ICP-MS. All determinations were based on measurement of $^{78}Se/^{74}Se$ and $^{82}Se/^{74}Se$ ratios for yeast samples [130].

In the selenium speciation analysis, GC is coupled with ICP-MS to obtained very low limit of detection. Actually, this combination has low detection limits for selenium species because condensation and losses of selenium are eliminated or minimized. Gaseous effluent that transports the selenium species is directed to inlet arm of the torch. Hence, sensitivity is improved because no nebulization is used [131]. Using GC-ICP-MS or other combination techniques different volatile species of selenium have been determined in different matrices in literature. Caruso et al. reported speciation of volatile selenium species in green coffee beans, roasted beans, and brewed coffee drink [132]. Solid phase microextraction (SPME) was used as a preconcentration technique. GC-ICP-MS measurements showed that the headspace of the supplemented coffee beans and brew contain many volatile selenium species, such as diethyl selenide, diethyl diselenide, ethylmethylselenosulfenate and ethyl methyl diselenide [132].

Lenz et al. performed selenium speciation in contaminated drinking, ground, or wastewaters using chromatographic and spectroscopic techniques [77]. It is emphasized that these methods are very suitable for selenium speciation in bioremediation processes. Using GC-Flame Ionization Detector, dimethylselenide and dimethyldiselenide formed during bioremediation of selenium contaminated waters were determined within 7.4 min. Absolute detection limits for dimethylselenide and dimethyldiselenide were 1 ng and 2 ng for 1.0 µL injection volume, respectively [77].

A.1.2.4.3. High Performance Liquid Chromatography (HPLC)

HPLC has been used for selenium speciation very often because most of the selenium species of interest are non-volatile; in addition, this technique has some advantages over other separation techniques. On the contrary to GC, HPLC does not require derivatization of selenium species. In addition, limit of detection obtained for selenium species using HPLC-ICP-MS are lower than those obtained using CE-ICP-MS. The reproducibility of separations is another advantage of HPLC in selenium speciation [117].

In the speciation analysis of selenium HPLC is generally combined with ICP-MS having very low detection limits. This combination is very popular in literature because sample solutions at all flow rates are easily pumped to a nebulizer where fine aerosols are obtained. It means that the flow rate of sample in the separation (HPLC) and detection (ICP-MS) systems are compatible. In both systems, flow rates can be used in the range of 0.01 to 2.00 mL/min. In addition, in this combination wide linear dynamic range can be obtained for different species of selenium [133]. In the speciation analysis like chromatographic system, one of the most important requirements is the constant flow rate. HPLC supplies this requirement with reciprocating pumps. Although this combination has been mostly used in literature, it has some limitations. For instance, the sensitivity of the ICP-MS system can be affected due to high salt or organic content of mobile phase used in HPLC. Sample and skimmer cones may be clogged due to salt or carbon deposition. In general, volatile organic chemicals containing low salt concentrations have been chosen to eliminate this deposition. In the case of using a high content of organic solvent in mobile phase forward power should be higher than 1500 W to eliminate cooling effect of solvent that causes depression in sensitivity [59].

Separation using different modes of HPLC such as reverse-phase, size-exclusion and ion-exchange (anion and cation exchange) has been used in literature for selenium speciation in different matrices [117].

A.1.2.4.3.1. Reverse-Phase HPLC System

Reverse-phase HPLC system has been widely used as a separation method in literature. In this system octadecyl (C18) or octyl (C8) chains are used as non-polar stationary phases. Non-polar stationary phases are bounded to the non-polar support material that is generally silica gel. Selenium species are partitioned between non-polar stationary phase and polar mobile phase carrying analytes. Reverse-phase ion-pairing chromatography is also used for selenium speciation. In this system, a counter ion is added to the mobile phase to permit the simultaneous separation of anionic, cationic and neutral molecules of selenium [134]. The earliest aim of ion-pairing chromatography was to make separation of organic compounds that can be ionized on reverse phase columns. Using a lipophilic counter ion in the mobile phase, this aim was easily achieved. Optimization of the experimental parameters is very crucial in the case

of ion-pairing reverse-phase chromatography. pH of the system, concentration of organic modifiers are some of the most important optimization parameters [135]. It is known that most of the seleno-amino acids are too hydrophilic to be separated in reverse phase columns. Hence, ion-pairing agent has been used as a mobile phase additive. Perfluorinated carboxylic acids such as Trifluoroacetic acid (TFA) [136, 137, 138], pentafluoropropanoic acid [137] and heptafluorobutanoic acid (HFBA) [101, 137] have been widely used for this aim [135]. Some of studies about selenium speciation using reverse-phase chromatography is shown in Table 8.

Table 8. Some of studies for selenium speciation using reverse-phase chromatography.

Selenium Species	Matrix	Column used	Mobile Phase	Ref.
Seleno-L-methionine, L-selenocystine, phenyl-L-selenocysteine, methyl-seleno-L-cysteine, methaneseleninic acid, selenate,selenocyanate and selenite	Diet supplements	A Luna C18 stationary phase	Mobile phase is a mixture of methanol and water both 0.05 % in trifluoroacetic acid for the separation of the organic species the while for the separation of inorganic species mobile phase is a mixture of methanol and tetrabutylammonium hydroxide 1.0 mM aqueous solution.	136
Se(IV), SeCys, MSC, and SeMet	Mushrooms	Alltima CS (250mm×4.6 mm, 5µm)	0.1% (v/v) heptafluorobutyric acid (HFBA), 5% (v/v) methanol, pH 2.5	93
Se(VI), As(V), As(III), and Se(IV)	Water samples, coal fly ash and sediment	Capcell C18 RP column	Mixed ion-pairing modifier containing 5 mM of butane sulfonic acid (BSA), 2 mM malonic acid, 0.30 mM hexane sulfonic acid (HSA) and 0.5% methanol of pH 2.5	139
SeMet	Antarctic krill	C18 Synergi 4µ Hydro-RP, 150x 1.0mm	60 % 15 mM ammonium acetate pH 6.0 40% methanol (v/v)	140
Se(VI), selenourea, selenomethionine, selenoethionine and trimethylselenonium ion	Human urine	A LiChrosorb RP 18 reversed-phase column	Mixed ion-pair reagents of 2.5 mM sodium 1-butanesulfonate and 8 mM tetramethylammonium hydroxide	141

A.1.2.4.3.2. Size-Exclusion HPLC System

Size-exclusion HPLC system has been used to characterize different selenium species in different matrices. In size-exclusion chromatography (SEC), separation of selenoproteins occurs in a porous non-absorbing stationary phase. Sizes of the pores are approximately the same as the analyte's effective solution dimensions. Interaction between stationary phase and analytes is minimized in order to obtain effective size separation. In general, there are two types of SEC; gel permeation chromatography (GPC) where organic mobile phase is used and gel filtration chromatography (GFC) in which aqueous mobile phase is used [117].

In literature, there are limited numbers of selenium speciation studies using GPC. Chatterjee et al. used a gel-permeation (polyvinyl alcohol-based resin) GS-220 column with 25 m*M* tetramethylammonium hydroxide and 25 m*M* malonic acid at pH 7.9 as mobile phase in order to separate selenite, selenate, selenomethionine, seneloethionine and trimethylselenonium ion in urine samples. In this study baseline separation was achieved for selenite, selenate, trimethylselenonium ion and unknown selenium compounds using a GS-220 column. In addition, measurements were free from interferences [142].

GFC has also many applications in the separation of different selenium species according to their sizes. Ogra and Suzuki used multi-mode gel filtration HPLC columns of capillary size to separate Se-methylselenocysteine and gamma-glutamyl-Se-methylselenocysteine in garlic, and selenosugar (1-beta-methylseleno-N-acetyl-D-galactosamine) and trimethylselenonium as selenometabolites in urine. In this study, two types of column were used. Narrower column (GS320A-M5D, 0.5 mm i.d. x 150 mm) was used to separate major selenocompounds found in selenized garlic and the larger column (GS320A-M8E, 0.8 mm i.d. x 250 mm) was used to separate the selenoamino acids [143].

Studies about selenium speciation using SEC can be seen in Table 9.

Table 9. Studies for selenium speciation using SEC.

Selenium Species	Matrix	Column used	Mobile Phase	Ref.
SeMetSeCys, Se(IV), Se(VI), SeMet, Se-containing proteins	Broccoli (*Brassica oleracea*)	Biosep SEC 2000 PRP-X100 Shodex Asahipax GS-220 HQ	50 mM Tris-HCl, 0.05 mM KCl pH 6.8 Ammonium citrate 10 mM, pH 5, 2% methanol 25 mM Ammonium acetate pH 6.7	144
Selenate, methane seleninic acid, selenite, selenocystine, selenomethylcysteine, selenomethionine. Se-containing proteins	Commercial Dietary Supplement	PL-Aquagel (300 × 7.5 mm)	Water/MeOH (92/8)	145
Se-containing proteins	Accumulator plant (Brassica juncea) andnonaccumulator plant (Helianthus annuus)	Superdex 75 HR 10/30 SEC (10 mm × 300 mm ×13 µm)	30 mM Tris–HCl, pH 7.5	146
Se-containing proteins	Cell Walls and Cell Membranes/Cytosol	Superdex 75 HR10/300 GL	Tris-HCl mobile phase,30 mmol L^{-1}, pH 7.0	147
Se-containing proteins (Ranges: 600–10 kDa, 70–3 kDa, 14–0.18 kDa)	Nut proteins	a) Superdex 200 b) Superdex 75 c) Superdex Peptide	a)150 mmol L^{-1} $(NH_4)HCO_3$ (pH 7.8) b) 50 mmol L^{-1} Tris/HCl (pH 8) c) 50 mmol L^{-1} Tris/HCl (pH 8)	92

A.1.2.4.3.3. Ion-Exchange (Anion and Cation) HPLC System

Ion-exchange HPLC system has been widely used for selenium speciation in different matrices. Using ion exchange system, not only the ions but also the easily ionized analytes can be separated from each other. The mechanism of ion exchange depends on equilibrium between stationary phase and mobile phase. Actually, separation takes place on surface ions of stationary phase. Oppositely charged ions in mobile phase are exchanged with surface ions [117]. There are two types of ion-exchange; cation and anion-exchange.

It is known that pH of the system is very important in the speciation studies, especially in separation part. The species distribution diagrams of selenic acid, selenous acid, selenomethionine, selenocystine in the pH range from 0 to 14 are shown in Figure 3 in the form of pH-pC and in Figure 4 in the form of potential-pH plots. Most of the selenoaminoacids are amphoteric compounds that can be present in the forms of cations, anions, or zwitterions. Hence pH of the mobile phase determines the charge of the analytes in the selenium speciation studies [148].

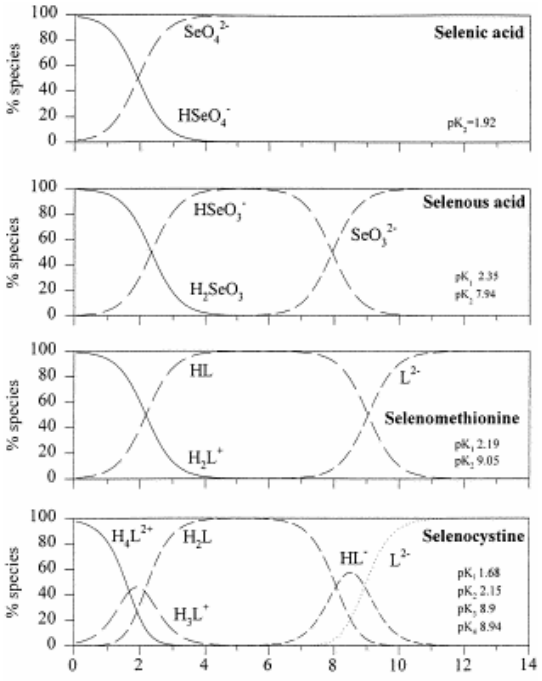

Figure 3. Species distribution diagrams of selenic acid, selenous acid, selenomethionine, selenocystine in the pH range from 0 to 14 [148].

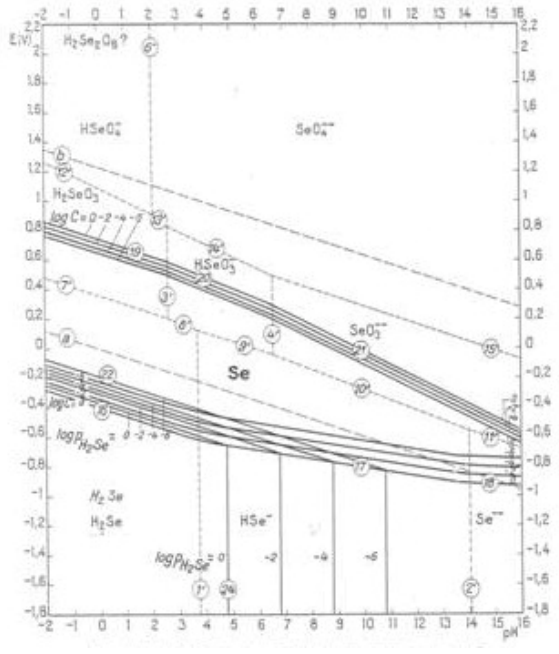

Figure 4. Potential-pH equilibrium diagram for the system selenium-water at 25.0 ^{O}C [149].

A.1.2.4.3.3.1. Anion-Exchange HPLC System

As it is seen in the Figure 3, anionic compounds of different selenium species can be obtained by changing pH of the system. Inorganic selenium species can be separated from each other in different pH medium using AE column. Citrate buffer has been widely used in AE studies. Most of the selenoaminoacids are amphoteric compounds and can be present in the forms of cations, anions, or zwitterions. Hence, pH of the mobile phase determines the charge of the analytes in the selenium speciation studies. The first proton of H_2SeO_4 is completely ionized. For this species, pK_2 value is 1.92 [148]. For H_2SeO_3, pK_1 and pK_2 values are 2.35 and 7.94, respectively. Selenomethionine has two ionizable protons. pK_1 and pK_2 values are 2.19 and 9.05, respectively. Selenocystine has 4 ionizable protons due to 2 carboxylic groups in the structure; pK values are pK_1 1.68, pK_2 2.15, pK_3 8.9 and pK_4 8.94 [148].

In literature, different mobile phases at different pH values have been used in the selenium speciation studies. Chandrasekaran et al. analyzed tube-well water samples in several parts of the Northeastern region of Indian Punjab for their Se(IV) and Se(VI) contents [150]. Ion-chromatography-ICP-MS method was developed for separation and detection of Se(IV) and Se(VI); separation of inorganic species was achieved within 11 minutes using an anion exchange column. Tirez et al. determined inorganic selenium species by ion chromatography with ICP-MS detection in microbial-treated industrial waste water [151]. In this study, speciation of the redox species was done using anion-exchange chromatography. 5 mM ammonium malonate and 100 mM NaOH+1% EtOH were separately used as mobile phases with the flow rates of 200 µL min^{-1}. Majez et al. made speciation of $SeCys_2$, SeMet, SeMeSeCys, Se(IV) and Se(VI) in leaves of different cultivars of chicory (*Cichorium intybus L.*), lamb's lettuce (*Valerianella locusta L.*), dandelion (*Taraxacum officinale Waggers*) and parsley (*Petroselinum crispum Mill.*). They used Hamilton PRP X100 column (250 mm × 4.1 mm × 10 µm) as an anion exchange column and 40 mM phosphate buffer (pH 6.0; 0.5 mL min^{-1}) as mobile phase [152]. Ochsenkuhn-Petropoulou et al. used strong anion exchange (SAX) chromatography coupled with ICP-MS for the selenium speciation in sediment. For this aim, a Dionex AS 11 (300mm x 4 mm) column was used [153]. Gradient elution was applied to obtain high separation efficiency. The eluents used in this study were *A* = 1.0 mM NaOH in 2% methanol, and *B* = 0.5 %

TMAH with the flow rate of 1.5 mL/min. McSheehy et al. applied anion exchange and reverse phase HPLC systems for the determination of methionine and selenomethionine in selenium-enriched yeast by species-specific isotope dilution with Liquid Chromatography–Mass Spectrometry and Inductively Coupled Plasma Mass Spectrometry detection [154]. In this study, ammonium acetate/acetic acid (pH 4.7) was used as mobile phase in gradient elution. Xu et al. also used anion exchange chromatography in the selenium speciation [155]. Inductively coupled plasma dynamic reaction cell-quadrupole mass spectrometry was coupled on-line with anion exchange chromatography for the quantification of selenium-tagged proteins in human plasma. Selenoprotein P, glutathione peroxidase, selenoalbumin and two unknown selenospecies (U1 and U2) in a pooled plasma sample from five healthy people were separated using anion exchange chromatography in this study [155].

Some studies about selenium speciation using anion-exchange HPLC system are given in Table 10.

Table 10. Some studies about selenium speciation using anion-exchange HPLC system.

Selenium Species	Matrix	Column used	Mobile Phase	Ref.
Selenite, selenate, selenocystine and selenomethionine	Sodium selenite supplemented feeds	Hamilton PRP-X100 column	10 mM citrate buffer (pH 5), 2 % methanol as mobile phase and a flow rate of 1.5 mL min^{-1}	156
Se(IV), $SeCN^-$ and Se(VI)	Wastewaters from an oil refinery	Polyestirene DVB copolymer	3 mM cyanuric acid adjusted with NaOH (final pH: 10.9), 2.5 mM perchlorate; 2 % acetonitrile	157
Selenomethylcysteine, selenocystine, selenite and selenomethionine	Selenious yeast tablet	AminoPac PA10 (Dionex) analytical column	Gardient elution using water, 1 M NaAc , 250 mM NaOH with the flow rate of 0.25 mL min^{-1}	158
$SeCys_2$, SeMet, Se(IV) and Se(VI)	Selenium enriched green onions (Allium fistulosum)	Hamilton PRP X-100 (4.1 mmx250 mmx10 µm)	Start buffer (A): 10 mM $NH_4H_2PO_4$, 1% (v/v) methanol, pH 5.0 Elution buffer (B): 50 mM $NH_4H_2PO_4$, 1% (v/v) methanol, pH 5.0 0–7 min: 100 % A, 7.1–21 min: 100 % B 21.1–26 min: 100 % A with flow rate of 1.5 mL min^{-1}	91
Methaneseleninic acid, selenite, selenate, methylselenocysteine, selenocystine, selenomethionine and its oxidized form	Selenium-enriched yeast and dietary supplement tablets	Hamilton PRP X-100 (4.1 mmx250 mmx10 µm)	10 mM citric acid pH 5, 2 % methanol	159

A.1.2.4.3.3.2. Cation-Exchange HPLC System

As it is seen in the Figure 3, cationic species of different selenium compounds can be obtained by changing pH of the system. Selenocystine and selenomethionine have positive charges at relatively low pH-values while selenous and selenic acids are negatively charged [148]. Hence, most of the selenium species can be separated using cation-exchange system. pH adjustment of mobile phase is very important as it is in the anion-exchange studies. In addition, some of the parameters like temperature and the ionic strength of the mobile phase that may influence the separation should be optimized to obtain good separation of the analytes using cation-exchange HPLC system [117].

Different pH values have been used in cation-exchange HPLC system. In general, pH of the mobile phase is set to value that is lower than 3 in order to obtain positively charged selenium species. Mazej et al. reported selenium speciation in leaves of chicory, dandelion, lamb's lettuce and parsley using a combination of anion and cation exchange column [152]. They determined the Se(IV), Se(VI), SeMet, SeMeSeCys and $SeCys_2$. In the cation exchange studies, 10 mM pyridine (pH 1.5; 1 mL/min) was used as mobile phase. Separated selenium species were determined using UV–HG-AFS as a detection system in optimized conditions [152]. Larsen et al. used cation exchange HPLC system for the separation of about 30 selenium species that occur in the hydrolysates [160]. In this separation, gradient elution using pyridinium formate as mobile phase was used; detections were performed using ICP-dynamic reaction cell-MS [160].

Some studies about selenium speciation using cation-exchange HPLC system are shown in Table 11.

Table 11. Some studies about selenium speciation using cation-exchange HPLC system.

Selenium Species	Matrix	Column used	Mobile Phase	Ref.
SeMet and $TMSe^{+}$	Lyophilised oysters and in their enzymatic extracts	Hamilton PRP X 200	4.0 mM pyridine formate (3 % methanol), pH=2.8	161
Selenite, selenate, selenomethionine, seneloethionine and trimethylselenonium ion	Urine	a)Silica-based LC-SCX cation-exchange column b)Gel-permeation (polyvinyl alcohol-based resin) GS-220 column	a)Mobile phase 20 m*M* pyridine, pH 1.9, flow rate 1.5 mL min^{-1} b)25 m*M* malonic acid at pH 7.9 as mobile phase	142
Se-methylselenomethionine	*Brassica juncea* roots	Strong cation-exchange column (Phenomenex Phenosphere SCX, 150 mm×3.2 mm i.d. with a 5 µm particle size)	Gradient elution; (A) 5.6 mM pyridinium formate (pH 3) and 3 % MeOH (B) 100 mM pyridinium formate (pH 3) and 3 % MeOH	162
SeMet and TMSe	Pretreated human urine	Dionex Ionpac CS5, 250 mm 64 mm id, 9 mm particle size	30 mM amonium formate, pH 3	163
Selenous acid, selenic acid, selenocystine, selenohomocystine, selenomethionine, selenoethionine, trimethylselenonium iodide, and dimethyl(3-amino-3-carboxy-1-propyl)selenonium iodide	Synthetic standard mixtures	Supelcosil LC-SCX cation-exchange column	Aqueous solutions of pyridine (20 mmol/L) in the pH range 2.0–5.7	164

A.1.2.5. Hyphenated Systems for the Determination of Selenium

In the speciation analysis, the last step is detection of analytes. Interfacing of several separation systems (HPLC, GC and CE) with proper detection systems have been used in selenium or other speciation procedures.

Chatterjee et al. coupled hydride generation AAS system with HPLC for the selenium speciation [165]. After HPLC separation, selenium hydride was produced using 0.3% of sodium tetrahydroborate in 0.2% NaOH and 3 M of hydrochloric acid. In addition, there are some other sensitive techniques including hydride generation, such as HG-atomic fluorescence spectrometry (AFS) [166,167], HG-ICP-MS [168], HG-ICP-OES [169] and HG-MIP-MS for selenium speciation [170]. In all these system, reduction of selenium species from a higher oxidation state to selenium hydride using $NaBH_4$ or other reductant are crucial [165].

GC-MS has also been used for speciation studies. Querdane and Mester studied the quantitative replacement of methionine by selenomethionine at >98% substitution, with up to 4940 µg of SeMet/g of yeast. Quantitation of SeMet and Met was performed by species-specific isotope dilution GC-MS [171].

ES-MS has also been used in literature for the detection and identification of selenium species [172,173]. Qualitative and quantitative measurements of selenium species can be done using ES-MS system. This is the main advantage of ES-MS over the other detection systems. Tastet et al. identified selenium-containing proteins in selenium-rich yeast aqueous extract by 2D gel electrophoresis, nanoHPLC–ICP MS and nanoHPLC–ESI MS/MS [174].

In literature, ICP-MS has been mostly conducted with HPLC [164, 175], GC [64, 130] and CE [116] for selenium speciation. It is stated that HPLC in conjunction with ICP–MS is known as one of the best combinations for selenium speciation. In HPLC-ICP-MS, a variation of mobile phase may be used for different separation problems. Solution carrying the separated analytes is sent to nebulizer where they are converted to fine aerosol. Larger particles are removed in spray chamber and analytes transported are ionized in the plasma. In addition, using this combination separation of organic, inorganic, and biological analytes regardless of their volatilities can be achieved. The most important advantages of HPLC-ICP-MS over other detection systems are following [133];

- Eluent flow rate in HPLC and ICP-MS (from 0.01 to 2.00 mL/min) are compatible
- Extremely low detection limits with respect to other detection systems
- Elemental specificity and wide linear dynamic range
- Fast sequential/multi element

The accuracy of the detection and all other steps in the speciation analysis should be carefully checked. The main tool in the speciation analysis for the accuracy check of each species of interest is the standard or certified reference materials. We need to analyze appropriate CRM or SRM in order to check the accuracy of the method. There are limited numbers of CRMs in the field of selenium speciation. Mester et al. certified selenized yeast reference material (SELM-1) for methionine, selenomethinone and total selenium content [176]. This CRM was produced by the Institute for National Measurement Standards, National Research Council of Canada (INMS, NRC). Certified species for SELM-1 were total selenium (2059 ± 64 mg/kg), methionine (5758 ± 277 mg/kg) and selenomethionine (3431 ± 157 mg/kg). Isotope dilution (ID) method was applied using ^{13}C-labelled SeMet and methionine [176]. In this method, different methods have been applied.

A.1.3. Selenium Studies in Egg

In literature, there are many studies about the selenium contents of different types of egg. In general, selenium concentration of egg was tried to be increased with selenium supplementation. Egg selenium content of species fed without supplementation was also studied. Golubkina and Papazyan studied the effect of egg mass of eight different avian species on Se distribution between egg components of species fed without Se supplementation [177]. Selenium in yolk, albumen, shell and shell membrane were determined separately. Se concentration in yolk–albumen complex was found to be 38.7 µg Se/100 g [177].

Sheng et al. found the selenium contents of eggs collected from supermarkets in Shanghai and Wuxi that are in along the lower reaches of the Changjiang River [178]. All the selenium measurements in egg white and yolk were done using spectrofluorometry. Concentrations of selenium were found to be in the range of 8–

16 µg per egg in the three regions. In addition, it was found that Se mainly deposited in the egg yolk [178].

Jiakui and Xiaolong made the determination of selenium in egg, blood, liver and kidney of laying hens fed with a diet containing high amounts of organic or inorganic selenium [179]. Sixty Leghorn laying hens were fed with diet including 0.23 mg Se/kg dry matter for 2 weeks. After this stage, the same basic diet without supplementation or with 0.51 mg Se/kg dry matter as sodium selenite or Se-malt was fed to hens. This study showed that by the effect of high selenium content of diet, Se content in egg, blood, liver and kidney was increased (P<0.05). Like the other studies, it was found that Se mainly deposited in the egg yolk [179].

Concentration of selenium in egg parts (egg white, yolk and shell) depends on not only the amount of selenium in diet but also the types of selenium in diets. Latshaw and Osman fed hens with and without selenite supplementation [180]. When selenite was added to diet, the selenium content of dried yolk increased. In addition, it was found that when selenomethionine was added to diet, increase in Se content was higher in egg white as compared with increase in egg yolk. By the effect of feeding selenocystine selenium in egg yolk was found to be higher than egg white. This study showed that selenocystine is metabolized to an inorganic selenium compound instead of incorporation into protein [180].

A.1.4. Selenium Studies in Selenium Supplement Tablets

Plants are the main source of selenium for human. If there is a deficiency of selenium in soil where plants are growing up, this directly affects the selenium levels in human. In this case, multi-vitamin drugs with mineral complexes or special nutritional dietary supplements have been used to supply selenium to body. Different selenium species are used in the supplements [181].

In literature, there are many studies about selenium supplement tablets. Veatch et al. analyzed Se supplement tablets (each of 15 different products representing 12 different brand names with most being sampled at two different times separated by approximately 30 months) for their selenium content [182]. Seleno-yeast and selenate were determined in 50, 100 and 200 µg /tablet dosages (seleno-yeast) and 25 and 200 µg/tablet. They found that one selenium tablet contained 2.5 times more selenium than

the stated one and supplements are less accurately labeled for their selenate content if it is compared to seleno-yeast. In addition, it was found that one of the most popular multivitamins that is labeled as 200 µg/tablet contained more than 300 µg/tablet for selenium content. Finally, it was concluded that subjects who use this supplement will be expected to exceed the 400 µg /day tolerable upper limit of intake for Se by the Institute of Medicine's Food and Nutrition Board [182].

Sun et al. applied capillary electrophoresis with direct UV detection and with a high-sensitivity detection cell for the simultaneous determination of arsenite [As(III)], arsenate [As(V)], monomethylarsonic acid, dimethylarsinic acid, selenate [Se(VI)], selenite [Se(IV)], selenocystine, selenomethionine and selenocystamine [121]. The method was applied for the speciation of arsenic in sediment and determination of Selenomethionine in a selenium nutrition supplement. In this paper, identification of selenomethionine peak was confirmed by spiking of SeMet to the sample. The SeMet concentration in this product was found as 1.81±0.57 mg/L (n=5) as Se while the label value is 2 mg/kg Se. As it is seen, RSD value was found to be high because of the fact that the concentration of SeMet in the diluted sample was close to the LOD level [121].

Wang et al. analyzed the pharmaceutical tablets for their selenium contents using either enzymatic hydrolysis or sodium dodecyl sulphate extraction [183]; ion chromatography coupled to ICP-MS was used. In the enzymatic extraction approach, proteolytic enzymes (protease/lipase) were used in a buffer containing 75 mM Tris–HCl at pH 7.5. It was found that the extraction recovery was more than 80% of total Se for pharmaceutical tablets where selenocystine, Se-methyl-selenocysteine and selenomethionine were extracted while chemical extraction of Se using SDS gave only 60% of total Se. It was found that the major Se species in the three types of selenium tablets were SeMet and $SeCys_2$. The lowest level of SeMet was found in Se-no-yeast tablets. Tablets of SeMC™ gave 37% Se-methyl-selenocysteine that is an important organically-bound Se source [183].

Goenaga-Infante et al. reported an international intercomparison study (CCQM-P86) to assess the analytical capabilities of national metrology institutes and selected expert laboratories worldwide to accurately quantitate the mass fraction of selenomethionine and total Se in pharmaceutical tablets of selenised-yeast supplements (produced by Pharma Nord, Denmark) [184].

In each selenium supplement tablet, different amounts of selenium are labeled, but some of these labels are not compatible with the experimental results of analysis. For instance, Sutton et al. analyzed ten commercially available dietary selenium supplements using a chiral column to identify and quantify the selenium species present [185]. ICP-MS detection was used in the identification. Total amounts of selenium in the samples were also determined in this study. All of the supplements they analyzed were in tablet form and the bottles gave the statements as to the source, species and quantity of the selenium in the tablets. None of the selenium results were matching with the labeled values. Most of the results found for the supplements were different in terms of either label species or label concentration. For the some brands, not only its species but also the amount was found to be completely different than the label information [185]. In a similar study, B'Hymer and Caruso analyzed six different brands of yeast-based selenium food supplements taken from local stores for their total selenium content and content uniformity; microwave nitric acid digestion was used for sample decomposition. These supplements were analyzed for the determination of the selenomethionine, a natural product of yeast when inoculated with selenium. It was found that six different brands of selenium supplements have near total selenium content with label values while each brand had dramatically different profiles for their selenium content in the supplement. Amoung the 6 brands, 2 brands had high levels of selenomethionine. In one brand, all selenium was found as inorganic selenium while half of total selenium was found in the inorganic form in another brand. In addition, recoveries of selenium species in two different brands were found to be low. This paper shows that labels in tablets showing the amount of selenium species may be inaccurate [186].

A.1.5. Poultry

A.1.5.1. Selenium in Poultry

Selenium is a very important element for poultry like other living organisms. Poultry animals take the selenium via feed. In general, feedstuffs for poultry are divided into two groups; a) Natural feedstuffs including corn or soybean meal b) Supplemental sources including sodium selenite. In the natural feedstuff, concentration of selenium

depends on not only the concentration of selenium in soil where plant grows up but also the plant type. Each plant has a different ability to accumulate selenium. If the accumulation of selenium is high in a plant, this is called as a Se-accumulator plant such as species of *Astragalus* and *Neptunia*. Although Se concentration is over 1,000 ppm in these types of plants, they are not commonly used in poultry diets. Selenium non-accumulator plants including corn, wheat, or oats are commonly used for animal diets [187]. It is reported that concentration of selenium that is mainly selenoamino acids, SeMet and SeCys, in natural animal feed ranges between 0.03-0.12 mg/kg. In order to eliminate any possible health problems caused by selenium deficiency, the feedstuffs are widely supplemented with various selenium sources. With selenium supplementation, the final Se levels reach 0.3 to 0.5 mg kg^{-1}. In general, organic and inorganic selenium, selenite or selenate, have been used for supplementation. Although the inorganic selenium species such as sodium selenite are still widely used in Central European countries, organic selenium supplementation has many advantages [188]. Advantages of organic selenium supplementation can be summarized as follows [189];

-Improved fertility
-Improved sperm quality
-Reduction in mortality, particularly in the first 10 days
-Improved feathering
-Improvement in egg color
-Better egg storage

Selenium is transferred to yolk for offspring. It is reported that supplying selenium via selenium containing yeast cause the enhancement in Se content in the form of SeMet in meat, milk and eggs. Hence, selenium supplementation is mostly done using SeMet [190].

A.1.5.2. Selenium Deficiency in Poultry

In the case of selenium deficiency, several detrimental conditions may appear. Exudative diathesis, pancreatic fibrosis and impaired reproduction are some of them [175]. Exudative diathesis is characterized by a general edema due to a typical permeability of the capillary walls. Pancreatic fibrosis causes atrophy of the pancreas, as well as poor growth. Impaired reproduction appears in female poultry. In that case,

egg production and hatchability were reduced in laying hens fed diets with reduced levels of Se. Some of these Se deficiency diseases like exudative diathesis and pancreatic fibrosis are Se form dependent that is needed to alleviate their deficiency signs [187]. In addition, it is reported that in the case of marginal deficiency of Se a delayed development of immune system of young chicks may occur. Hence, subsequent energy losses, increased number of dead birds, reduced egg production, and increased embryonic mortality may appear [188]. Hence, form of the selenium in the feed of poultry is very important.

Se deficiency diseases are summarized as follows [190];

-Pancreatic entropy in chicks
-Muscular dystrophy in chicks
-Reduced egg production
-Increased dead-in-shell chicks
-Low chick weights at hatching
-Retained placenta and weak or stillborn calves.

A.1.5.3. Selenium Studies in Poultry

There are many approaches to improve the selenium status of human. In general, natural foods containing relatively high selenium content have been recommended [191]. Selenium enriched yeast used for the production of bread can be used for this aim. Actually, this approach is very useful due to high consumption of bread in many countries including Turkey. In some countries, selenium supplemented products have been added to wheat flour to improve the selenium content of bread. For instance, Se-enriched mushroom extract is added to wheat flour by fortification. Actually, it is very difficult to eliminate the global Se deficiency. Poultry industry is another way to minimize global Se deficiency. Se-enriched eggs and meat have been produced to minimize this problem [191].

Speciation studies of selenium in the chicken samples are still scarce in literature due to difficulties in the determination. Speciation of selenium needs suitable and sensitive analytical methods. Bierla et al. has developed a method for the simultaneous determination of selenomethionine and selenocysteine in a variety of meat samples including chicken and lamb muscles and different offal tissues such as

heart, liver and kidney [192]. Purification was done using size-exclusion liquid chromatography. Ion-paring reversed-phase HPLC–ICP-MS was applied to make speciation analysis. The method developed was applied to selenoproteins containing SeCys and other Se-containing proteins including SeMet in tissues of animals during supplementation studies (dose–effect and tolerance) [192]. In the tolerance study, animals were fed a diet containing 6.9 mg/kg Se for 3 months. Concentrations of both total Se and selenoaminoacids were measured after 3 months and after the following 42 days. In the dose-effect study the animals were fed a diet with total selenium in the range from 0.20 to 0.45 mg/kg in the dry weight for 112 days [192].

Daun et al. studied about molecular weight distribution in the water-soluble fraction of muscles of chicken, turkey, duck, ostrich, lamb, cattle and pig [193]. Size-exclusion chromatography–ICP-MS was used throughout the study. In this study, glutathione peroxidase and selenoprotein W were claimed to account for 70–100% of the water-soluble selenium [193].

In literature, there are many studies about improvement of selenium content in poultry using different methods. Sevcikova et al. studied the effect of dietary supplementation of selenium in organic form on performance, carcass traits and selenium content in tissues of broiler cockerels Ross 308 [194]. In this study, broilers were divided into 3 groups; Group 1: Control, Group 2: Se-enriched yeast group (0.3 mg Se/kg) and Group 3: Se-enriched alga Chlorella (0.3 mg Se/kg). This study showed that with the selenium supplementation in both experiment groups increased mortality. Indeed, Se-enriched alga was found as the best feed conversion. In breast and thigh muscles of broiler fed with selenium enriched food, selenium concentrations were found to be higher than the control group. Selenium content of the broiler chickens fed with Chlorella was higher as compared to the group fed with Se-enriched yeast. In addition, selenium content in liver was found to be higher in both experimental groups as compared to the control values [194].

Yoon et al. studied the effects of the source and concentration of Se on growth performance and Se retention [195]. For this aim, 360 one-day-old Jumbo Cornish Cross broiler chicks were used. Broilers were divided into different groups fed with diet containing 0.0, 0.1, 0.2 and 0.3 ppm of supplemental Se from SelenoSource AF (Se yeast A, Diamond V Mills, Cedar Rapids, IA), 0.3 ppm of Se from Sel-Plex (Se yeast B, Alltech, Nicholasville, KY), or 0.3 ppm of Se from sodium selenite. Starter diets were fed

for the first 21 d (6 replicates of 10 broilers per treatment). This study shows that selenium supplementation did not affect the growth performance of broilers. Indeed, concentration of selenium in blood and GPX activities increased ($P < 0.05$) with the higher concentration of selenium in diet. Selenium retention in organic supplementation was found to be greater when compared with inorganic Se. In this paper, it was concluded that bioavailability of Se from the organic source is higher than sodium selenite [195].

Singh et al. studied the effects of selenium and vitamin E supplementation on some immune parameters in broilers [196]. For this purpose, broilers were fed with diets containing different concentrations of vitamin E (0-200 mg/kg) and selenium (0-0.2 mg/kg). Selenium and vitamin E were given either alone or in combinations from 1 to 42 day of age. Heavier spleen was observed in the chicks given 200 mg vitamin E/kg and 0.2 mg/kg selenium. In addition, it was observed that vitamin E and selenium have synergistic effects on immune responses [196].

Pappas et al. studied about effect of selenium intake on progeny of female chicken [197]. In this study, hens were fed with control or Se-supplemented diets, containing 0.027 and 0.419 µg Se/g, respectively. Using a diet containing 0.419 µg/g selenium, selenium content in hens' eggs was elevated 7.1-fold. In addition, selenium content of liver, breast muscle and whole blood of the chicks originating from the high-Se parents were, respectively, found to be 5.4, 4.3 and 7.7-fold higher than the chicks originating from low-Se parents. Glutathione peroxidase activity of tissue was higher in chicks originating from the high-Se parents for 2–4 weeks post-hatch [197].

Turker et al. studied not only the effect of selenium-enriched feed on the selenium content of chicken meat and eggs but also the effect of digestion procedures on the determination [198]. In this study, hydride-generation atomic absorption spectrometry was used in the detection step. It was figured out that selenium content of both chicken meat and eggs fed with selenium fortified feed was higher than those fed without fortified feed [198].

PART A

CHAPTER 2

A.2. EXPERIMENTAL

A.2.1. Reagents and Apparatus

All the chemicals used in all parts of this study were of analytical grade or high purity. While the stock solutions of seleno-DL-methionine (Sigma, SIS 3875) and seleno-DL-cystine (Sigma, SIS 1650) were prepared in 0.1 M HCl, stock solutions of Se(IV) (Aldrich, seleniumdioxide, 99.8 %) and Se(VI) (Ventron, sodium selenate) were prepared in de-ionized water. For the further dilutions of all these standards, de-ionized pure water from a Milli-Q Water Purification System was used. All the standards were prepared daily. Pepsin (Merck, 107185), Trypsin (Pancreas Protease) (Merck, 108367), Bacillus Subtilis (Sigma, SIB 4006), Driselase (Sigma, SID 9515), Pancreatin (Sigma, SIALP 1750), Proteinase K (Sigma, SIP 2308), Protease XIV (Sigma, SIP 5147) were used as enzymes in the extraction step and stored at -20 ^{O}C. Phenylmethanesulfonylfluoride (Fluka, FL 78830), Tris (hydroxymethyl)amino methane hydrochloride (Fluka, FL93363), Tetramethy ammonium hydroxidepentahydride (Sigma, SIT 7505), Ammonium acetate (Fluka, FL 73594), Sodiumdodecylsulfate (Sigma, L4509) were also used in the extraction step as chemicals. Argon used in ICP-MS for the production of plasma was supplied from Habaş Industrial and Medical Products, Ankara. All glass apparatus have been kept permanently full of 2.0 M nitric acid when not in use.

In the HPLC studies, heptafluorobutyric acid (Aldrich, AL 164194), trifluoroacetic acid (Fluka, FL 73645), methanol (Merck), pyridine (Riedel, RH 16037), citric acid (Sigma, SIAL 251275) were used as reagents in mobile phases.

A.2.2. Instrumentation

A Heto FD 8 freeze-drying unit was used to dry samples. Samples were firstly keep at -85 ^{0}C throughout 24 hours, and then sample was located in freeze-drying instrument where temperature was adjusted to -55 ^{0}C. Drying process was continued throughout 48 hours.

Dionex HPLC system equipped with a binary HPLC pump was used. Gradient elution was possible using this instrument. The HPLC system was connected to ICP-MS using 85.0 cm tubing having 1.0668 mm i.d. and 1.6764 mm o.d. This tubing connects the output of HPLC column and the nebulizer of ICP-MS. Anion exchange (Spheris S5 SAX), C18 (Dionex C18), C8 (Alltima C8) and Cation exchange (Spheris S5 SCX) columns were used for the separation of 4 selenium species, selenite, selenate, selenomethionine and selenocystine.

Selenium species were analyzed using Thermo X Series ICP-MS system where there is no collision cell technology for the elimination of spectral interferences on ^{80}Se caused by ^{80}ArAr (^{40}Ar^{40}Ar).

Ethos Plus Milestone microwave oven system equipped with temperature controller unit was used to digest the samples for the determination of total selenium in samples.

Millipore Stirred ultrafiltration cell (8400 Model) was used to filtrate extracts. This cell contains a cap (Nylon), membrane holder (Polysulfone), body (Polysulfone), magnetic stirrer assembly (Acetal, polysulfone), retaining stand (Nylon), O-rings (Silicone rubber) and tube fitting assembly (Nylon). Cell capacity is 400 mL. Maximum operating pressure should be lower than 75 psi (5.3 kg/cm^2). Pure argon gas was used for pressurizing the cell because compressed air can cause large pH shifts due to dissolution of carbon dioxide. Hence, in the solutions, oxidation may also occur, leading to conversion of analytes to each other. 10.0 KDa ultrafiltrasyon membrane (Filter Code: YM10 Dia: 63.5 mm, 28.7 cm^2) was used in the filtration of extraction solutions. This filter is made from polyethersulfone. Stirred Ultrafiltration Cell used in the study can be seen in Figure 5.

In addition to these instruments, Elma, Elmasonic S 40 H brand sonication instrument and shaker were used in the extraction studies. Sigma 2-16 (D-37520

Osterode am Harz, Germany) brand ultracentrifuge instrument was used to separate supernatant.

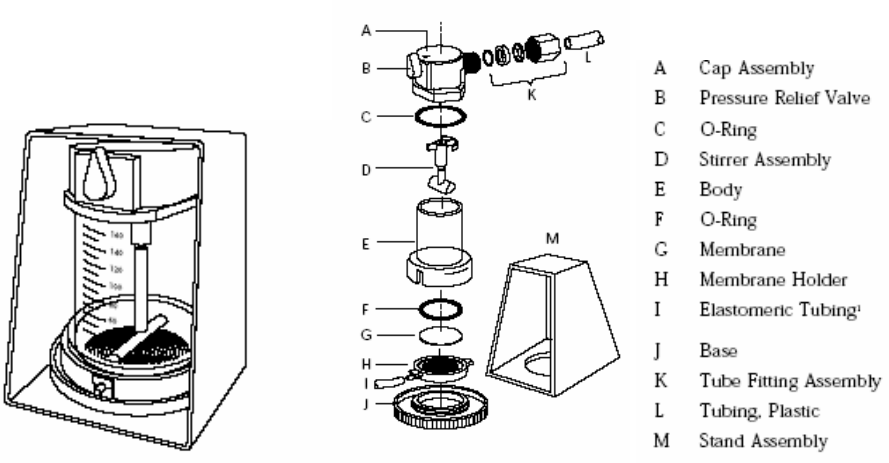

Figure 5. Stirred Ultrafiltration Cell used in the study [199].

A.2.3. Sample Treatment

Egg which was produced by chicken fed using selenium-added food was taken from market place in Ankara. In the egg studies, egg samples were broken to separate yolk and white part from shell. In the chicken studies, chicken buttock and chicken breast samples were taken from chicken fed with selenium-added food in the marketplace of Kayseri and Bursa. Breast and buttock samples were transported to laboratory in bag where the temperature was about 0 ^{O}C. Samples were firstly washed with de-ionized water and then cut using a blender where a titanium blade was used. In the selenium supplement chicken study, chicken were taken from Bursa. Same procedure with other chicken samples was applied to all these samples.

A.2.3.1. Freeze-Drying Unit

Egg yolk and white mixture were first kept at -85 ^{0}C throughout 24 hours, and then sample was placed in freeze-drying instrument where temperature was adjusted to -55 ^{0}C. Drying process was continued for 48 hours. After the freeze-drying process, lyophilized egg samples were powdered in order to increase surface area and then placed in desiccator to protect from moisture.

In chicken studies, chopped samples were kept at -85 ^{O}C for 24 hours, and then they were located in freeze-drying instrument where temperature was adjusted to -55 ^{O}C. Drying process was continued for 36 hours. After the freeze-drying process, lyophilized breast and buttock samples were powdered in mortar in order to increase surface area and then placed in desiccator to protect from moisture. The same method was applied to dry up the chicken breast samples throughout this thesis.

A.2.3.2. HPLC System

Chromatographic conditions including flow rate of mobile phase, mobile phase composition, pH of mobile phase were optimized to obtain a good separation of analytes. C18 (Dionex C18) and C8 (Alltima C8) were chosen as columns for the analysis of the selenoamino acids; heptafluorobutyricacid (HFBA) and trifluoroaceticacid acid (TFA) were selected as the ion-pairing reagents. HFBA and TFA were used as a mobile phase at natural pH. Cation exchange (Spheris S5 SCX) HPLC system was used for the separation of selenium species, selenite, selenate, selenomethionine and selenocystine. Pyridine in different concentration buffered to different pH was used as the mobile phase in cation exchange HPLC system. Inorganic selenium species were also tried to be separated using anion exchange (Spheris S5 SAX) HPLC system. Citrate buffer containing CH_3OH was used as a mobile phase in anion exchange system.

A.2.3.3. ICP-MS System

In ICP-MS system, the Protective Ion Extraction and Infinity II ion optics, based upon a hexapole design with chicane ion deflector was used to have a low background. Peltier cooling system was used to improve the S/N ratio by cooling the spray chamber. The simultaneous analog/PC detector with real time multi-channel analyzer electronics was used for both continuous and transient signal analysis. The instrument and accessories are fully computer controlled using PlasmaLab software. Extraction Lens Voltage, Lens 1 Voltage, Lens 2 Voltage Focal Lens Voltage, 1. Diffraction Aperture Voltage, 2. Diffraction Aperture Voltage, Quadrupole Voltage, Hegzapole Voltage, Argon Flow Rate in Nebulizer, Lens 3 Voltage, Horizontal Position of Torch, Vertical

Position of Torch, 3. Diffraction Aperture Voltage, Argon Flow Rate to Cool Torch, Argon Flow Rate to Produce Plasma, Sampling Depth and Forward Power were optimized to obtain best sensitivity for selenium. Dwell time was used as 10.0 ms in the measurements.

Singly charged cations of selenium isotopes ^{74}Se ^{76}Se ^{77}Se, ^{78}Se and ^{82}Se were monitored during all measurements. In all calculations throughout this thesis, peak area (ICPS) values were used. Selenium was determined using masses of 78 and 82 to check whether spectral interferences appeared on one of the isotope traces. In the extraction studies, tris HCl was used, so there might be some interference in $^{82}Se^+$ due to $^{12}C^{35}Cl_2^+$. Another possible interference for this mass is $^{81}Br^1H^+$. In addition, it is known that $^{40}Ar^{38}Ar^+$ has interference effect for $^{78}Se^+$ [117]. In the $^{78}Se^+$, effect of $^{40}Ar^{38}Ar^+$ is constant, but should be checked by calculating isotopic ratios. In all analyses, baselines of selenium signals were controlled. In addition, $^{78}Se/^{82}Se$ ratio was measured and compared with ratio of natural abundances of these isotopes. In all measurements, it was found that there were no big changes between experimental and theoretical isotopic ratios (lower than 3%). This shows that there were no detectable interferences on these isotopes and ^{78}Se can be used in all measurements. ^{82}Se was mostly used in calculations; another calibration curve was also obtained using ^{78}Se to check if there is any interference. There was no change observed in both results obtained by using ^{78}Se and ^{82}Se. This also indicates that there may be no interferences in both of these isotopes.

A.2.3.4. Microwave Digestion System

In the reference cell, temperature at the inside of cell is measured using thermocouple to control the inside temperature. Temperature program and acid mixture used were optimized to obtain an effective digestion. HNO_3-H_2O_2 (1+1, volume) was used as acid mixture. In the optimization of microwave conditions, 0.30 g sample was used. Temperature program used in the digestion can be seen in Table 12.

Table 12. Temperature program of microwave digestion system for the egg and chicken breast samples.

Period, Minute	Temperature, ^{0}C
5	↑ 100
10	→ 100
5	↑ 150
10	→ 150
5	Ventilation

A.2.4. Samples

In the egg analyses, 3 different brands were bought from markets in Ankara. It was claimed that two of these brands contained high amount of selenium because chickens produced these eggs were fed with selenium enriched diets. One brand that is not claimed to have high amount of selenium was also analyzed for its selenium content as control sample.

In the vitamin tablet study, six brands of selenium supplement tablet were analyzed for their selenium contents. They were purchased from a drug store. Statement value on the tablets shows the total selenium amount in the samples. 5 brands claim that there must be selenomethionine in the tablets while 1 brand states that selenium presents in the tablets in the form of Se(VI).

In the chicken studies, all of the optimization was performed using the samples taken from Bursa (2 samples) and Kayseri (2 samples). Samples were sent from Bursa and Kayseri market place and they claim that these chicken samples contained high amount of selenium. Chickens were cut in Bursa and Kayseri, and breast and buttock parts of chickens were taken. Samples were transported to METU Chemistry Laboratory in plastic bag while the temperature was kept at about 0 ^{O}C.

In inorganic and organic selenium fed chicken study, chicks were randomly categorized into three groups including control group, inorganic selenium fed group and organic selenium fed group. 225 chicks (Ross 508) were brought up in this study. Each group contained 5 sub-experiment groups each containing 15 chicks. 5 chicks were randomly selected in each sub-experiment group and analyzed for their selenium

species content. Hence, 75 chicks were analyzed. Control group premix contained no selenium. For the inorganic selenium fed group, chickens were fed with the diet containing 0.15 mg Se/kg in the form of Na_2SeO_3. In addition, for the organic selenium fed group, chickens were fed with a diet containing 0.15 mg/kg Se(selenomethionine) in Sel-plex. For the homogenization of the selenium in diet, selenium species were firstly added to premix; after the homogenization, premix was mixed with basal diet to obtain 1000 kg of final diet. Sel-Plex contains organic selenium yeast produced by Saccharomyces cerevisiae CNCM I-3060. In literature, Sel-Plex has been widely used. Selenium in Sel-Plex is more digestible and better retained; this allows chickens to build nutrient reserves [200].

Newly hatched chicks were grown up throughout 6 weeks (42 days) in Vocational School at Balıkesir University. Newly hatched chicks were brought up at coop where the temperature, humidity and light were optimum. NRC standards were applied throughout growing up. In the period of 0-3 weeks, starter diet was used for feeding. The content of diet used in feed of chicks can be seen in Table 13 and Table 14. At the end of 3^{rd} week, grower diet was administrated until the end of 6^{th} week. At the end of 42^{nd} day, chickens were cut in a clean room of Vocational School at Balıkesir University, and breast and buttock parts of chickens were taken. Samples were transported to METU Chemistry Laboratory in plastic bag while the temperature was kept at about 0 ^{O}C.

Table 13. Content of premix used in feed of chicks.

Content	In 2500 g of Premix
Vitamin A	15000000 IU
Vitamin D3	3000000 IU
Vitamin E	50000 mg
Vitamin K3	4000 mg
Vitamin B1	3000 mg
Vitamin B2	6000 mg
Vitamin B6	5000 mg
Vitamin B12	30 mg
Niacin	40000 mg
Cal-D-Pant	15000 mg
Folic Acid	1000 mg
D-Biotin	75 mg
Choline Chloride	400000 mg
Vitamin C	50000 mg
MnO	80000 mg
$FeSO_4$	60000 mg
ZnO	60000 mg
$CuSO_4$	5000 mg
Iyodine	2000 mg
Cobalt	500 mg
Aroma	25000 mg

Table 14. Structure and composition of basal rations (basic diet).

	% (w/w) Ingredient	
Raw materials	Starter (0-3 week)	Grower (4-6 week)
Corn	51.65	57.85
Soybean meal, 46%	17.65	14.05
Full fat soybean, 18%	15.00	11.05
Meat bone meal, 30%	3.00	3.00
Poultry meal, 50 %	3.50	3.50
Sunflower meal, 34 %	2.50	--
Boncalit	2.50	5.00
Acid oil	1.73	3.00
Animal fat	--	1.00
Limestone	0.41	0.275
Salt	0.24	0.205
Sodium bi carbonate	0.23	0.20
Vit Min premix[1]	0.25	0.25
Mono calcium phosphate, 22.7%	0.35	0.25
L- Lysine	0.375	0.09
L- Thrionine	0.085	--
Liquid Methionine, 88%	0.365	0.145
Anticoccidial	0.050	0.05
Phytase	0.115	0.085
TOTAL	100 %	100 %
Calculated Values		
Metabolizable energy, kcal/kg	3068	3155
Crude Protein (CP), %	22.50	21.00
Methionine, %	0.60	0.50
Methionine+Cysteine, %	0.94	0.80
Lysine, %	1.35	1.11
Calcium, %	0.90	0.80
Phosphorus, %	0.43	0.37
Analysis Values, %		
Dry matter	87.64	87.71
Crude protein	22.30	21.10
Crude fat	4.34	4.94
Crude cellulose	2.55	2.50
Crude ash	5.70	6.15

PART A

CHAPTER 3

A.3. RESULTS and DISCUSSION

A.3.1. Optimization of ICP-MS Parameters

ICP-MS conditions were optimized in order to improve the sensitivity of ICP-MS for selenium determination. For this aim, 100.0 ng/mL of Se(IV) prepared from Na_2SeO_3 was used. In the optimization procedure, one parameter was optimized while others were kept constant. In the first part of optimization, estimated parameters were selected and then optimizations were repeated until finding the best results. Sensitivity of system was checked before measurements. In the case of any significant reduction in the sensitivity, ICP-MS parameters were re-optimized. Any reduction of sensitivity higher than 30% warranted a new optimization; for smaller variations recalibration was used. Baseline of the selenium varied in the case of changing in HPLC and ICP-MS parameters. Throughout the all studies, cones of ICP-MS system were periodically cleaned to eliminate clogging possibility. Typical optimization results for ICP-MS can be seen in Table 15.

Table 15. Optimization results of ICP-MS.

Parameter	Optimum Value
Extraction Lens Voltage, V	3.0
Lens 1 Voltage, V	-220.0
Lens 1 Voltage, V	-21.2
Focus Lens Voltage, V	17.0
1. Diffraction Aperture Voltage, V	-41.6
2. Diffraction Aperture Voltage, V	-151.0
Quadrupole Voltage, V	-3.5
Hegzapole Voltage , V	5.1
Argon Flow Rate in Nebulizer, L/min	0.85
Lens 3 Voltage, V	-197.6
Horizontal Position of Torch	63.0
Vertical Position of Torch	619.0
3. Diffraction Aperture Voltage, V	-30.6
Argon Flow Rate to Cool Torch, L/min	13.0
Argon Flow Rate to Produce Plasma, L/min	0.9
Sampling Depth, relative units	100
Forward Power, W	1400

In literature, different optimization parameters for ICP-MS instrument have been used for the selenium speciation [132, 145, 157]. Differences in the optimization results depend on not only the instrument used but also the matrix of sample.

After obtaining optimum values for the ICP-MS, sample volume used in ICP-MS measurement was optimized. In order to have minimized sample consumption and a minimum contamination of especially MS part, flow injection (FI) was used. It is known that one of the factors that affects the lifetime of cones is the sample volume injected. Hence, using this optimization procedure, it was aimed to minimize the sample volume. For this aim, 0.10, 0.25, 0.50 and 1.00 mL loops were prepared. Performance of each loop was determined using 1.00 mg/L of Se(IV). The continuous signal of 1.00 mg/L of

Se(IV) in ICP-MS is seen in Figure 6. Using this signal, we tried to decide the proper loop size for Se determination in ICP-MS.

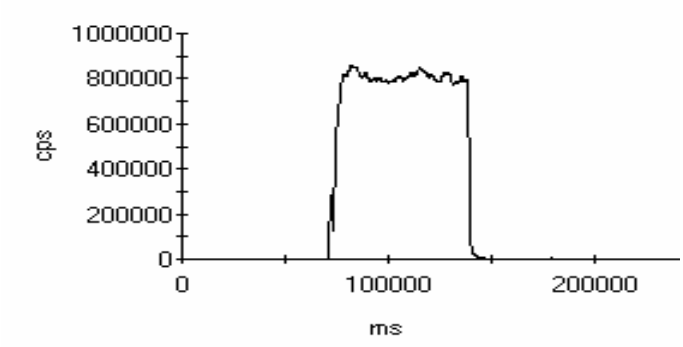

Figure 6. ICP-MS signal of 1.00 mg/L Se(IV) in continuous flow.

Although sensitivity of signal obtained by using 0.25 mL of loop is 15% lower than signal obtained in continuous flow, 0.25 mL of sample volume was selected in the further studies due to considering shape and small sample volume. As it is known, many different organic solvent are used in the extraction process. Hence, a sample volume of 0.25 mL was selected in order to minimize contamination of cones due to high organic content of matrix.

A.3.2. Freeze–Drying of Samples

Freeze-drying procedure given in “A.2.3.1.” part was applied to samples. After drying process, water content of egg was calculated using initial and final weights of samples. Water content of egg samples is given in Table 16.

Table 16. Water content of egg (N=3).

Sample	Amount
Total egg, g	58.548 ± 2.563
Egg shell, g	7.654 ± 1.231
Egg White+yolk, g	50.894 ± 2.104
After lyophilization, g	11.516 ± 0.931
Water content for egg white and yolk %	77.37 ± 3.13

A.3.3. Determination of Total Selenium in Egg

In order to determine the relative extraction efficiencies in the further studies, total Se content of egg was determined. For this aim, different acid or acid mixtures in different volumes were used for digestion. These were added to freeze dried egg sample to find the best digestion procedure. Reagents used for digestion of egg samples are shown in Table 17.

Table 17. Acid and acid mixtures used in digestion procedure.

Egg used	Reagent
0.306 g	8 mL of HNO_3 (Conc.)
0.302 g	8 mL of (HNO_3+H_2O_2) (1+1, volume) (Conc. Reagents)
0.306 g	8 mL of (HNO_3+H_2O_2) (2+1, volume) (Conc. Reagents)

Proper amounts of freeze-dried egg sample shown in Table 17 were digested by using the microwave oven system. Temperature program of microwave oven is shown in Table 18.

Table 18. Temperature program of microwave oven for egg samples.

Time, Minute	Temperature, ^{0}C
5	100 ↑
5	100 →
5	150 ↑
5	150 →
5	180 ↑
10	180 →
5	Cool

After the digestion procedure, solutions cooled to room temperature were diluted to 10.0 mL and then put into PTFE containers. For each digestion procedure, 3 solid sample replications were applied.

In the optimum conditions of ICP-MS, linear calibration plot shown in Figure 7 for Se was obtained by using 0.25 mL of standard solutions. In the calibration plot, peak area (ICPS) of signal was used.

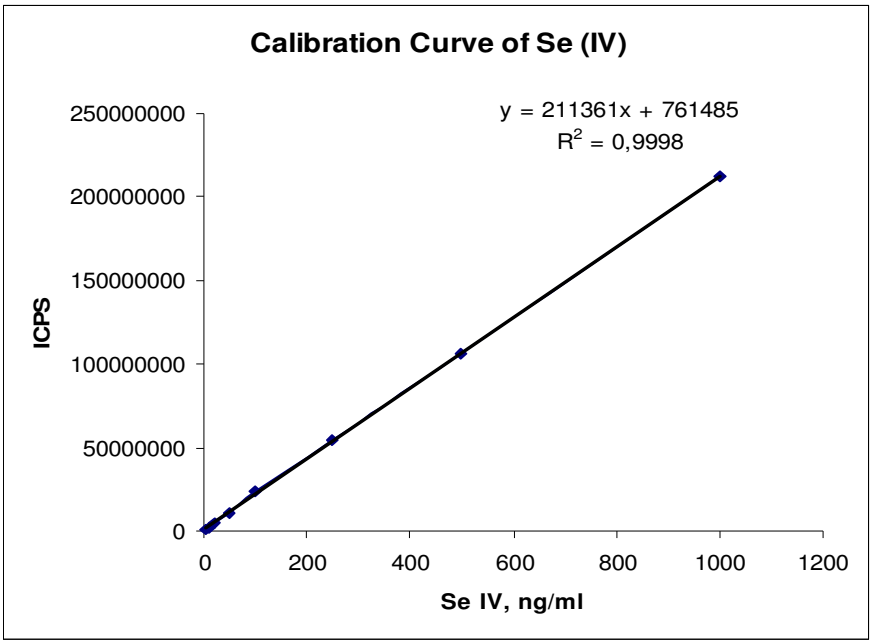

Figure 7. Linear calibration plot of Se(IV) by monitoring ^{78}Se.

Digested egg samples were analyzed using ICP-MS and Se content of egg were calculated using the linear calibration plot. Se results of egg obtained using different digestion procedures are seen in Table 19.

Table 19. Total amount of Se in egg samples using different acid or acid mixtures.

Procedure	Acid or Acid Mixture	Se, ng/g (Dry) (N=2)
1	8.0 mL of HNO_3 (Con.)	677 ± 20
2	8.0 mL of (HNO_3+H_2O_2) (1+1, volume) (Con.)	816 ± 25
3	8.0 mL of (HNO_3+H_2O_2) (2+1, volume) (Con.)	752 ± 23

As seen in Table 19, 1+1 mixture of HNO_3-H_2O_2 gives a higher result as compared with others. In order to check the accuracy of results, 1566b Oyster Tissue as an CRM was digested by using the digestion procedure 2. Concentration of Se in 1566b Oyster Tissue was found to be around 25% lower than the certified value. Same

experiments were performed using a different microwave oven in a different laboratory to find out whether problem is due to microwave instrument in our laboratory or not. For the CRM sample, selenium concentration was found to be lower than certified value. In addition, a different temperature program was tried. In the new digestion procedure, 10.0 mL of HNO_3-H_2O_2 (1+1, volume) was used. Temperature program used in new digestion procedure is shown in Table 20.

Table 20. New temperature program of microwave oven.

Time, Minute	Temperature, ℃
5	100 ↑
10	100 →
5	150 ↑
10	150 →
5	Cool

Using the temperature program shown in Table 20, 1566b Oyster Tissue as an CRM and lyophilized egg samples were digested and analyzed by ICP-MS. Direct calibration method and standard addition method were applied to the egg samples. It was observed that the slope of standard addition method was different from the slope of direct calibration plot. In order to find the source of this problem some experiments were performed. For this purpose, aqueous selenium standard,100 ng/mL, was digested with the same acid mixture, 10.0 mL of HNO_3-H_2O_2 (1+1) (Con.), and then analyzed using ICP-MS. The same concentration of selenium was also prepared in HNO_3-H_2O_2 (1/1) without digestion. It was observed that a lower ICPS value was obtained using the digested selenium standard. ICPS value of selenium prepared in HNO_3-H_2O_2 (1/1) was also found to be lower than that obtained selenium standard prepared in water. This shows that decrases in selenium signal is caused by acid mixture used. Hence, in the further studies, standard addition method was applied to samples.

Results for CRM and lyophilized egg sample using the optimum parameters and standard addition method can be seen in Table 21.

Table 21. Se results SRM and lyophilized egg sample after digestion procedure.

Sample	Acid Mixture	Certified Result, mg/kg	Result Found, N=3, mg/kg
1566b Oyster Tissue	10.0 mL HNO_3-H_2O_2 (1/1)	2.06 ± 0.15	1.93 ± 0.08
Egg	10.0 mL HNO_3-H_2O_2 (1/1)	-	1.19 ± 0.05

As it is clear in Table 21, Se result obtained using new temperature program is in good agreement with certified one.

In the selenium supplement tablet study, a method that was used in literature was applied for the total determination of selenium in the tablets [185]. In this method, tablets from each supplement brand were digested using concentrated nitric acid for the quantification of total selenium. Each supplement tablet was placed in a beaker and 30 mL of concentrated HNO_3 was added. Samples were heated to boiling on a hot-plate. The samples were then kept at the boiling-point for 2.0 h. At the end of digestion procedure, samples were filtered using 0.45 micron nylon filters. The solution was then made up to 100.0 mL using de-ionized water. Standard addition method was applied to sample to eliminate the possible matrix interferences. Three points were used in the standard addition method. In the standard addition method, Se(VI) was used because all the selenium species should be converted to highest oxidation state after HNO_3 digestion protocol. Standard addition calibration plot for Brand B can be seen in Figure 8.

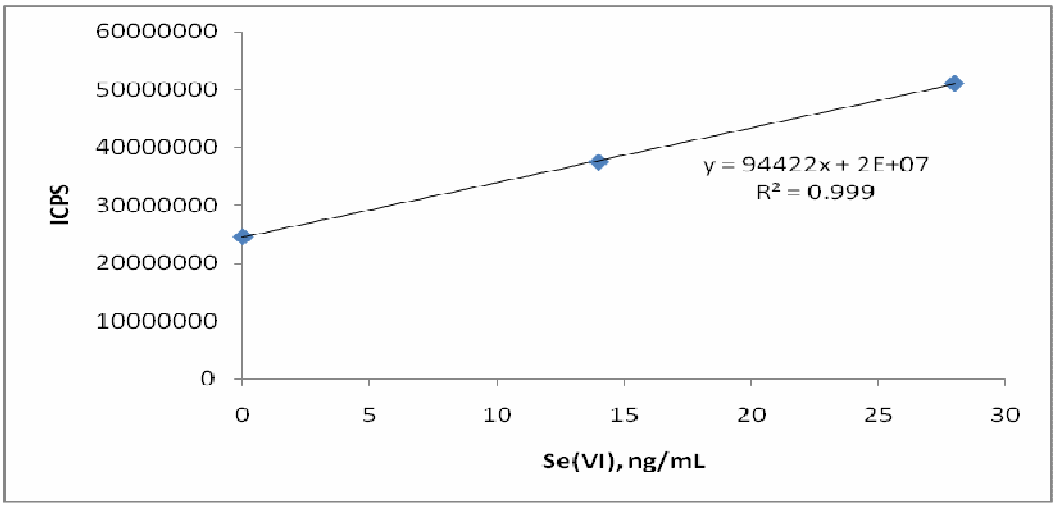

Figure 8. Standard addition calibration plot of Brand B for total selenium determination by monitoring ^{82}Se.

As seen in the figure, R^2 value is very close to 1.00. All selenium results for supplement tablets are given in Table 40.

The same procedure with egg samples given above was also applied the chicken breast samples for the determination of total selenium concentration. Lyophilized chicken breast samples were digested using HNO_3-H_2O_2 (1/1) as acid mixture. Clear solutions obtained after digestion procedure were analyzed using ICP-MS. It was observed that the slope of standard addition method was different from slope of direct calibration plot. Hence, standard addition method was applied to samples to eliminate interferences coming from matrix. Signal profiles of one of the chicken samples obtained using standard addition method can be seen in Figure 9.

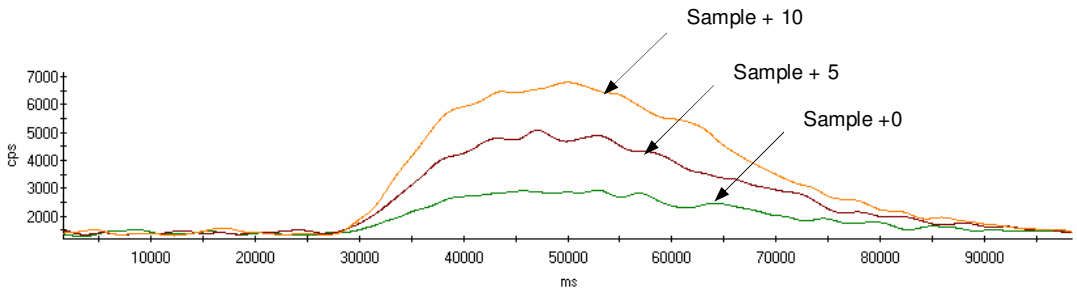

Figure 9. Signals of one of the chicken samples obtained using standard addition method by monitoring ^{82}Se.

The same digestion method was also applied to DOLT-4 as a CRM to check the accuracy of the method. Standard addition method with three points based on peak area was used to measure the concentration of selenium. Result was found as 9.05 ± 0.45 while the certified value is 8.3 ± 1.3. It is obvious that result found was in good agreement with certified value. This digestion and standard addition methods were applied to all chicken samples to determine the concentration of total selenium.

A.3.4. Extraction Studies

A.3.4.1. Extraction of Selenium from Egg Samples

The most crucial step in speciation is the extraction procedure. In this step, all selenium species should be taken from matrix to the solution without any alterations in concentration distributions for species. Although some of selenium species can be dissolved in water, there should be some selenium attached to the proteins and these can only be taken with hydrolysis. Enzymatic hydrolysis should be used instead of an acidic one. In Table 22, 17 different extraction procedures used in this study are given to find the relatively best one for selenium. In the extraction step, 0.20 g of lyophilized egg samples were taken and placed into a closed graduated plastic tube and then 10.0 mL of extraction solution containing different chemicals and enzymes were added. Tube was shaken for 24 hours. Clear solution could not be obtained by using ultracentrifuge instrument. Hence, extract was filtrated using Millipore Stirred ultrafiltration cell (8400 Model) in order to obtain a clear solution containing different selenium species; this solution was then analyzed using ICP-MS to find total Se concentration. In the ICP-MS, slopes of direct calibration and standard addition methods were also compared to each other. It was observed that the slopes of two methods were sufficiently close to each other. Hence, direct calibration method was used in the extraction studies.

Table 22. Reagents used in extraction step and extraction results of egg sample, dry mass (24 h extraction period).

Procedure	Reagents	Se, ng/g (N=3)
1	H_2O	134 ± 4
2	0.20 M HCl containing 10% of CH_3OH	369 ± 8
3	0.10 M HCl	< 50
4	0.10 M NaOH	279 ± 8
5	Tris HCl (pH 7.2) containing 4.0% of SDS	< 50
6	90% CH_3OH - 10% H_2O	181 ± 5
7	0.020 M of Tris HCl – 0.15 M NH_4CH_3COO (pH 7.0)	< 50
8	30 mM of Tris HCl (pH 7.2) containing 20.0 mg of Pepsin (4230 units/mg)	145 ± 4
9	30 mM of Tris HCl (pH 7.2) containing 20.0 mg of Tripsin (1670 units/mg)	217 ± 7
10	0.80 g TMAH - 30 mM Tris HCl (pH 7.2)	417 ± 10
11	30 mM of Tris HCl (pH 7.2) containing 20.0 mg of Protease XIV (4.5 units/mg)	664 ± 20
12	30 mM of Tris HCl (pH 7.2) containing 20.0 mg of Proteinase K (7.5 units/mg)	452 ± 14
13	30 mM of Tris HCl (pH 7.2) containing 20.0 mg of Driselase	50 ± 2
14	30 mM of Tris HCl (pH 7.2) containing 20.0 mg of Bacillus Subtilis	110 ± 3
15	30 mM of Tris HCl (pH 7.2) containing 20.0 mg of Pancreatin	139 ± 4
16	30 mM of Tris HCl (pH 7.2) containing 20.0 mg of PMSF	< 50
17	30 mM of Tris HCl (pH 7.2)	89 ± 4

As it is seen in Table 22, the relative best extraction was obtained using Protease XIV (4.5 units/mg) as an enzyme while the result obtained using Proteinase K was 452 ± 14. In the cases of PMSF, Tris HCl–NH_4CH_3COO buffer, SDS and 0.10 M of

HCl, selenium was found to be lower than 50 ng/g. The signal obtained using Protease XIV (4.5 units/mg) can be seen in Figure 10.

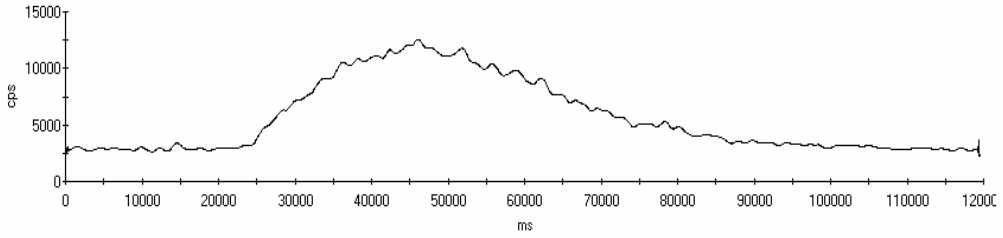

Figure 10. The ICP-MS signal of extract obtained using 30 mM of Tris HCl (pH 7.2) containing 20 mg of Protease XIV (4.5 units/mg).

ICPS value of this peak is 5050100. The extraction efficiency of procedure using 10% of CH_3OH in 0.20 M HCl was calculated by considering the effect of CH_3OH on nebulization efficiency. For this aim, the signal of 10.0 ng/mL of Se(IV) seen in Figure 11 was compared with the signal of 10.0 ng/mL of Se(IV) prepared in 10% of CH_3OH in 0.20 M HCl.

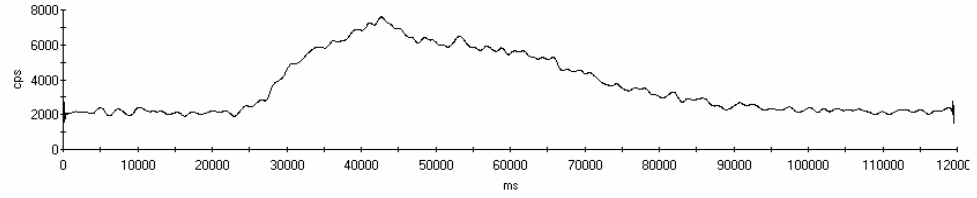

Figure 11. ICP-MS signal of 10.0 ng/mL of Se(IV) prepared using water.

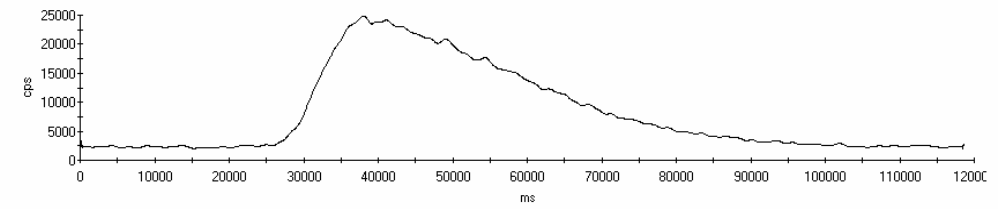

Figure 12. ICP-MS signal of 10.0 ng/mL of Se(IV) prepared using 10% CH_3OH in 0.20 M HCl.

The ICPS values of signal in Figure 11 and Figure 12 are $2.9x10^6$ and $9.4x10^6$, respectively. As it is seen Figure 12, selenium signal increases about 3.28 fold using 10.0% of CH_3OH in 0.2 M HCl instead of water. The effect of CH_3OH was therefore taken into account in order to calculate the selenium content after extraction procedure (10.0% of CH_3OH in 0.20 M HCl). For this aim, calibration standards were prepared in the same solution with the extraction procedure that is 10% of CH_3OH in 0.2 M HCl. The similar observations were obtained in the procedure of 90% of CH_3OH-10% of H_2O. ICPS value of the signal obtained for 10.0 ng/mL Se(IV) in 90% of CH_3OH -10% H_2O is $4.0x10^6$ which is about 1.41 fold higher than that obtained in H_2O. As it is seen in Table 22, concentration of selenium after extraction was found to be 417 ± 10 using 0.80 g of TMAH-30 mM of Tris HCl (pH 7.2). It was observed that hydroxyl group of TMAH improves the nebulization efficiency in ICP-MS system. ICP-MS signals of 10.0 ng/mL of Se(IV) in 0.80 g TMAH - 30 mM of Tris HCl (pH 7.2) and 10.0 ng/mL of Se(IV) in H_2O are given in Figure 13. In the Figure 13, it is clear that signal obtained using 0.80 g of TMAH - 30 mM Tris HCl (pH 7.2) is about 2 times higher than that in H_2O medium. Due to the differences in the nebulization efficiencies, calibration standards were prepared in the same medium with the extraction procedure that is 0.80 g of TMAH - 30 mM Tris HCl (pH 7.2).

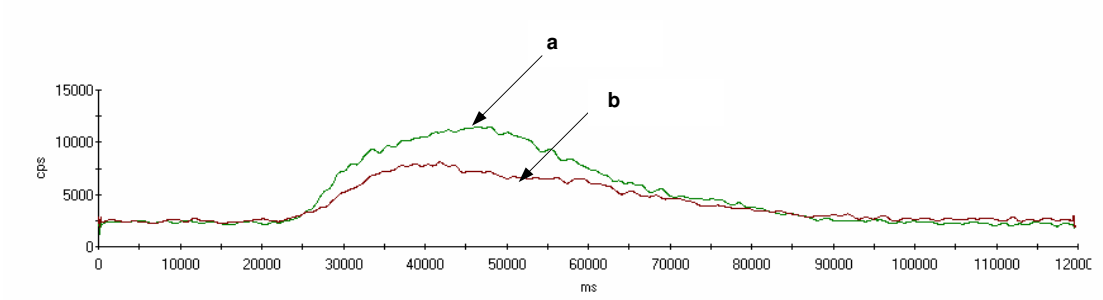

Figure 13. The ICP-MS signals of 10.0 ng/mL of Se IV in 0.80 g TMAH - 30 mM Tris HCl (pH 7.2) (a) and 10.0 ng/mL of Se IV in H_2O (b).

In addition, the effect of 30 mM of Tris HCl (pH 7.2) mostly used in extraction procedures was also checked in order to find whether the nebulization efficiency was affected by the presence of Tris HCl. For this aim, Se(IV) was also prepared in 30 mM of Tris HCl (pH 7.2). The ICPS value for this signal is $2.8x10^6$. This value is very close to result obtained using H_2O. Hence, it is obvious that Tris-HCl does not affect the nebulization efficiency. In addition, blank analyses were done in all extraction procedures and selenium was not found in chemicals used in the extraction.

A.3.4.1.1. Optimization of Extraction Period for Egg

In the extraction study, extraction period was optimized to obtain the highest extraction efficiency. In general, extraction step is known as the most time consuming step in speciation studies. In this study, the aim was not only to find the best extraction period but also to minimize the time spent. For this aim, about 0.20 g of lyophilized egg sample was taken and 30 mM of Tris HCl (pH 7.2) containing 20.0 mg of Protease XIV (4.5 units/mg) was added to the sample. Samples were placed in the shaker. Tubes were shaken for 4, 8, 12, 24 and 28 hours. At the end of each period, two of the samples were taken and then extraction solutions were filtrated using 10.0 KDa ultrafiltration membrane (Filter Code: YM10 Dia: 63.5 mm) and Millipore Stirred ultrafiltration cell (8400 Model) in order to not only reduce the matrix content but also obtain a clear solution. After the filtration, clear solutions were analyzed using ICP-MS. In addition, total amount of selenium in the egg sample was determined. For this aim, 0.20 g of egg sample taken from Kayseri was lyophilized and then digested using 10.0

mL of HNO_3-H_2O_2 (1/1) for the determination of total amount. In the microwave oven, temperature program given in Table 20 was used. Concentration of selenium in egg was found to be 1637 ± 125 ng/g (N=3).

Selenium results obtained after each extraction period are shown in Figure 14.

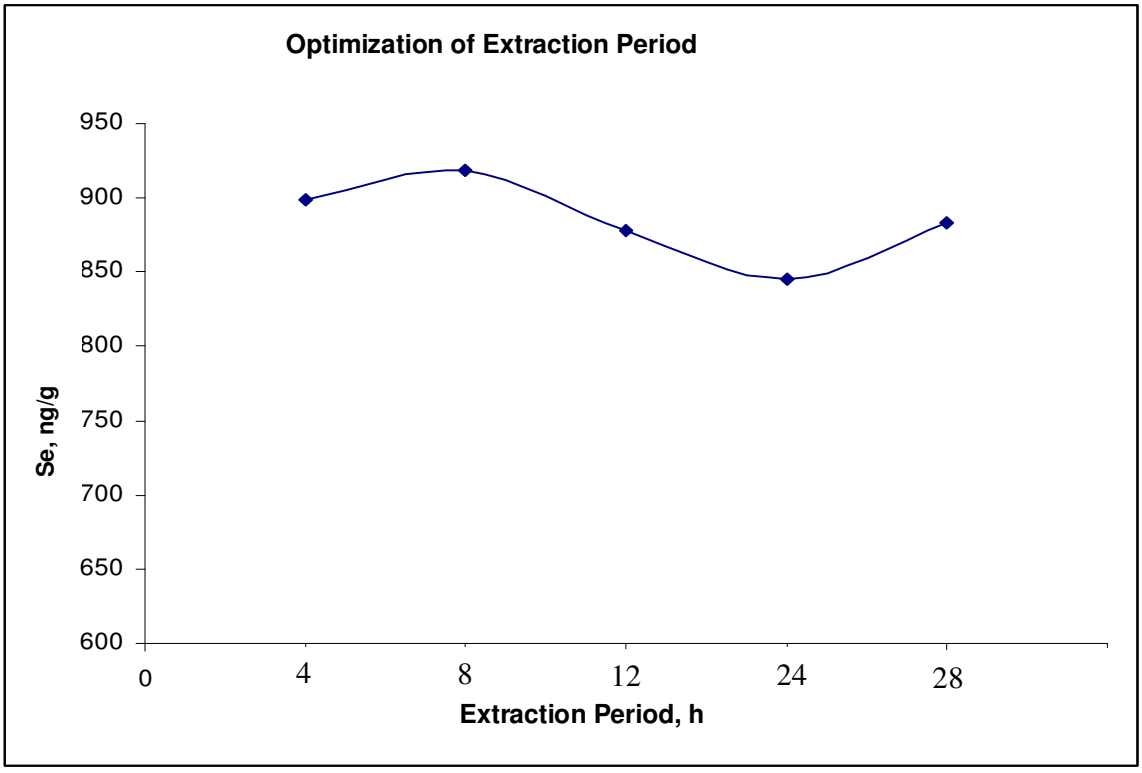

Figure 14. Optimization of extraction period for egg.

As it is seen in Figure 14, extraction efficiency of selenium from the egg sample slightly varied with the different extraction periods. Hence, 4 hours of extraction period was selected as the optimum one for further egg studies to minimize the time consumption.

A.3.4.1.2. Optimization of Enzyme Amount Used in Extraction of Se from Egg

Different enzymes have been used to extract the selenium from different matrices without uniformation [93, 100, 113]. As it is stated in Table 22, the best

extraction was achieved using protease XIV in 30 mM Tris HCl. Hence, this enzyme was used for further studies. Optimization of enzyme amount was also made to find the optimum Sample/Enzyme (w/w) ratio in the extraction step. For this aim, egg/enzyme ratios were set to 40, 20, 10, 5 and 3.3. Selenium in the each sample was extracted in 30 mM of Tris HCl (pH 7.2) containing fifferent amount of Protease XIV (4.5 units/mg) for 4 hours. At the end of 4 hours, samples were taken out of the shaker and then extraction solutions were filtrated using 10.0 KDa ultrafiltration membrane (Filter Code: YM10 Dia: 63.5 mm) and Millipore Stirred ultrafiltration cell (8400 Model) in order to obtain clear solution. After the filtration, clear solutions were analyzed using ICP-MS with direct calibration method.

Selenium results obtained after each extraction are given in Figure 15.

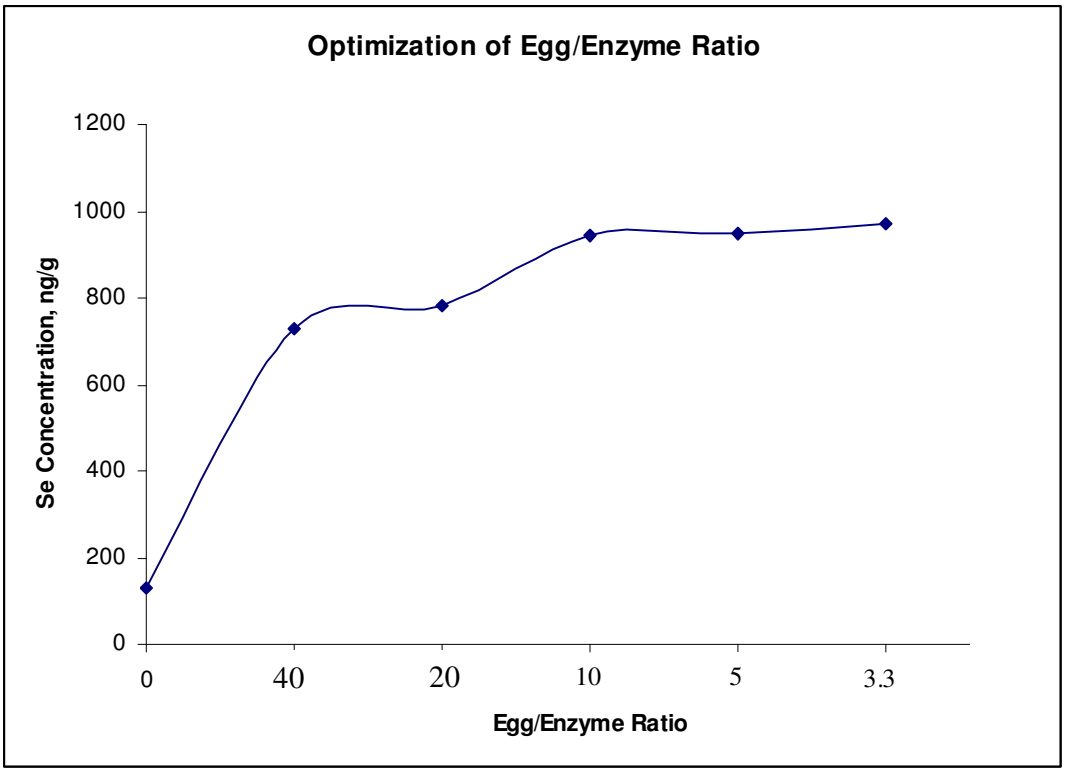

Figure 15. Optimization of egg/enzyme ratio.

25.0 mg of enzyme for 200 mg egg sample was selected as the optimum one for the further studies because this amount of enzyme is present in the plato. This amount is equal to 8 as egg/enzyme ratio.

In the CRM study, about 0.20 g of 1566b Oyster Tissue was taken and 30 mM of Tris HCl (pH 7.2) containing different amount of of Protease XIV (4.5 units/mg) were added to the sample. Same extraction procedure given above under the optimum conditions was applied to CRM in the extraction. Certified and found values were 2.06 ± 0.15 and 1.38 ± 0.05, respectively. Extraction efficiency was found to be 67 ± 2.5 % for 1566b Oyster Tissue while this value was 57 ± 5 % for egg samples (N=8). Matrix of 1566b Oyster Tissue is different from the egg sample. Hence, extraction efficiencies were found to be different from each other.

A.3.4.2. Extraction Studies for Selenium in Selenium Supplement Tablets

In order to find the best method for the extraction of selenium species from the matrix, 3 different extraction procedures were applied to selenium supplement tablets.

A.3.4.2.1. Extraction Method 1

Selenium supplement tablet was weighed into a 100 mL beaker and then 100 mL of de-ionized water were added. Samples were sonicated for 120 min in Elma, Elmasonic S 40 H brand sonication instrument at room temperature (24-27 ^{o}C), and then centrifuged for 20.0 min at 15000 rpm using Sigma, 2-16 brand ultracentrifuge instrument. Supernatant was decanted into a clean 15 mL centrifugation tube. Supernatant was filtered using 0.20 micron filter into a clean vessel. The extraction solution obtained was diluted 50 times using de-ionized water and then total selenium was determined using ICP-MS.

A.3.4.2.2. Extraction Method 2

5.0 M HCl was used as an extraction reagent. In this method, tablets were weighed and transferred into 100 mL of beakers and then 100.0 mL of 5.0 M of HCl was added. Samples were sonicated for 120 min at room temperature (24-27 ^{o}C), and then

centrifuged for 20 min at 15000 rpm. Supernatant was decanted into a clean 15 mL centrifugation tube. Supernatant was filtered using 0.2 micron filter into a clean vessel. The extraction solution obtained was diluted 50 times using de-ionized water and then used for the total selenium determination.

A.3.4.2.3. Extraction Method 3

Protease XIV was used as an enzyme in the extraction protocol. In this method, tablets were weighed into 50 mL of centrifuge tube and then 50 mg of Protease XIV was put into the centrifuge tube. 25.0 mL of 30.0 mM of Tris HCl adjusted to pH 7.2 were added. Samples were shaken using the shaker for 24 h. After 24 h, sample was poured into a 100 mL beaker and 75.0 mL of 30.0 mM of Tris HCl were added. Samples were sonicated for 120 min at room temperature (24-27 ^{o}C), and then centrifuged for 20 min at 15000 rpm. Supernatant was decanted into clean 15 mL of centrifugation tube. After the centrifuging step, supernatant was filtered using 0.20 micron filter into a clean vessel. The extraction solution obtained was diluted 50 times using de-ionized water and then sent to ICP-MS system for the total selenium determination.

Selenium signals obtained by extraction solutions of Brand A using 3 methods mentioned above are shown in Figure 16.

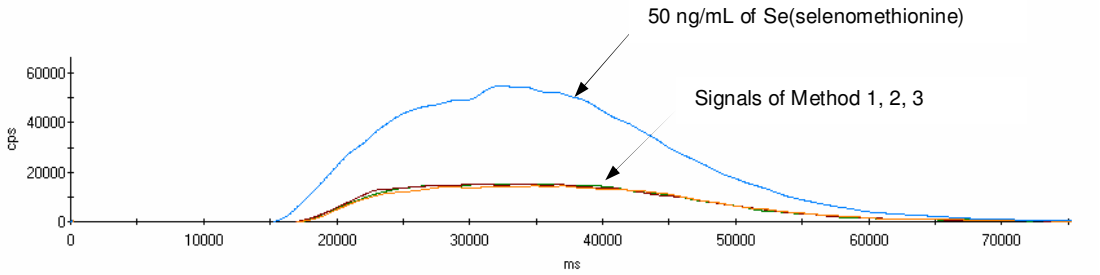

Figure 16. ICP-MS signals of extraction solutions of Brand A by using 3 methods.

In the extraction studies to find the best extraction method, Brand A having high amount of selenomethionine was used. As seen in Figure 16, there was no difference in the signals obtained by using the three methods. Results are very meaningful because there is no selenium yeast added to selenium supplement tablets. In case that selenium yeast is used as a source of selenomethionine, we should use enzyme to extract

selenomethionine bonded to proteins. On the bottles of the tablets, it is written that selenium is added to tablets in the form of either selenomethionine or selenate. Hence, there is no need to use Method 2 and 3. We decided to use Method 1 for the further selenium supplement tablet studies because we did not observe any differences in the results from Method 1, 2 and 3. After applying Method 1, proper dilutions were done for each selenium supplement tablet brand.

A.3.4.3. Extraction of Selenium from Chicken Tissue Samples

The similar extraction studies with egg samples were also performed for chicken samples. As it is mentioned in the introduction part, selenium is added to poultry diet to increase the selenium levels in chicken parts and egg. All samples were firstly freeze-dried and lyophilized samples were powdered in order to improve surface area. Water contents of samples were calculated using the weights measured after and before drying process. Water contents of samples are given in Table 23.

Table 23. Water contents of samples.

Sample	**%Water, (w/w), (N=4)**
Chicken Breast	70.7 ± 2.6
Chicken Buttock	70.9 ± 5.5

As it is seen in Table 23, water content of chicken breast was found as 70.7 ± 2.6 while this value was 70.9 ± 5.5 for chicken buttock. It is clear that water contents of two tissues are very close to each other.

Buttock and breast parts of chickens were analyzed to decide which part of chicken was proper for further chicken studies by concidering cconcentration of selenium. For this aim, 10.0 mL of HNO_3-H_2O_2 (1+1, volume, conc.) was used for about 0.30 g of sample. In the digestion procedure, the parameters given in Table 20 were applied. Standard addition method was used throughout this study. The concentrations of selenium in each part of chicken are given in Table 24.

Table 24. Total selenium concentration in buttock and breast parts of chicken (N=3).

Sample	Se, mg/kg (Dry mass)
Chicken breast 1 taken from Kayseri	0.51 ± 0.02
Chicken breast 2 taken from Kayseri	0.48 ± 0.02
Chicken buttock 1 taken from Kayseri	0.53 ± 0.03
Chicken buttock 2 taken from Kayseri	0.50 ± 0.03
Chicken breast 1 taken from Bursa	2.22 ± 0.11
Chicken breast 2 taken from Bursa	1.79 ± 0.09
Chicken buttock 1 taken from Bursa	2.16 ± 0.10
Chicken buttock 2 taken from Bursa	1.73 ± 0.09

It is seen in Table 24 that concentration of selenium in buttock and breast parts of chicken taken from Bursa were about 4 times higher than those obtained from Kayseri. Although there were no changes in the concentration of selenium in breast and buttock parts, we decided to continue with chicken breast for the further chicken studies due to better lyophilization of breast as compared to buttock.

A.3.4.3.1. Optimization of Extraction Period for Chicken Breast

In the extraction study, extraction period was optimized to obtain the best extraction efficiency. “Chicken breast 1” form Bursa was used in the optimization study. Aim was not only to find best extraction period but also to minimize the time consumed. For this aim, about 0.20 g of lyophilized chicken breast sample was taken and 30 mM of Tris HCl (pH 7.2) containing 20.0 mg of Protease XIV (4.5 units/mg) was added to the sample. Samples were placed into the shaker. Tubes were shaken for 2, 4, 8, 12 and 24 hours. At the end of each period, two of the samples were taken and then contents were filtrated using 10.0 KDa ultrafiltration membrane and Millipore Stirred ultrafiltration cell in order to obtain a clear solution. After the filtration, clear solutions were analyzed using ICP-MS. Selenium results are given in Figure 17.

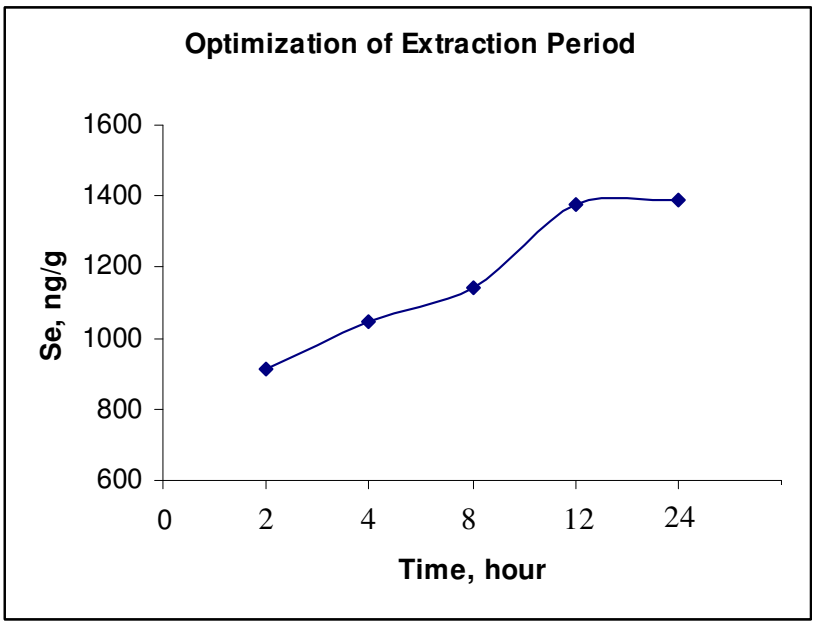

Figure 17. Optimization of extraction period for chicken breast.

As it is seen in Figure 17, extraction efficiency increases with longer extraction periods until using 24 h extraction period. The results obtained from 12 and 24 hours of extraction periods were very close to each other. Hence, 14 hours that was in the plateau was selected as the optimum extraction period. This period was different than value found for egg samples. This may be due to differences in sample matrices.

A.3.4.3.2. Optimization of Enzyme Amount Used in Extraction of Se from Chicken Breast

Optimization of enzyme amount was done to find out the optimum Sample/ Enzyme ratio in the extraction step. Similar with the egg studies, Protease XIV was used in the extraction studies. For this aim, chicken breast/enzyme ratios (w/w) were set to 40, 20, 10, 5 and 3.3. Selenium in the each sample was extracted using 30 mM of Tris HCl (pH 7.2) containing different amount of Protease XIV (4.5 units/mg) for 14 hours found as the optimum extraction period. At the end of 14 hours, samples were taken and then contents were filtrated using 10.0 KDa ultrafiltration membrane and

Millipore Stirred ultrafiltration cell in order to obtain a clear solution. After the filtration, clear solutions were analyzed using ICP-MS.

Selenium results obtained after each extraction are given in Figure 18.

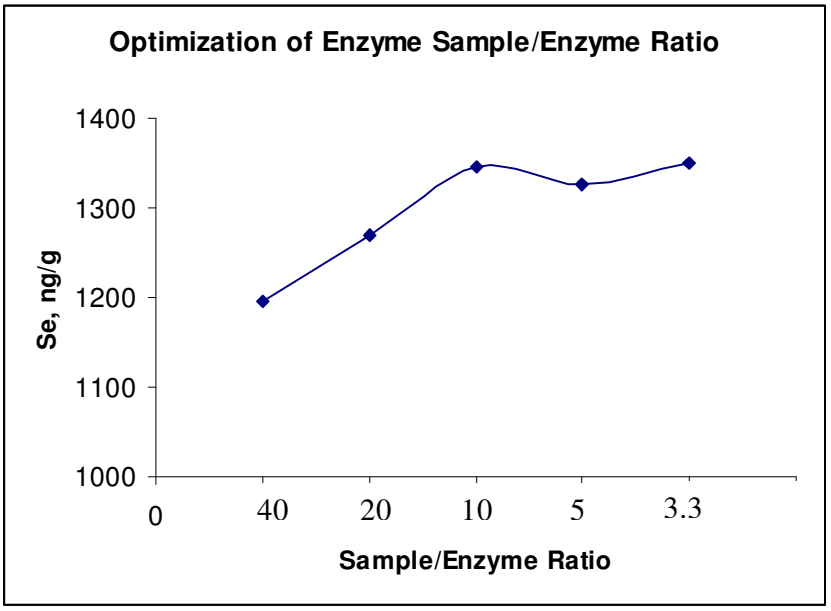

Figure 18. Optimization of chicken breast/enzyme ratio (Extraction period was 14 h).

20.0 mg enzyme was selected in further experiments for 200.0 mg of lyophilizied chicken breast sample. Average extraction efficiency was found as 61 ± 7 (N=8) using optimum parameters for chicken breast samples.

A.3.5. High Performance Liquid Cromatography (HPLC) Studies

In the HPLC studies, selenomethionine, selenocystine, Se(IV) and Se(VI) were tried to be separated from each other using different columns. Anion exchange, cation exchange, C18 and C8 column were tested to find the best separation conditions.

A.3.5.1. Anion Exchange (AE) Column Studies

Anion exchange (AE) column is one of the mostly used columns for the separation of selenium species from each other [156, 157, 158, 159]. In the AE studies, an S5 SAX (25cm x 4.6 mm) column was used. The output of column was connected to

nebulizer using 85.0 cm of tubing having 1.0668 mm i.d. and 1.6764 mm o.d. Although the abundance of ^{80}Se is the highest when it is compared with others, ^{78}Se and ^{82}Se were used throughout the studies. ^{80}Se was not used in the experiments due to $^{80}Ar\text{-}Ar^{+}$ interference that can not be eliminated without collision cell; in our ICP-MS, this technolgy is not present. For the accuracy and sensitivity checks, ^{74}Se, ^{76}Se, ^{77}Se and ^{82}Se isotopes were monitored throughout the studies. Major isotopes of selenium are subject to severe interference from $^{40}Ar^{36}Ar^{+}$, $^{40}Ar^{38}Ar^{+}$, and $^{40}Ar_2^{+}$. The molecular ions $^{40}Ar^{37}Cl^{+}$ and $^{81}Br^{1}H^{+}$ interfere with ^{77}Se and ^{82}Se, respectively when the sample contains high chloride and bromide content. Careful selection of Se isotopes is essential for the determination of Se compounds [142]. As mentioned before, the possible interferences in *m*/*z* 78 were monitored using other selenium isotopes, and no interferences were observed in *m*/*z* 78 for the sample used. Although baseline of ^{78}Se signal is higher than ^{82}Se, it does not show variation with time since the source of $^{40}Ar^{38}Ar^{+}$, namely Ar gas, is continuously present in plasma. Hence, ^{78}Se signal can be safely used in the measurements.

A.3.5.1.1. Mobile Phase AE-MP1

The first separation conditions for AE column can be seen in Table 25.

Table 25. Isocratic chromatography separation conditions for anion exchange HPLC-ICP-MS using AE-MP1.

Parameters	
Column	Spheris S5 SAX Anion Exchange Column
Mobile Phase, AE-MP1	10.0 mM of Citrate buffer in 10.0% (v/v) CH_3OH, pH 5.00 adjusted by NH_3
Flow Rate	1.0 mL/min
Loop Volume	91 µL

100.0 ng/mL of each selenium species was injected to Anion Exchange-HPLC-ICP-MS system using the parameters given in Table 25. Signals obtained can be seen in Figure 19.

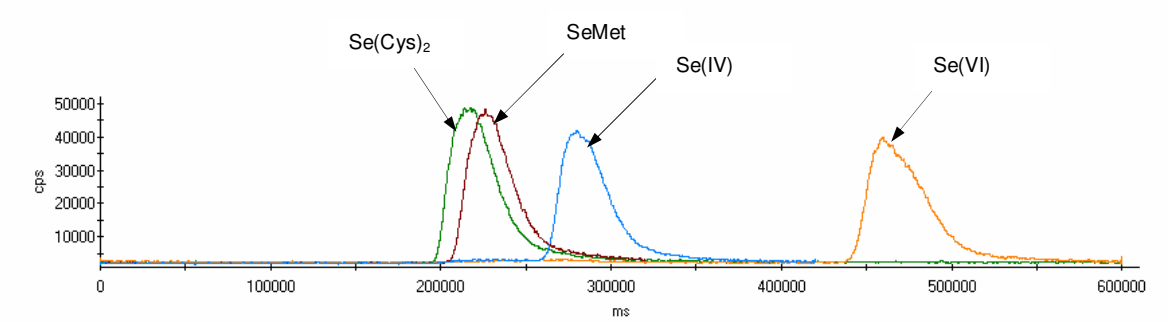

Figure 19. ICP-MS signals of 100 ng/mL of Se(VI) Se(IV), Se(selenocystine) and Se(selenomethionine) injected to AE-HPLC-ICP-MS system using parameters given in Table 25.

In Figure 19, it is shown that selenomethionine and selenocystine were not separated from each other. In addition, the signal of Se(IV) was slightly affected from the signals of selenomethionine and selenocystine. While the retention times for selenomethionine and selenocystine were found to be about 220 seconds, these values for Se(IV) and Se(VI) were 280 and 465 seconds, respectively.

pK_2 value of H_2SeO_4 is 1.92. Hence, Se(VI) is present in the form of SeO_4^{2-} at the pH of 5.0. Se(IV) is present in the form of $HSeO_3^-$ at this pH because pK_1 and pK_2 values of Se(IV) are 2.35 and 7.94, respectively. Retention properties of SeO_4^{2-} is higher than $HSeO_3^-$, resulting in a longer retention time in anion exchange column than that for $HSeO_3^-$.

The chromatogram obtained from the mixed standard solution containing the 100.0 ng/mL of each selenium species can be seen in Figure 20. Parameters given in Table 25 were used to obtain this signal.

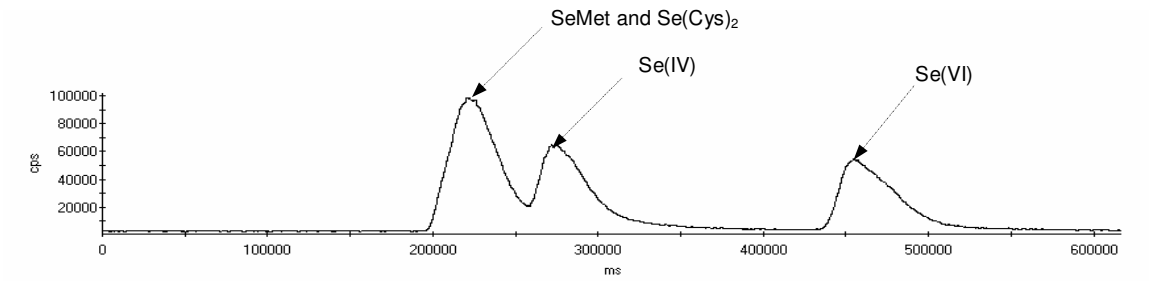

Figure 20. HPLC-ICP-MS chromatogram of mix solution containing 100 ng/mL of Se(VI), Se(IV), Se(selenocystine) and Se(selenomethionine) using parameters given in Table 25.

As it is seen in Figure 20, signal of Se(IV) is affected from the selenomethionine and selenocystine species due to very close retention times. In addition, there is a small shoulder on Se(VI) signal. Testing the values of retention times by using both single analyte solutions and a mixed solution was always applied during HPLC studies. Retention time of signals was automatically calculated via software of the instrument.

A.3.5.1.2. Mobile Phase AE-MP2

Concentration of citrate in buffer solution was decreased because separation of species was not good using Mobile Phase AE-MP1. All of the parameters used can be seen in Table 26.

Table 26. Isocratic chromatography separation conditions for anion exchange HPLC-ICP-MS using AE-MP2.

Parameters	
Column	Spheris S5 SAX Anion Exchange Column
Mobile Phase, AE-MP2	5.0 mM of Citrate buffer in 10.0% (v/v) CH_3OH, pH 5.00 adjusted by NH_3
Flow Rate	1.0 mL/min
Loop Volume	91 µL
Concentration of selenium	100 ng/mL

As it is seen in Table 26, amount of CH_3OH in buffer solution was also changed to 10.0 % in addition to concentration of citrate buffer as compared to AE-MP1. Chromatogram of mixed selenium solution containing 100 ng/mL of Se(VI), Se(IV), Se(selenocystine) and Se(selenomethionine) using parameters given in Table 26 is shown in Figure 21.

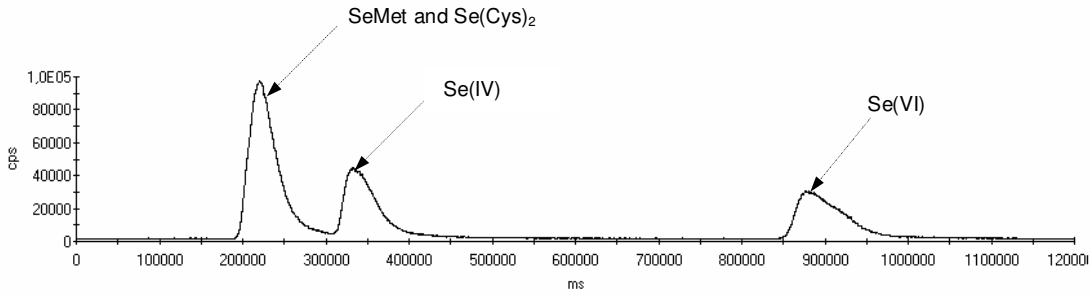

Figure 21. HPLC-ICP-MS chromatogram of mix selenium solution containing 100 ng/mL of Se(VI), Se(IV), Se(selenocystine) and Se(selenomethionine) using parameters given in Table 26.

Although inorganic selenium species can be separated from selenomethionine and selenocystine using AE-MP2, time required for the separation is rather long. This is the disadvantage of AE-MP2. Retention time of Se(VI) was especially found to be very high, about 900 seconds. Therefore, we decided to change the flow rate of the AE-MP2 in order to reduce the separation time without decreasing the resolution. For this aim, flow rate of the mobile phase was increased to 1.5 mL/min. All the parameters used can be seen in Table 27.

Table 27. Isocratic chromatography separation conditions for anion exchange HPLC-ICP-MS using AE-MP2 and 1.5 mL/min flow rate.

Parameters	
Column	Spheris S5 SAX Anion Exchange Column
Mobile Phase, AE-MP2	5.0 mM of Citrate buffer in 10.0% (v/v) CH_3OH, pH 5.00
Flow Rate	1.5 mL/min
Loop Volume	91 µL
Concentration of selenium	100 ng/mL

Anion Exchange-HPLC-ICP-MS chromatogram of mixed selenium standard containing 100 ng/mL of Se(VI), Se(IV), Se(selenocystine) and Se(selenomethionine) can be seen in Figure 22.

Retention times of all species were changed by altering the flow rate of mobile phase from 1.0 mL/min to 1.5 mL/min. The retention times of selenomethionine and selenocystine was shifted from 230 s to 150 s. In addition, retention time of Se(IV) was changed from 345 s to 225 s while the retention time of Se(VI) was decreased from 880 s to 660 s. Using this flow rate, separation was completed in 880 s.

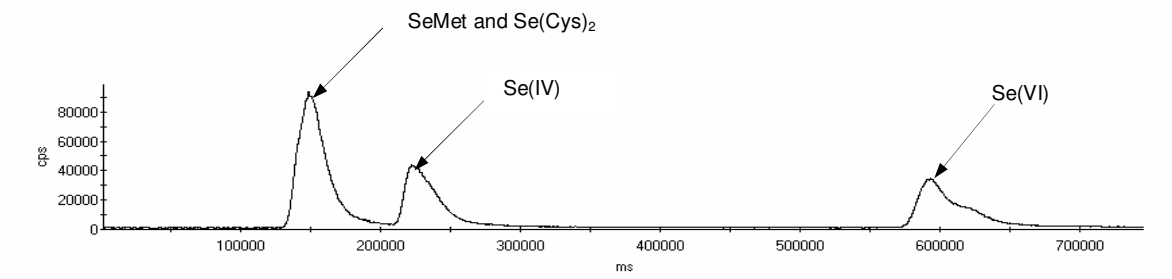

Figure 22. HPLC-ICP-MS chromatogram of mixed selenium standard containing 100 ng/mL of Se(VI), Se(IV), Se(selenocystine) and Se(selenomethionine) using 1.5 mL/min flow rate and parameters given in Table 27.

Under the these conditions, inorganic selenium species, Se(IV) and Se(VI), were separated from selenomethionine and selenocystine in 700 seconds.

A.3.5.1.2.1. Analytical Performance of Anion Exchange-HPLC-ICP-MS System

Using the Anion Exchange-HPLC-ICP-MS system, separations of inorganic selenium species from each other and from organometallic selenium species, SeMet and $Se(Cys)_2$, were achieved. Hence, analytical performances for Se(VI) and Se(IV) were investigated.

A.3.5.1.2.1.1. Analytical Performance of Se(IV)

A calibration plot was constructed using signals of 2.0, 5.0, 10.0, 20.0, 50.0 and 100.0 ng/mL of Se(IV) in Anion Exchange HPLC-ICP-MS as shown in Figure 23. Retention times of all concentrations were found to be same. This shows that stability of anion exchange column is satisfactory.

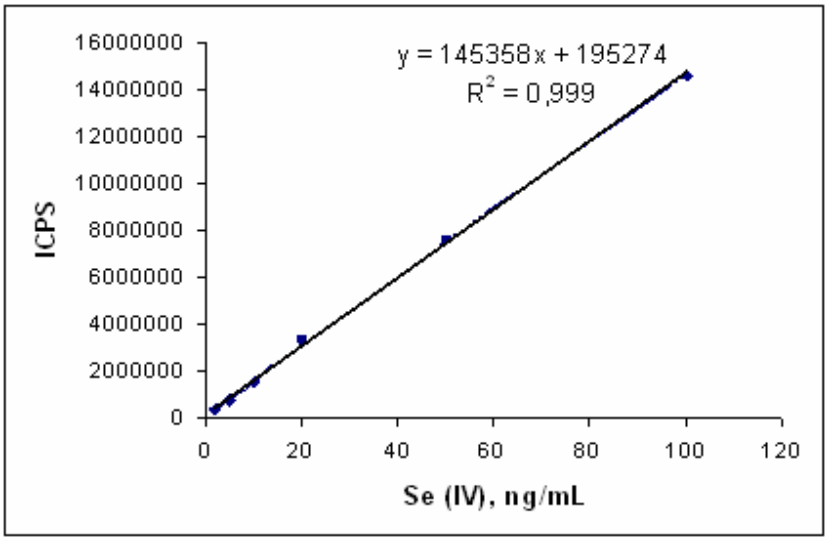

Figure 23. Calibration curve of Se(IV) in Anion Exchange-HPLC-ICP-MS using 1.5 mL/min flow rate and parameters given in Table 27.

Linear range for Se(IV) was found in the range of 2.0-100.0 ng/mL. R^2 value was 0.999. Five replicates were at least used throughout the all LOD and LOQ calculations. In the calculation of ICPS values, start and end points of the signal were considered. These selected points did not change for individual measurements. The replicate

signals of 5.0 ng/mL of Se(IV) sent to Anion Exchange-HPLC-ICP-MS system are shown in Figure 24.

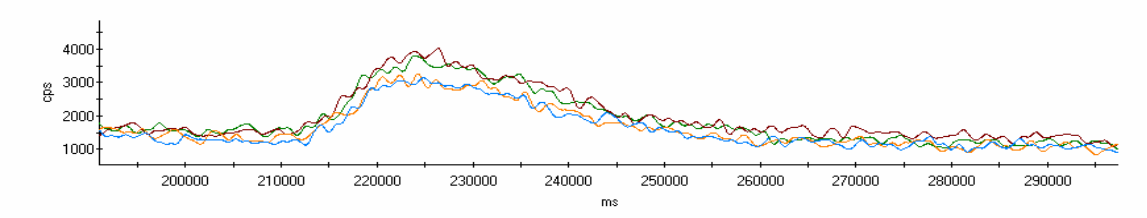

Figure 24. Replicate signals of 5.0 ng/mL Se(IV) injected to Anion Exchange HPLC-ICP-MS system using 1.5 mL/min flow rate and parameters given in Table 27.

Similarly signals were obtained to calculate LOD values.

A.3.5.1.2.1.2. Analytical Performance of Se(VI)

Linear calibration plot of Se(VI) can be seen in Figure 25 using the parameters given in Table 27**.**

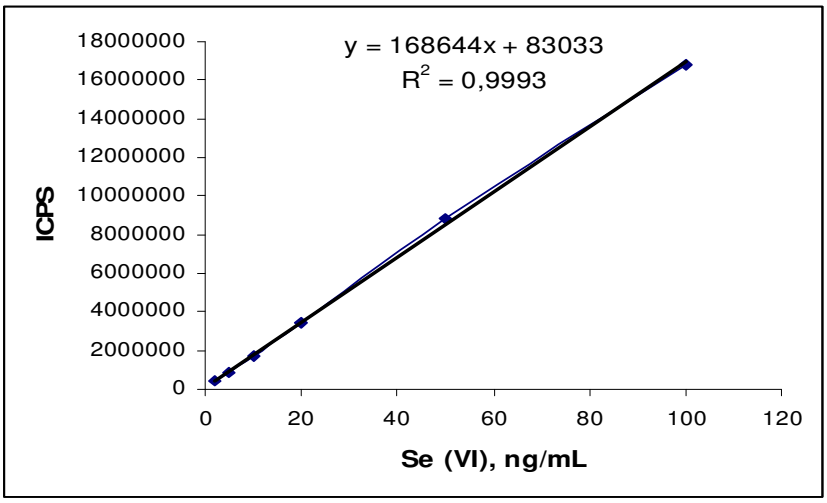

Figure 25. Linear calibration plot of Se(VI) obtained in Anion Exchange-HPLC-ICP-MS.

Linearity for Se(VI) was found to be in the range of 2.0-100.0 ng/mL. R^2 value was 0.9993. Limit of detection (LOD) and limit of quantitation (LOQ) values of Se(VI)

were calculated using standard deviation results of ICPS values of 5.0 ng/mL of Se(VI).

A.3.5.1.2.1.3. LOD and LOQ for Se(IV) and Se(VI) in Anion Exchange HPLC-ICP-MS system using Separate Standard Solutions

In the calculation of LOD and LOQ values of Se(IV) and Se(VI), 5.0 ng/mL analyte solutions were used. Parameters in Table 27 was used and the following formulation was applied.

LOD **=** 3**x**Standard Deviation (5.0 ng/mL of Se) / Slope
LOQ **=** 10**x**Standard Deviation (5.0 ng/mL of Se) / Slope

LOD and LOQ results of Se(IV) and Se(VI) are given in Table 28.

Table 28. LOD and LOQ results of Se(IV) and Se(VI) in Anion Exchange HPLC-ICP-MS system using separate standard solutions.

	Se(IV)	**Se(VI)**
Limit of Detection, LOD, ng/mL	0.85	0.68
Limit of Quantitation, LOQ, ng/mL	2.84	2.29

It is clear that Anion Exchange-HPLC-ICP-MS is a sensitive method for the speciation of inorganic selenium species.

A.3.5.1.3. Solvent Program, AE-SP1

Using the mobile phase 2 at the flow rate of 1.5 ml/min, separation of inorganic selenium species from each other and from organometallic selenium species were good, but not perfect. Hence, mobile phase 3 was applied to AE-HPLC-ICP-MS system to improve the resolution for the separation of inorganic selenium species. In addition, peak shape of Se(VI) was not symmetric; there is a shoulder in this signal. Gradient elution was applied to improve resolution. All parameters used can be seen in Table 29. Gradient elution was applied to obtain not only a good separation but also better peak

shapes.

Table 29. Solvent programming chromatographic separation conditions for anion exchange HPLC-ICP-MS using AE-SP1 and 1.5 mL/min flow rate.

Parameters	
Column	Spheris S5 SAX Anion Exchange Column
Solvent program, AE-SP1	0-6.0 min 5.0 mM Citrat Buffer in 10.0% CH_3OH (v/v), pH 4.50 6.0-10.0 min 5.0 mM Citrat Buffer in 10.0% CH_3OH (v/v), pH 6.00 10.0-20.0 min 5.0 mM Citrat Buffer in 10.0% of CH_3OH (v/v), pH 4.50
Flow Rate	1.5 mL/min
Loop Volume	91 µL

Using the conditions given in Table 29, a mixed selenium standard containing 50.0 ng/mL of Se(VI), Se(IV) and 25.0 ng/mL of Se(selenocystine) and Se(selenomethionine) was also injected to Anion Exchange-HPLC-ICP-MS system and the chromatogram shown in Figure 26 was obtained.

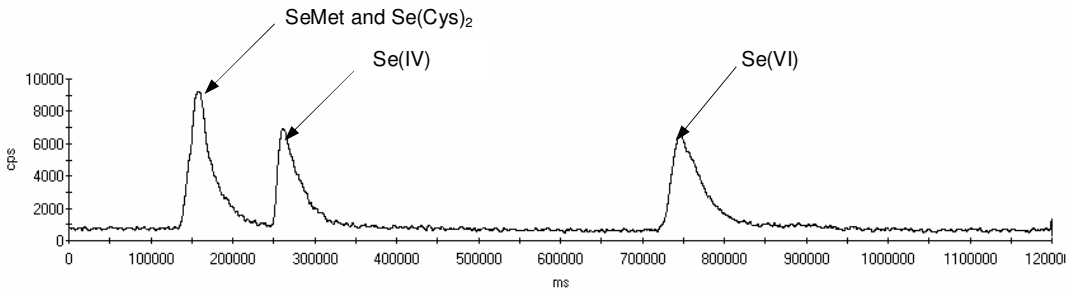

Figure 26. AE-HPLC-ICP-MS chromatogram of mix selenium solution containing 50.0 ng/mL of Se(VI) and Se(IV) with 25.0 ng/mL of Se(selenocystine) and Se(selenomethionine) using 1.5 mL/min flow rate and parameters given in Table 29, AE-SP1.

There was no differences in the retention times of Se(IV) and Se(VI) in pure and

mixed standard solutions. Resolution of Se(IV) and Se(VI) is high enough to make qualitative and quantitative measurements while selenomethionine and selenocystine have same retention times.

Recovery of the selenium species from the column was also investigated. For this aim, a mixed standard containing 1.0 mg/L for each of Se(IV), Se(VI), Se(selenomethionine) and Se(selenocystine) was injected to AE-HPLC-ICP-MS under the optimum conditions. After the anion exchange column, eluent was collected throughout the gradient elution for 15 min. The same experiment was also performed using no column. A mixed standard containing 1.0 mg/L for each of Se(IV), Se(VI), Se(selenomethionine) and Se(selenocystine) was injected to HPLC using the same loop and eluent was collected before the column. Both collected solutions were aspirated to ICP-MS system and signals shown in Figure 27 were obtained.

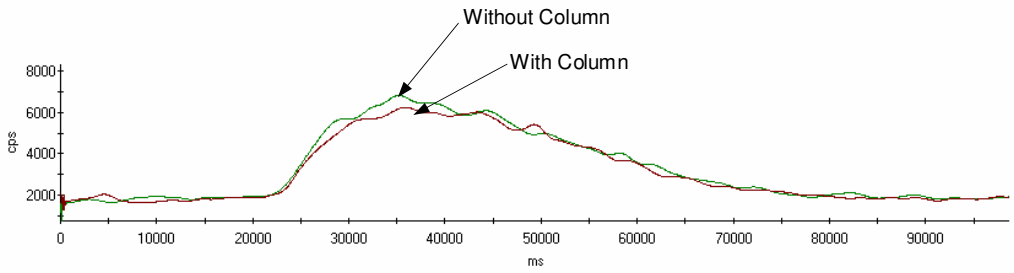

Figure 27. Signals for 91 µL of 1.0 mg/L mixed selenium standard with and without column using 1.5 mL/min flow rate and parameters given in Table 29.

In Figure 27, it is shown that there is no significant difference in the signals of solutions obtained with and without using column. This means that recovery from the column is high enough for quantitative measurements for selenium species; in other words, there is no loss in column.

A.3.5.1.3.1. Analytical Performance of Se(IV) and Se(VI) in Anion Exchange-HPLC-ICP-MS System using Mixed Standard Solution

Selenium mix standards at different concentration were injected to AE-HPLC-ICP-MS to get linear calibration plots for Se(IV) and Se(VI). Signals can be seen in Figure 28.

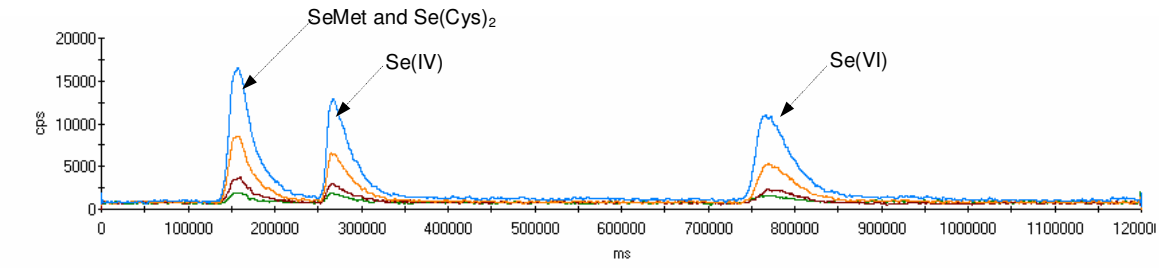

Figure 28. HPLC-ICP-MS chromatogram of mixed selenium standards containing 100.0, 50.0, 20.0, 10.0 ng/mL of Se(VI), Se(IV) and 50.0, 25.0, 10.0, 5.0 ng/mL of Se(selenocystine) and Se(selenomethionine) using the 1.5 mL/min flow rate and parameters given in Table 29, EA-SP1.

Linear calibration plot for Se(IV) and Se(VI) obtained can be seen in Figure 29-Figure 30. As seen in figures, linearity of calibration plots is sufficient.

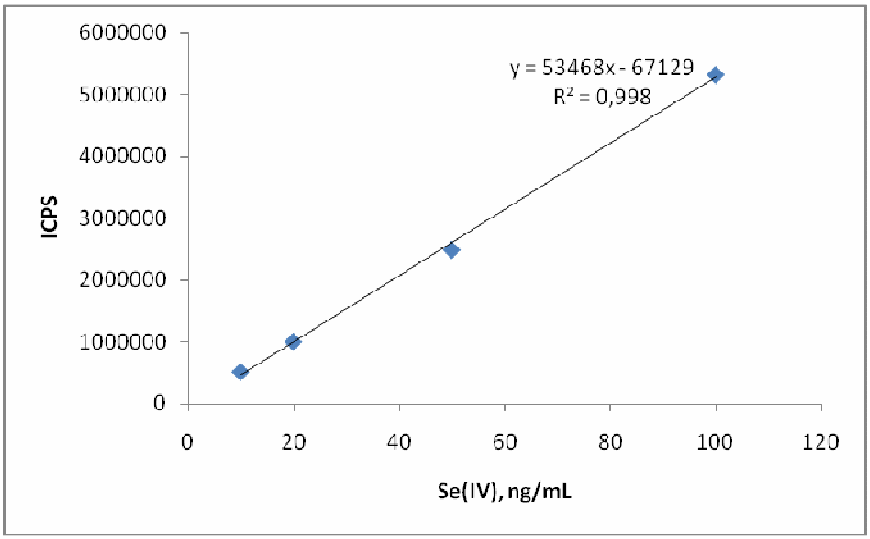

Figure 29. Linear calibration plot of Se(IV) obtained using Anion Exchange-HPLC-ICP-MS.

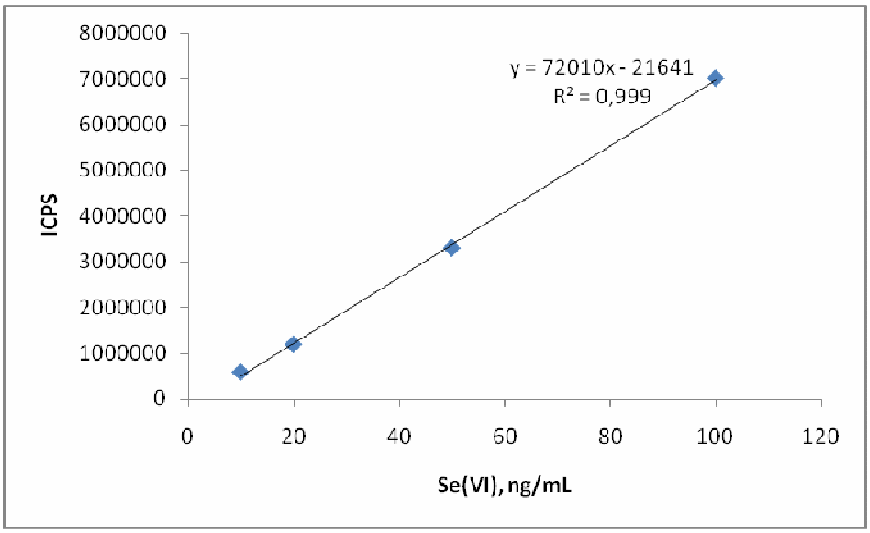

Figure 30. Linear calibration plot of Se(VI) obtained using AE-HPLC-ICP-MS.

For the LOD and LOQ calculation, a mixed standard solution containing 5.0 ng/mL of Se(IV), Se(VI) and 2.5 ng/mL of Se(selenocystine) and Se(selenomethionine) was injected to system 5 times. Peak areas were used in all calculations.

The same formulas with previous study were applied for LOD and LOQ calculations. Standard deviations obtained using the areas of signals (ICPS) were put to formulas in the calculations. LOD and LOQ results of Se(IV) and Se(VI) can be seen in Table 30.

Table 30. LOD and LOQ results of Se(IV) and Se(VI) in AE-HPLC-ICP-MS system using mixed standard solution.

	Se(IV)	**Se(VI)**
Limit of Detection, LOD, ng/mL	0.75	0.80
Limit of Quantitation, LOQ, ng/mL	2.51	2.67

As seen in Table 30, LOD and LOQ values for Se(IV) and Se(VI) are low enough to make quantitative measurements. In addition, the values are not much different than those given in Table 28.

A.3.5.2. C8-C18 Studies by Reversed Phase HPLC-ICP-MS

Reversed phase column that uses non-polar substances for the stationary phase has been used in selenium speciation study. It has been widely used in literature to separate Se species. Ion pairing reagents make the analytes uncharged. Hence, separation of analytes takes place in the column according to their polarities. For this purpose, heptafluorobutiric acid [93], trifluoroacetic acid [136], and pentafluoropropanoic acid [137] have been mostly used as ion pairing reagents. These perfluorinated carboxylic acid reagents provide sufficient separation for Se species in different chemical structures.

The experiments with different parameters given below have been performed for the separation of Se(IV), Se(VI) selenomethionine and selenocystine using different mobile phases and columns. Elution regimes for RP-HPLC system can be seen in Table 31.

Table 31. Elution regimes tested for RP-HPLC-ICP-MS separation of selenium species using 91.0 µL loop volume.

Elution Regime	Column and flow rate	Given Name	Mobile Phase and/or Solvent Program
Isocratic	Alltima C8, 1.0 mL/min	C8-MP1	5.0% CH_3OH
Isocratic	Alltima C8, 1.0 mL/min	IP-MP2	0.1% TFA in 5.0% of CH_3OH, pH Natural
Isocratic	Alltima C8, 1.0 mL/min	IP-MP3	0.05% of HFBA in 5.0% CH_3OH, pH natural
Isocratic	Alltima C8, 1.0 mL/min	IP-MP4	0.20% of HFBA in 2.5% of CH_3OH, pH Natural
Isocratic	Alltima C8, 1.0 mL/min	IP-MP5	0.10% of HFBA in 5.0% of CH_3OH, pH Natural
Solvent Programming	Alltima C8, 1.0 mL/min	IP-SP1	5.0-15.0 min 50% of 0.10% of HFBA in 5.0% CH_3OH, pH Natural 50% of 5.0% of CH_3OH
			15.0-18.0 min 0.10% of HFBA in 5.0% CH_3OH, pH Natural
			5.0-15.0 min 50% of 0.10% of HFBA in 5.0% CH_3OH, pH Natural 50% of 5.0% of CH_3OH
Solvent Programming	Alltima C8, 1.0 mL/min	IP-SP2	0-5.0 min 0.10% of HFBA in 5.0% CH_3OH, pH Natural
			5.0-15.0 min 25% of 0.10% of HFBA in 5.0% CH_3OH, pH Natural 75% of 5.0% of CH_3OH
			15.0-18.0 min 0.10% of HFBA in 5.0% CH_3OH, pH Natural
Isocratic	Dionex C18,1.5 mL/min	IP-MP6	0.12% of HFBA in 10.0% of CH_3OH (v/v), pH Natural

The retention times of Se(IV) and Se(VI) were found to be very close to each other due to no retention in C8 column using C8-MP1. Using single analyte solution, it was observed that the retention time for selenocystine was 110 seconds while it was 160 seconds for selenomethionine. Difference between the retention times of selenomethionine and selenocystine was about 70 seconds. Four selenium species were not completely separated from each other using C8-MP1 as mobile phase. Retention times of Se(VI), Se(VI) and selenocystine are very close to each other. Hence, these species could not be separated from each other and gave a signal having wide peak width.

In IP-MP2 system, retention times of Se(VI) and selenomethionine were 95 and 255 seconds, respectively while retention times of Se(IV) and selenocystine were found to be very close to each other, 120 seconds. Since the retentions of inorganic selenium species and selenocystine under these conditions are very close, they were not separated efficiently.

Retention times of Se(VI) and Se(IV) were found to be 90 and 95 seconds, respectively, using IP-MP3. In addition, retention times for selenocystine and selenomethionine were 115 and 250 seconds, respectively. When compared to Mobile phase C8-MP1, the retention time of selenocystine slightly increased. In addition, the retention time of selenomethionine increased to 250 seconds. Under these conditions only selenomethionine was separated from the other species. Selenocystine, Se(IV) and Se(VI) could not be separated.

Using IP-MP4, retention time of Se(IV) was found to be 100 seconds while it was 110 seconds for Se(VI). The resolution between the peaks of these two species is getting better. Retention times for selenocystine and selenomethionine were found to be 200, 980 seconds, respectively. Selenomethionine and selenocystine species were successfully separated from inorganic selenium species. Because the retention time of selenomethionine is too long, its peak got broadened. Peak width of SeMet was about 175 s. This situation could cause problems during the analysis of real samples. Therefore, it was decided that this mobile phase was not proper for the separation of selenium species for the real sample analysis.

In IP-MP5 mobile phase system, chromatographic separation of inorganic species Se(IV) and Se(VI) was found as the best one among the other cases. The retention times of Se(IV) and Se(VI) was found to be 85 and 110 seconds, respectively.

The retention time of selenocystine was 225 seconds while it was 680 seconds for selenomethionine. The separation between Se(IV) and Se(VI) was partly achieved. The small signal that was very close to selenocystine peak was disappeared in this concentration of HFBA. This mobile phase is proper to separate selenocystine from inorganic Se species. Long retention time of selenomethionine in this column causes the broadening in selenomethionine peak. The signal from a single analyte solution of selenomethionine was also rather broad but symmetrical. This system was not selected for determination due to doubling in selenomethionine signal in mixed standard solution.

Using IP-SP1, the retention time for selenomethionine decreased 580 seconds and the broadening was eliminated noticeably by changing mobile phase composition during separation. In order to evaluate the change in retention by solvent programming, a second program was applied. There were no changes in the retention times using two solvent programs. In addition, it was observed that stability of C8 column was not good regarding variation in retention time in the case of solvent program. Although a good separation was obtained, reproducibility of the retention times of selenium species detoriated by repeated use. Therefore, it was decided that this solvent program, IP-SP2, could not be used for further studies.

In the mobile phase IP-MP6, C18 column was applied to separate selenium species instead of C8 column. Selenomethionine and selenocystine species were separated from each other whereas inorganic selenium species were not separated effectively. It was found that the retention time of Se(VI) was around 75 seconds while this value was 90 seconds for Se(IV). It is clear that retention times of inorganic species are very close to each other. Retention time of selenocystine and selenomethionine were found to be 220 and 740 seconds, respectively. Peak width of selenomethionine was about 100 seconds due to the longer retention time when compared with other selenium species. There were no tailing in the $Se(Cys)_2$ and SeMet signals and resolution of these two species were high enough for qualitative and quantitative measurements. Hence, it was decided to use these conditions only for the determination of selenomethionine and selenocystine species in real sample measurements; the conditions were not suitable for separation of inorganic selenium species.

A.3.5.2.1. The Analytical Performance of C18-HPLC-ICP-MS System

It was clear that C18 is the most suitable column type for the separation of selenium species among the reverse phase systems given above. The analytical performance of this system was shown by the calculation of LOD and LOQ values. Since it is possible to separate selenomethionine and selenocystine species by using C18 column in our system, the analytical performances of these species only were determined. For this aim, 2.0, 5.0, 10.0, 20.0, 50.0 and 100.0 ng/mL of standard solutions of each selenium species were prepared and injected to the C18-HPLC-ICP-MS system under the conditions indicated in Table 31, IP-MP6.

In order to obtain the calibration curve, the peak areas (ICPS) were used. The calibration plot obtained by using the peak areas is shown in Figure 31 for selenomethionine.

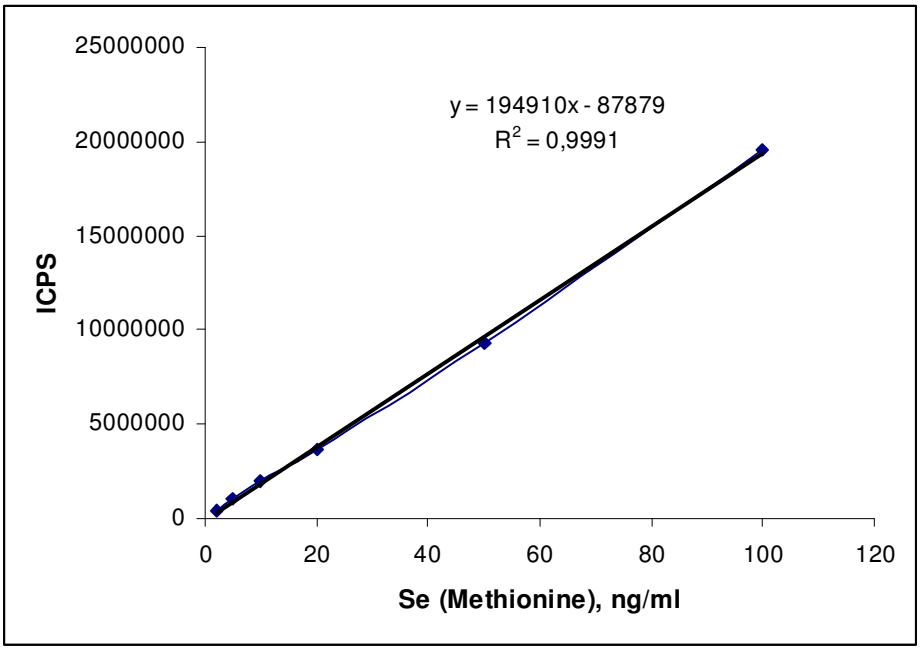

Figure 31. Calibration plot of Se(selenomethionine) using C18-HPLC-ICP-MS system.

Linear range for Se(selenomethionine) was found to be in the range of 2.0-100.0 ng/mL. Higher concentrations were not injected to system to minimize the contamination of column. R^2 value was 0.9991.

The linearity of the calibration plot obtained from selenocystine species having the retention time of around 215 seconds was found to be in the range of 2.0-100.0 ng/mL.

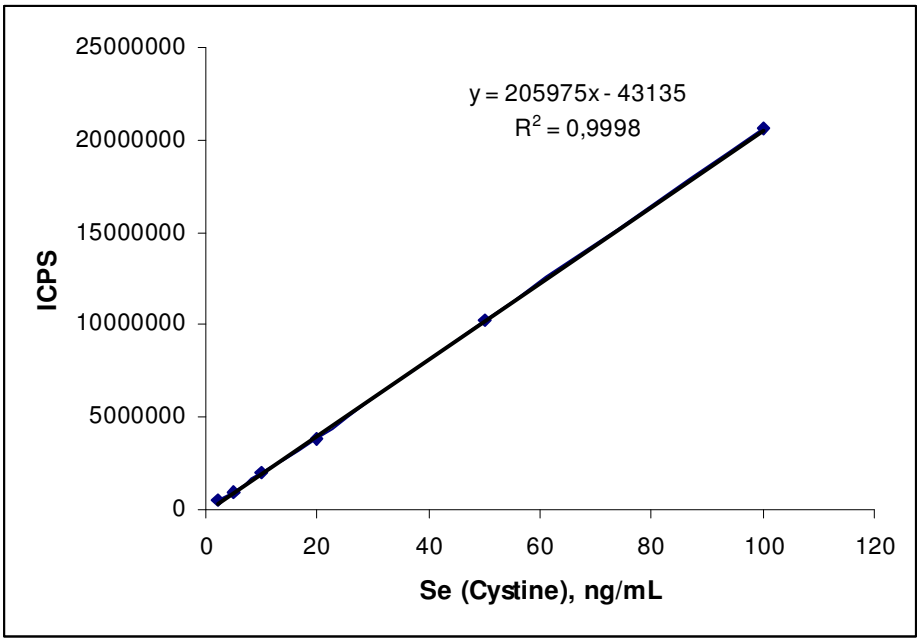

Figure 32. Calibration curve of Se(selenocystine) using C18-HPLC-ICP-MS system.

In the calculations of LOD and LOQ values of selenomethionine and selenocystine species the signals from 5.0 ng/mL (N=5) were used. The standard deviation values obtained from the peak areas, ICPS; and LOD and LOQ values for these species were calculated. LOD and LOQ values of Se(selenomethionine) and Se(selenocystine) species are shown in Table 32.

Table 32. LOD ve LOQ values of Se(selenomethionine) and Se(selenocystine) species.

	Se(selenomethionine)	Se(selenocystine)
Limit of detection, LOD, ng/mL	0.76	0.38
Limit of quantitation, LOQ, ng/mL	2.53	1.25

It is seen that selenocystine is two times more sensitive than selenomethionine.

This may be because of broadening in the selenomethionine signal due to longer retention time.

A.3.5.3. Cation Exchange (CX) Column Studies

Cation exchange column is used for the separation of positively charged selenium species having a low molecular weight. This technique commonly used in literature use the cationic groups present in the structure of selenium species. Inorganic selenium species can not be separated from each other by using a cation exchanger column because of the anionic character of the structures. In addition to this, when pH is adjusted to 3.0 selenite (SeO_3^{2-}) is protonated and retained more in the cation exchange column in the form of $HSeO_3^-$. When the pH value is lower than 3.0, the retention time of Se(IV) in the cation exchange column increases as a result of proton concentration [164].

Different mobile phases were tried to find best separation condition using Cation Exchange-HPLC-ICP-MS system.

A.3.5.3.1. Mobile Phase CX-MP1

In this system, 20.0 mM of pyridine in 5.0% of CH_3OH adjusted by HCl to pH 1.65 was used as mobile phase. All parameters for this mobile phase system can be seen in Table 33.

Table 33. Isocratic chromatographic separation conditions for cation exchange HPLC-ICP-MS using Cation Exchange-MP1.

Parameter	Value
Column	Spheris S5SCX cation exchange column
Mobile phase	20.0 mM of Pyridine in 5.0% CH_3OH, pH 1.65
Flow rate	1.0 mL/min
Loop Volume	91 µL

The chromatogram obtained from each selenium species injected to the cation

exchange column separately are shown in Figure 33.

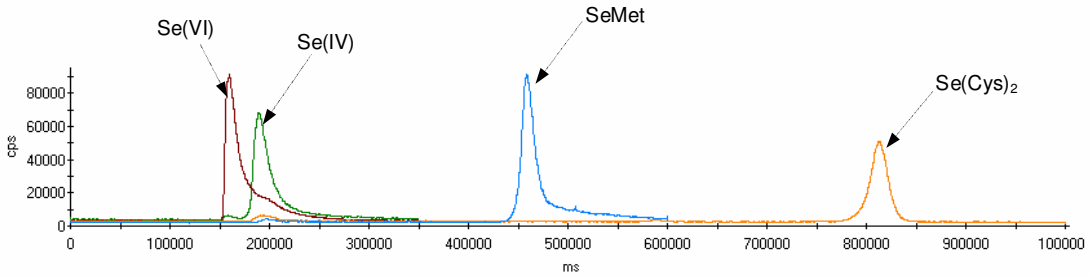

Figure 33. Signals obtained using 100.0 ng/mL of Se(VI), Se(IV), Se(selenocystine) and Se(selenomethionine) species injected to cation exchange-HPLC-ICP-MS system by using the parameters given in Table 33.

The retention times of Se(VI) , Se(IV) , selenomethionine and selenocystine in the cation exchange column using Mobile Phase CX-MP1 were found to be 165, 200, 460 and 815 seconds, respectively. As shown in Figure 33, all of the selenium species were separated from each other using the conditions given in Table 33. Although the separation between inorganic selenium species was not complete, the signal of Se(IV) did not affect the 75.0% of the signal of Se(VI). Although the separation between inorganic species was improved with increasing the acidity of the mobile phase, this approach was not selected in order to prevent the deformation of the filling material of the cation exchange column. The ICP-MS signals were obtained by the injection of the solution containing 100.0 ng/mL of each selenium species to the cation exchange column under the conditions given in Table 33; the chromatogram is shown in Figure 34.

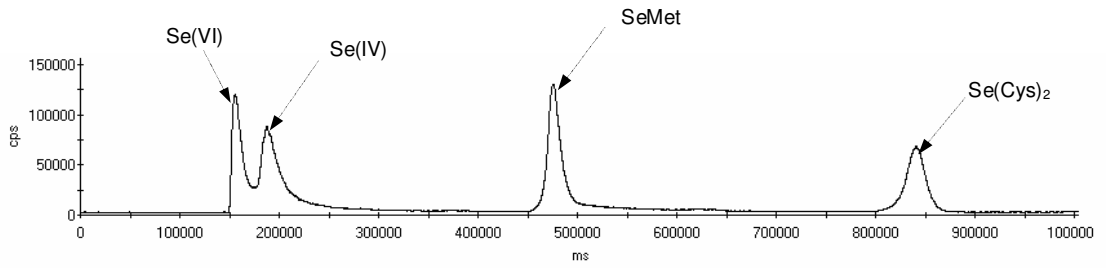

Figure 34. HPLC-ICP-MS chromatogram of mixed selenium standard containing 100.0 ng/mL of Se(VI), Se(IV), Se(selenocystine) and Se(selenomethionine) using 1.0 mL/min flow rate and parameters given in Table 33.

As shown in Figure 34, selenomethionine and selenocystine species can be successfully separated in a period of 900 seconds and resolution of Se(VI), Se(IV) species was partially satisfactory. It was decided that the conditions indicated in Table 33 were most suitable for separation of Se(VI), Se(IV), selenomethionine and selenocystine among all the columns and mobile phases used so far in this study. In addition, time required was also lower than those obtained using other columns and mobile phases.

Recovery of selenium species from cation exchange chromatography was also performed. There was no difference between the signals obtained with and without column. This shows that recovery of selenium from cation exchange column used in this experiment was high enough for quantitative measurment. Results of analytical performance for this system are given in Table 36 and Table 37.

A.3.5.3.2. Mobile Phase CX-MP2

In addition to the first separation system using CX-HPLC-ICP-MS, another mobile phase was applied to separate selenium species. In this system, 10.0 mM of pyridinium formate in 5.0% of CH_3OH (pH 2.12) was used as mobile phase. In the pH adjustment, formic acid was used. All parameters for Mobile Phase 2 system can be seen in Table 34.

Table 34. Isocratic chromatographic separation conditions for cation exchange HPLC-ICP-MS using CX-MP2.

Parameter	**Value**
Column	Spheris S5SCX cation exchange column
Mobile phase CX-MP2	10.0 mM of pyridinium formate in 5.0% CH_3OH (v/v), pH 2.12
Flow rate	1.5 mL/min
Loop Volume	91 µL

Under the conditions mentioned in Table 34, a mixed selenium standard was injected to HPLC-ICP-MS system and chromatogram seen in Figure 35 was obtained.

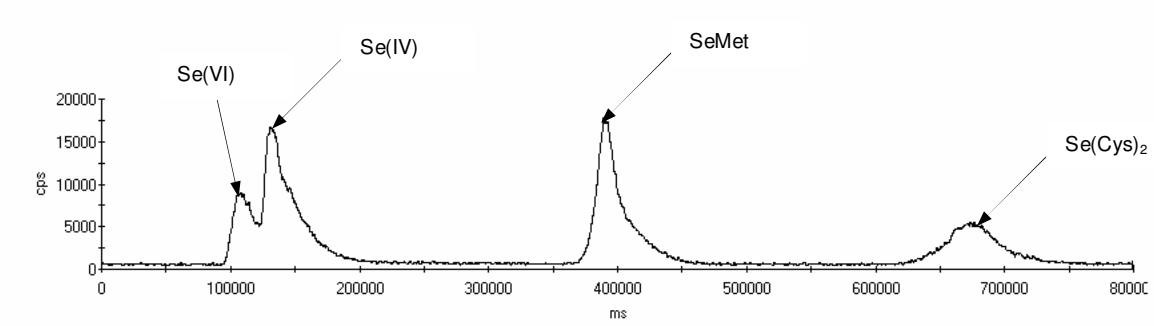

Figure 35. HPLC-ICP-MS chromatogram of mix selenium solution containing 100.0 ng/mL of Se(selenomethionine), Se(IV) and 50.0 ng/mL of Se(VI), Se(selenocystine) using 1.5 mL/min flow rate and parameters given in Table 34.

At the beginning, each selenium species was injected to system one by one to figure out retention time of each species. It was observed that there were no changes in the retention times of selenium species in the mixture and pure solutions. As seen in Figure 35, SeMet and $Se(Cys)_2$ can be separated from inorganic species and from each others while separation was not good enough to make quantitative measurements for Se(IV) and Se(VI). Results of analytical performance for this system are given in Table 36 and Table 37.

A.3.5.3.3. Mobile Phase CX-MP3

Mobile phase CX-MP3 was also applied to CX-HPLC-ICP-MS. In this system, 10.0 mM of pyridinium formate in 5.0% CH_3OH (pH 1.20) was used as mobile phase. The parameters for Mobile Phase CX-MP3 can be seen in Table 35. In this system, two different flow rates were tried. There was no change in the resolution of selenomethionine and selenocystine using 1.0 mL/min and 1.5 mL/min as mobile phase flow rates. Hence, 1.5 mL/min was finally selected to reduce the elution time.

Table 35. Isocratic chromatographic separation conditions for cation exchange HPLC-ICP-MS using CX-MP3.

Parameter	**Value**
Column	Spheris S5SCX cation exchange column
Mobile phase CX-MP3	10.0 mM of pyridineformate in 5.0% CH_3OH (v/v), pH 1.20
Flow rate	1.5 mL/min
Loop Volume	91 µL

Under the optimum conditions given in Table 35, mixed selenium standard was injected to CX-HPLC-ICP-MS system and chromatogram seen in Figure 36 was obtained.

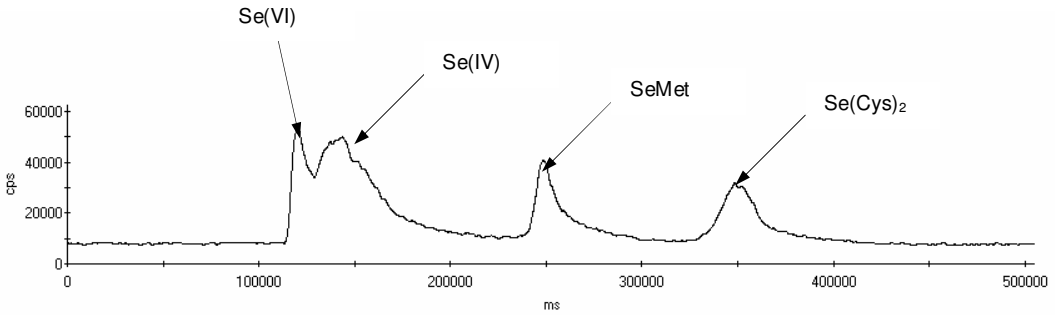

Figure 36. HPLC-ICP-MS chromatogram of mix selenium solution containing 50 ng/mL of Se(VI), Se(IV) and 25 ng/mL of Se(selenomethionine), Se(selenocystine) using 1.5 mL/min flow rate and parameters given in Table 35.

Each selenium species was injected to system one by one to determine retention time of each species. There were no changes in the retention times of selenium species in the mixture and pure solutions. As seen in Figure 36, SeMet and $Se(Cys)_2$ can be separated from inorganic species and each other. Hence, this system is proper to make qualitative and quantitative measurements of these species.

Recoveries of the selenium species from the column were also determined like the other separation system. For this purpose, 1.0 mg/L of Se(IV), Se(VI), Se(selenomethionine) and Se(selenocystine) was used under the optimum conditions given in Table 35. No difference was observed in the chromatograms of solutions obtained with and without column experiments. Analytical performance results for this system are given in Table 36 and Table 37.

A.3.5.3.4. Analytical Performance Data for CX-HPLC-ICP-MS

Calibration plots were constructed using single analyte solutions of 2.0, 5.0, 10.0, 20.0, 50.0 and 100.0 ng/mL Se for each species. Concentration higher than 100.0 ng/mL were avoided to minimize contamination of the system. Best line equation and correlation coefficients were computed by using regression analysis. For LOD and LOQ values, at least 5 replicate signals of 2.0 or 5.0 ng/mL Se standard solution for each species were used. Results are given in Table 36 and Table 37.

Table 36. LOD* and LOQ* values for Se species using CX-HPLC-ICP-MS and several elution regimes.

Elution regime and conditions	LOD and LOQ, ng/mL Se			
	Se(IV)	Se(VI)	Se(SeMet)	$Se(Se(Cys)_2)$
Isocratic, CX-MP1, Table 33	0.35 1.16	0.38 1.16	0.41 1.36	0.44 1.47
Isocratic, CX-MP2, Table 34	-	-	0.84 2.80	0.99 3.30
Isocratic, CX-MP3, Table 35	-	-	0.55 1.81	0.46 1.54

*For LOD and LOQ, 2.0 or 5.0 ng/mL was used for each species (N=5 at least)

Table 37. Working range, best line equation and R^2 for calibration plots using CX-HPLC-ICP-MS and several elution regimes**.**

Elution regime	Working range (ng/mL), best line equation and R^2			
	Se(IV)	Se(VI)	Se(SeMet)	Se(Se(Cys)$_2$)
Isocratic, CX-MP1, Table 33	2.0-100.0 y=103389x + 604466 0.9995	2.0-100.0 y=257506x + 271124 0.9997	2.0-100.0 y=339719x - 115021 0.9992	2.0-100.0 y=176881x + 97316 0.9997
Isocratic, CX-MP2, Table 34	-	-	5.0-100.0 y=60396x + 57625 0.9990	5.0-100.0 y=66950x + 74622 0.9990
Isocratic, CX-MP3, Table 35	-	-	2.0-100.0 y=20088x - 14794 0.9990	2.0-100.0 y=21168x - 4064 0.9990

A.3.6. General Evaluation of HPLC-ICP-MS Optimization

Single analyte solution and mixed solutions were used to verify that retention times did not show any variations in these two cases. Often, both ^{78}Se and ^{82}Se chromatograms were used to verify the analytical behavior and lack of spectral interferences. In order to check whether any analyte is adsorbed and lost in the column, analyses of injected analytes collected before introduction to ICP-MS were performed with and without using the column; no losses were obtained.

Considering the data in Table 36 and Table 37, it could be observed that LOQ values are sufficiently low, so that the working ranges reported are justified for each species. Among the several alternative considered and studied, the following conditions were selected for final speciation analysis of vitamin tablet, chicken breast from Bursa and Kayseri, egg and selenium fed chicken breast samples in this thesis. Chromatographic separation conditions used in speciation of selenium are shown in Table 38.

Table 38. Chromatographic separation conditions used in speciation of Se.

	Chromatographic Condition	
	Cation Exchange	Anion Exchange
Egg	CX-MP1	-
Vitamin Tablets	CX-MP2	AE-SP1
Chicken breast from Bursa and Kayseri	CX-MP1	-
Selenium fed chicken breast samples	CX-MP3	AE-SP1

A.3.7. Selenium Speciation in Egg Samples

Extraction of egg samples was done under the optimum conditions given in experimental part. Following the extraction procedure, samples were injected to system in less than 10 min to eliminate or minimize the oxidation of SeMet. Concentrations of Se species in egg samples were determined by using CX-MP1 in Cation Exchange-HPLC-ICP-MS system.

Cation Exchange-HPLC-ICP-MS chromatograms of extraction solution of Egg A and mixed standard solution containing Se(VI), Se(IV), Se(selenocystine) and Se(selenomethionine) are shown in Figure 37

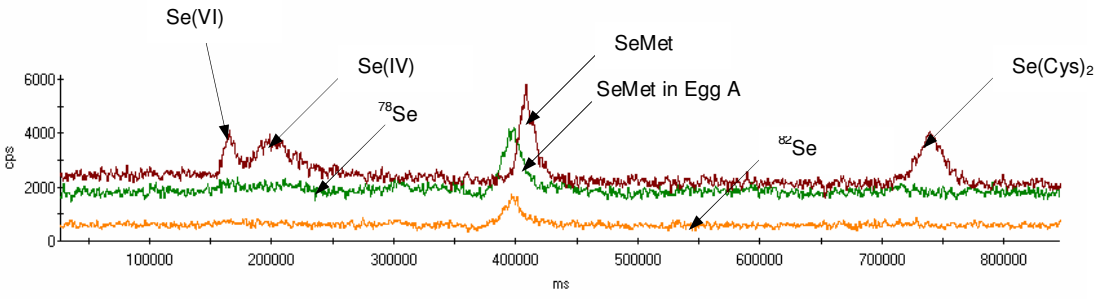

Figure 37. Chromatograms of extraction solution of Egg A and 2.5 ng/mL of mixed standard solution containing Se(VI), Se(IV), Se(selenocystine) and Se(selenomethionine) obtained using the parameters given in Table 33, CX-MP1.

As seen in Figure 37, concentration of selenomethionine was found to be the highest among other species. In addition, concentrations of Se(IV), Se(VI) and

Se(selenocystine) were found to be lower than LOD values.

Eggs produced by chickens those were fed with normal diet was used as control sample. A selected sample was named Sample B. The same sample preparation procedure with the other egg samples was applied to these egg samples. After the extraction procedure, filtered solution was injected to Cation Exchange-HPLC-ICP-MS system with CX-MP1 and signals were obtained. Selenomethionine signal of egg extract was found at about 390 seconds that was 20 seconds before than signal obtained from standard solution of selenomethionine. In order to decide whether this signal belongs to selenomethionine or not, selenomethionine standard was spiked to egg extract. Cation Exchange-HPLC-ICP-MS chromatograms of extraction solution of Egg B and selenomethionine spiked Egg B are shown in Figure 38.

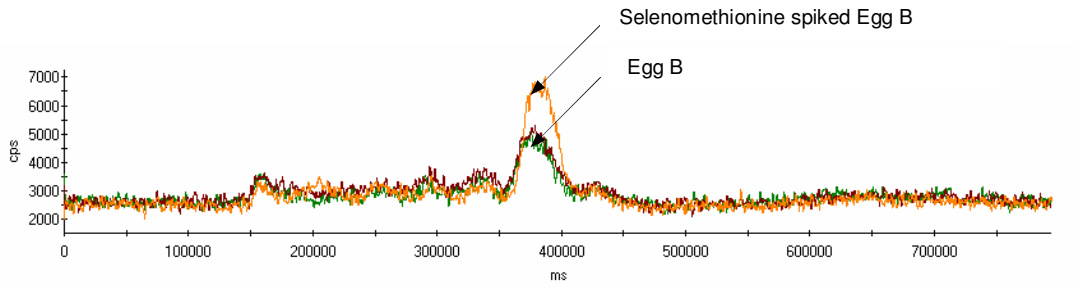

Figure 38. Chromatograms of extraction solution of Egg B and selenomethionine spiked Egg B obtained using the parameters given in Table 33, CX-MP1.

As seen in Figure 38, retention times of Egg B and selenomethionine spiked Egg B are same. This shows that the signal obtained at 390 seconds is due to selenomethionine. This shift in retention time is most probably caused by matrix.

Another sample purchased from market with a claim of Se-rich diet was called Egg C. Cation Exchange-HPLC-ICP-MS chromatograms of extraction solution of Egg C and selenomethionine spiked Egg C are given in Figure 39.

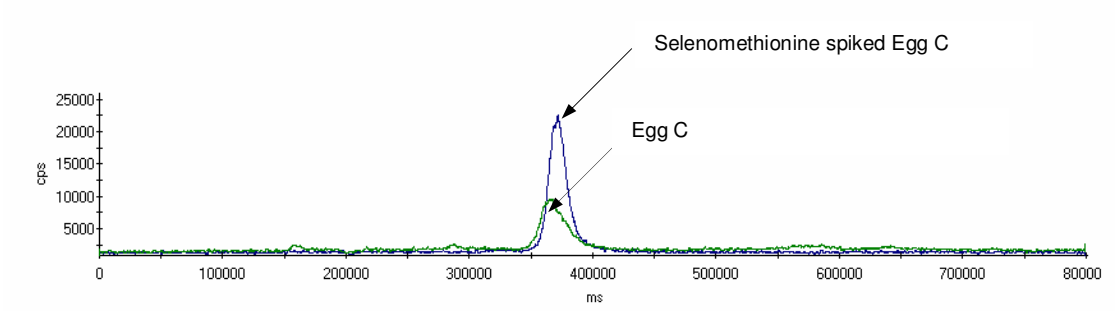

Figure 39. Chromatograms of extraction solution of Egg C and selenomethionine spiked Egg C obtained using the parameters given in Table 33.

As seen in Figure 39, a symmetric signal was obtained for selenomethionine in Egg C sample. Chromatographic features were similar to Egg B. Concentrations of Se(IV), Se(VI) and Se(selenocystine) were found to be lower than LOD values like the Egg A and B. Similar with Egg B, selenomethionine peak shifted to 380 seconds from 410 seconds. In order to decide whether this signal is belonging to selenomethionine or not, selenomethionine standard was spiked to Egg C extract. It was observed that this peak is belonging to selenomethionine.

The results of total Se and Se speciation analyses are given in Table 39.

Table 39. Concentration of total selenium and Se(IV), Se(VI), Se(selenomethionine) and Se(selenocystine) species in egg samples.

Sample	Se found in sample, Mean ± S.D., ng/g (dry mass), N=2				
	Total Se	Se(IV)	Se(VI)	Se(SeMet)	Se(Se(Cys)$_2$)
Egg A	926 ± 39	N.D.	N.D.	381 ± 34	N.D.
Egg B, Control	774 ± 46	N.D.	N.D.	321 ± 10	N.D.
Egg C	2080 ± 104	N.D.	N.D.	1011 ± 61	N.D.

N.D.: Not detected

As seen in Table 39, concentration of total selenium was found to be lowest in control sample. Concentration of Se(IV), Se(VI) and selenocystine were not detected in all samples. Selenomethionine concentration was found to be the highest in Brand C when compared with Sample A and B.

A.3.8. Selenium Speciation in Selenium Supplement Tablets

In this study, anion and cation exchange chromatographic methods were used for the identification and quantification of inorganic selenium species and two selenoamino acids in conjunction with HPLC-ICP-MS in selenium supplement tablet samples. Speciation information was compared with the value given on the tablets and the total levels of selenium measured with ICP-MS.

Total selenium contents of tablets are determined using the method given in experimental part. In the extraction study, all optimum parameters given in experimental part were applied to tablet samples. Proper dilutions were done for each brand and extraction solution were injected to HPLC-ICP-MS system for quantitative measurement.

A.3.8.1. HPLC-ICP-MS Studies for the Speciation of Selenium in Selenium Supplement Tablets

Extraction of selenium species from selenium supplement tablets were done using the Extraction Method 1 described before. Three sample replicates were used for each brand. Extraction solution of tablets were injected to Cation Exchange-HPLC-ICP-MS system with CX-MP2 for the determination of organic selenium species, selenomethionine, and Anion Exchange-HPLC-ICP-MS system with AE-SP1 for inorganic selenium species, Se(VI). Chromatogram of mixed selenium standard containing selenomethionine, selenocystine, Se(VI) and Se(IV) can be seen in Figure 40 for CX-HPLC-ICP-MS and in Figure 41 for AE-HPLC-ICP-MS systems. It can be seen that determination of organic and inorganic species can be performed by using CX and AE coupled to ICP-MS, respectively. All selenium isotopes were monitored throughout selenium supplement study.

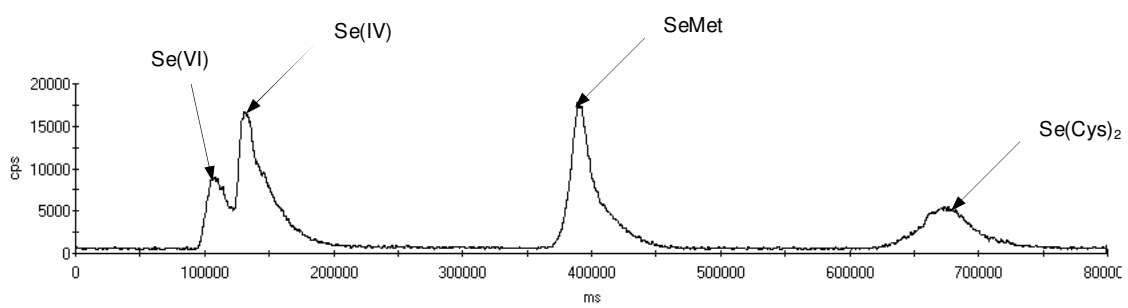

Figure 40. CX-HPLC-ICP-MS chromatogram of mix selenium solution containing 100 ng/mL of Se(selenomethionine), Se(IV) and 50 ng/mL of Se(VI), Se(selenocystine) using the 1.5 mL/min flow rate and parameters given in Table 34, CX-MP2.

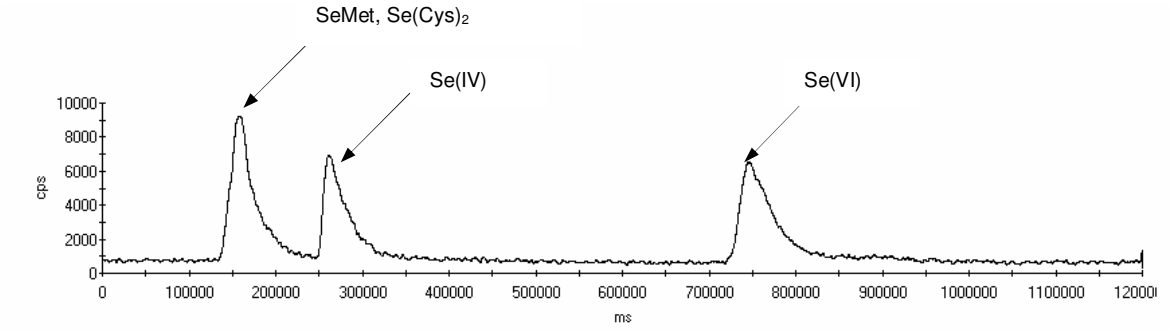

Figure 41. AE-HPLC-ICP-MS chromatogram of mix selenium solution containing 50.0 ng/mL of Se(IV), Se(VI) and 25.0 ng/mL of Se(selenocystine) and Se(selenomethionine) using the 1.5 mL/min flow rate and parameters given in Table 29, AE-SP1.

A.3.8.1.1. Brand A

It is written on the bottle of Brand A that each tablet includes 100 µg Se in the form of selenomethionine. It was found that total amount of selenium in Brand A was 109.1 ± 1.5 µg using nitric acid digestion method. This result found agrees reasonably well with the amounts of selenium reported on the bottle label of Brand A.

In order to make speciation analysis of selenium in the Brand A, extraction solution of Brand A was injected to CX-HPLC-ICP-MS system with CX-MP2 to figure out whether the reported value on the bottle as selenomethionine is accurate or not. The chromatograms of three replicates of Brand A are shown in Figure 42

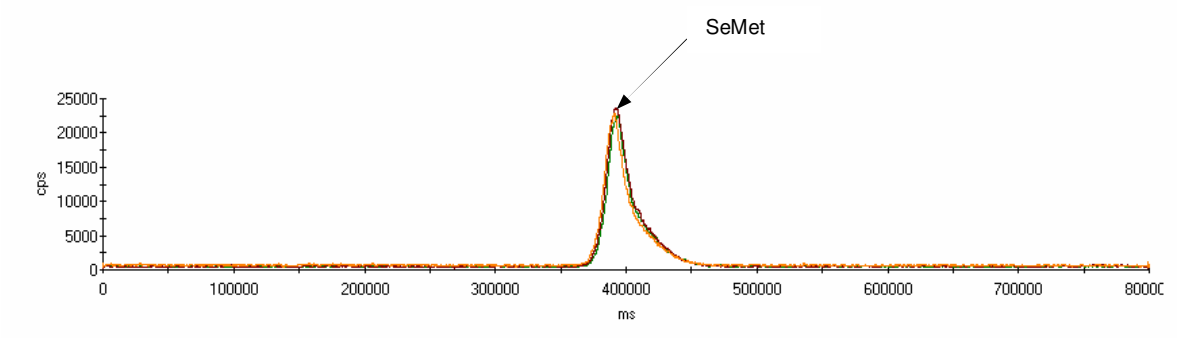

Figure 42. CX-HPLC-ICP-MS chromatograms of 3 replicates of Brand A using the parameters given in Table 34, CX-MP2.

As seen in the figure, retention times of all signals are exactly the same and the peaks are sharp and symmetric. There is no other signal observed in the chromatograms except the selenomethionine peak. Chromatograms of Brand A and mixed selenium standards containing 100.0 ng/mL of Se(selenomethionine), Se(IV) and 50.0 ng/mL of Se(selenocystine), Se(VI) are shown in Figure 43.

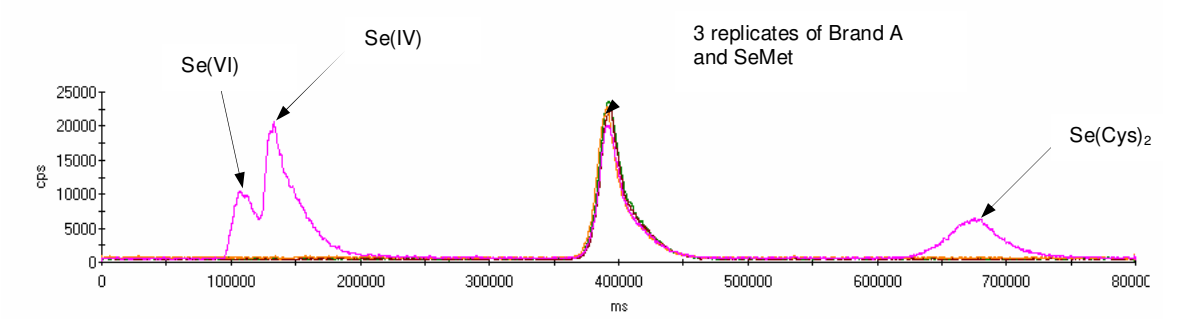

Figure 43. CX-HPLC-ICP-MS chromatograms of 3 replicates of Brand A and mixed standard containing 100.0 ng/mL of Se(selenomethionine), Se(IV) and 50.0 ng/mL of Se(selenocystine), Se(VI) parameters given in Table 34, CX-MP2.

It is clear in Figure 43 that retention time of the signals of Brand A extracts are matching with the retention time of selenomethionine in the mixed standard solution. In order to make sure there are no matrix interferences to affect selenomethionine signal, standard addition method was also applied to Brand A. Standard addition chromatogram of Brand A is shown in Figure 44.

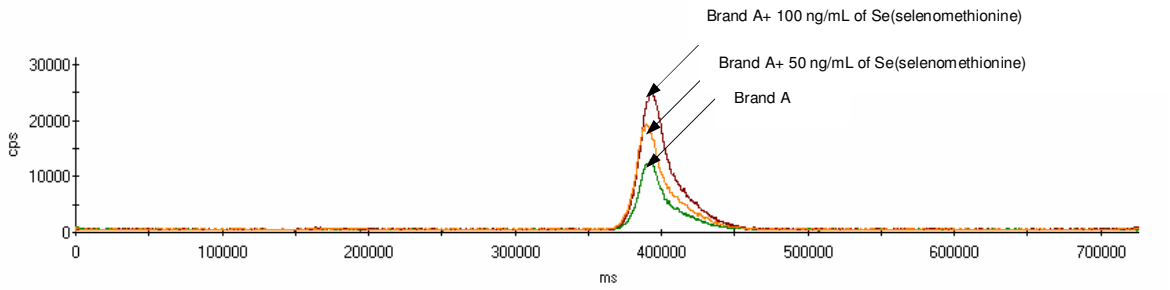

Figure 44. CX-HPLC-ICP-MS chromatograms of +0 Brand A, +50.0 ng/mL of Se(selenomethionine) added Brand A and +100.0 ng/mL of Se(selenomethionine) added Brand A using the parameters given in Table 34, CX-MP2.

External calibration method and standard addition method results are in good agreement. Hence, external calibration method was applied for further selenium supplement tablet studies unless there is a problem in the retention time, peak shape and other features of signal. Selenium amount in the form of selenomethionine per tablet was found as 114.1 ± 8.2 µg. These results are compatible with the label information.

A.3.8.1.2. Brand B

Brand B claims that each selenium supplement tablet includes 50 µg Se in the form of selenomethionine. Total amount of selenium in Brand B was found to be 54.4 ± 5.4 µg. It is clear that the result found agrees reasonably well with the label information regarding total Se content.

Extraction solution of Brand B was injected to CX-HPLC-ICP-MS system to find out whether reported value on the bottle as selenomethionine is compatible with experimental result. Chromatograms of Brand B and mixed selenium standards containing 100.0 ng/mL of Se(selenomethionine), Se(IV) and 50.0 ng/mL of Se(selenocystine), Se(VI) are shown in Figure 45.

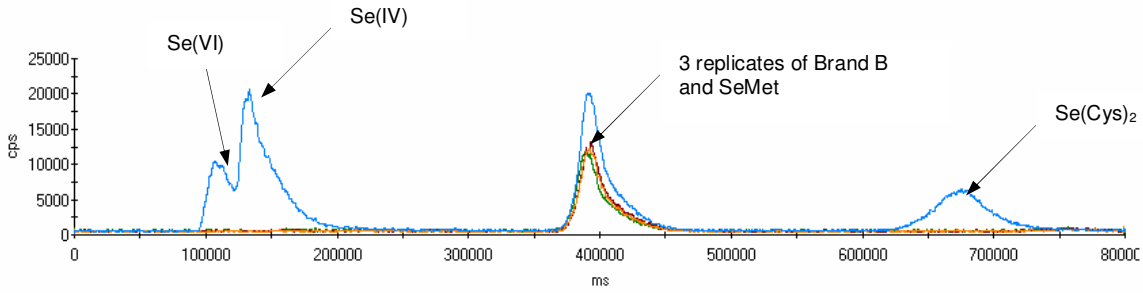

Figure 45. CX-HPLC-ICP-MS chromatograms of 3 replicates of Brand B and mixed selenium standards containing 100.0 ng/mL of Se(selenomethionine), Se(IV) and 50.0 ng/mL of Se(selenocystine), Se(VI) using the parameters given in Table 34, CX-MP2.

Retention times of the signals were exactly the same and peak shapes were not distorted. No other signal was observed in the chromatograms of Brand B except the selenomethionine peak. External calibration method was used for the quantitative measurement of selenomethionine. Selenium in the form of selenomethionine per tablet was found as 50.4 ± 2.6 µg. The results are compatible with the label information.

A.3.8.1.3. Brand C

In the bottle of Brand C, it is claimed that each selenium supplement tablet includes 25 µg Se in the form of selenomethionine. Determination of total selenium was done and total selenium amount per tablet was found to be 23.7 ± 1.1. Result found agrees reasonably well with the amounts of selenium reported on the bottle labels of Brand C.

Speciation analysis was performed using the extraction procedure and CX-HPLC-ICP-MS system with CX-MP2. As seen in Figure 46, there is a shift in the retention time of selenomethionine to shorter retention times. That is most probably due to interferences coming from the tablet matrix. In order to make sure that the signal in the retention time of 305 seconds belongs to selenomethionine, some experiments were performed. For this aim, Brand C was diluted with de-ionized water and injected to CX-HPLC-ICP-MS. In addition, Brand C extract was diluted and spiked with selenomethionine. Dilution factors for both experiments were same. All chromatograms can be seen in Figure 46.

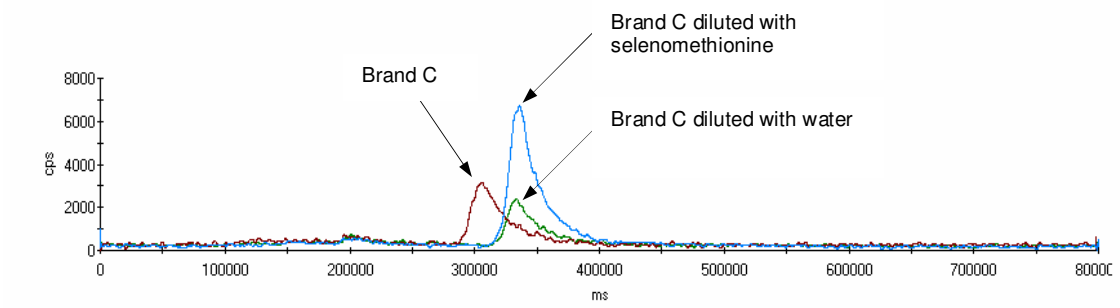

Figure 46. CX-HPLC-ICP-MS chromatograms of Brand C, Brand C diluted with water and Brand C diluted with selenomethionine standard using the parameters given in Table 34, CX-MP2.

As seen in Figure 46, selenium signal shifts to longer retention times after dilution with either water or selenomethionine standard. Diluted sample solutions have the same retention times. In order to eliminate matrix interference, standard addition method was applied to Brand C. All chromatograms obtained using standard addition method are shown in Figure 47.

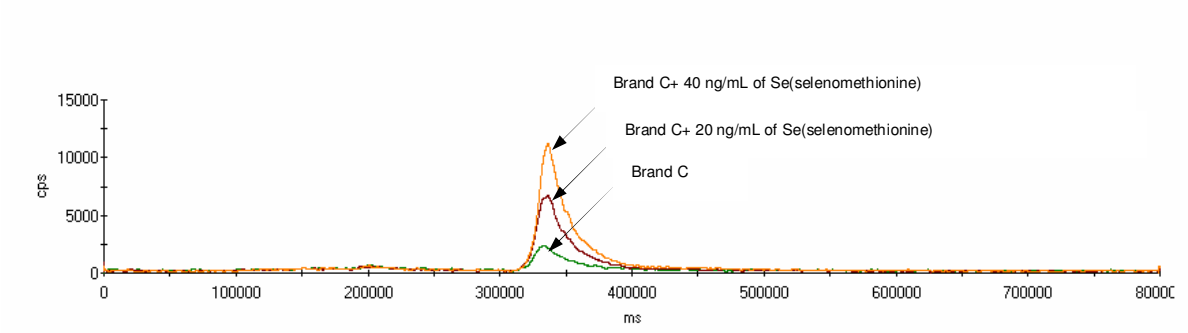

Figure 47. CX-HPLC-ICP-MS chromatograms of +0 (Brand C), +20.0 ng/mL of Se(selenomethionine) added Brand C and +40.0 ng/mL of Se(selenomethionine) added Brand C using the parameters given in Table 34, CX-MP2.

Peak areas of the signals were used to draw the calibration plot. Selenomethionine was the only species to be detected in Brand C. Amount of selenium per tablet was found as 23.9 ± 0.4 in the form of selenomethionine. This value agrees with the label value given on the bottle of Brand C.

A.3.8.1.4. Brand D

Brand D is one of the multivitamin tablets and it is stated that there is 25 µg Se in the form of selenomethionine per tablet. Amount of total selenium per tablet was determined and found to be 32.8 ± 1.6 µg. It is clear that the result found is higher than reported value on the bottle labels of Brand D. Extraction of Brand D was performed under the optimum conditions and the extract was injected to CX-HPLC-ICP-MS with CX-MP2 to find out whether selenomethionine is present in Brand D or not. Chromatogram can be seen in Figure 48.

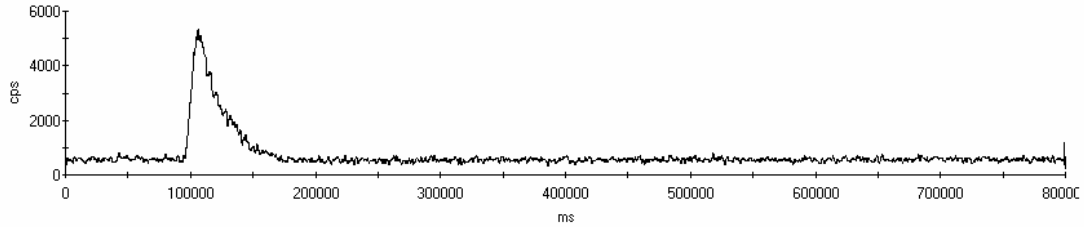

Figure 48. CX-HPLC-ICP-MS chromatogram of Brand D using the parameters given in Table 34, CX-MP2.

As seen in the Figure 48, there is no signal in the retention time of selenomethinone while there is a signal in the retention time of inorganic selenium. The both chromatograms of the mixed standard solution and Brand D are shown in Figure 49.

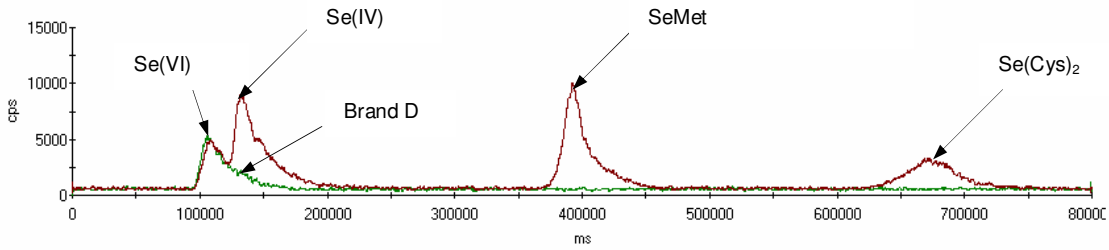

Figure 49. CX-HPLC-ICP-MS chromatograms of Brand D and 50.0 ng/mL of Se(selenomethionine), Se(IV) and 25.0 ng/mL of Se(selenocystine), Se(VI) using the parameters given in Table 34, CX-MP2.

Retention time of the signal in the chromatogram of Brand D is matching with retention time of Se(VI) in mixed standard. Resolution of Se(IV) and Se(VI) is not high enough for especially quantitative measurement. Hence, anion exchange chromatography was used to make quantitative measurement of selenium species. For this aim, extraction solution of Brand D was injected to AE-HPLC-ICP-MS system with AE-SP1, and the chromatogram shown in Figure 50 was obtained. In addition, Se(VI) is spiked to Brand D extract in order to make sure that the signal is due to Se(VI). As seen in Figure 50, retention times of both signals are exactly the same. Therefore, the signal obtained from Brand D extract is due to Se(VI).

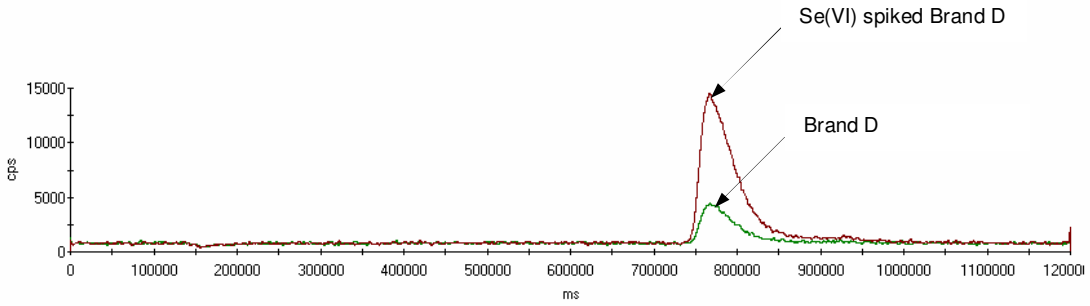

Figure 50. AE-HPLC-ICP-MS chromatograms of Brand D and Se(VI) spiked Brand D using the 1.5 mL/min of flow rate and parameters given in Table 29, AE-SP1.

One of the chromatograms of Brand D and mixed selenium standard containing 50.0 ng/mL of Se(VI), Se(IV) and 25.0 ng/mL of Se(selenocystine), Se(selenomethionine) are shown in Figure 51.

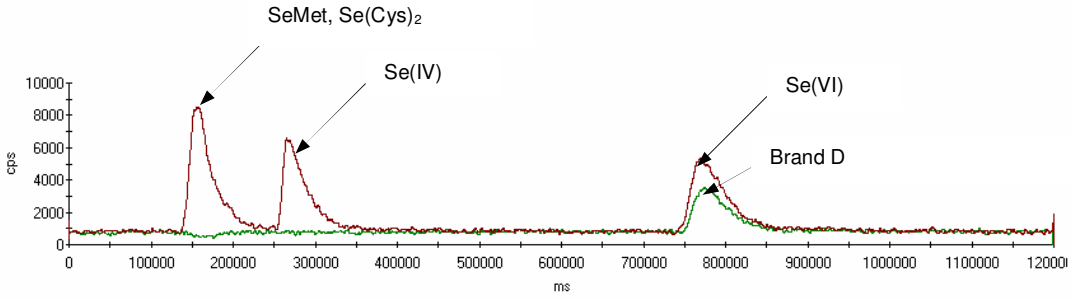

Figure 51. AE-HPLC-ICP-MS chromatograms Brand D and a mixed standard solution containing 50.0 ng/mL of Se(VI), Se(IV) and 25.0 ng/mL of Se(selenocystine), Se(selenomethionine) using the parameters given in Table 29, AE-SP1.

On the bottle of this brand, sample was reported to contain selenium in the form of selenomethionine, but no selenomethionine was detected in both CX-HPLC-ICP-MS and AE-HPLC-ICP-MS studies. Instead, 29.5 ± 2.0 µg selenium per tablet in the form of Se(VI) was found. The results are not compatible regarding the selenium species present.

A.3.8.1.5. Brand E

On the label of Brand E, it is reported that there is 25 µg Se in the form of selenate per tablet. Total selenium per tablet was determined found to be 26.0 ± 0.8 µg. The result found is in good agreement with the reported value on the bottle labels of Brand E. Extraction of Brand E was performed and the extraction solution was injected to CX-HPLC-ICP-MS with CX-MP2 to figure out which selenium species is present in Brand E. The one of the replicate chromatograms can be seen in Figure 52.

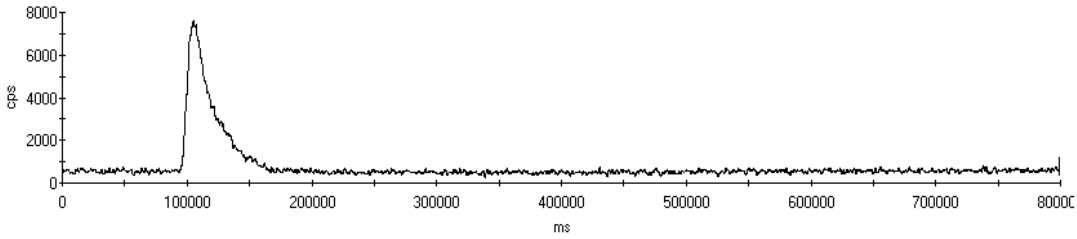

Figure 52. CX-HPLC-ICP-MS chromatograms of Brand E using the parameters given in Table 34, CX-MP2.

In Figure 52, it is shown that Se(VI) is present in the Brand E. There is no organic selenium species having the higher retention times in the chromatogram. The chromatograms obtained by both Brand E and mixed standard solution are shown in Figure 53.

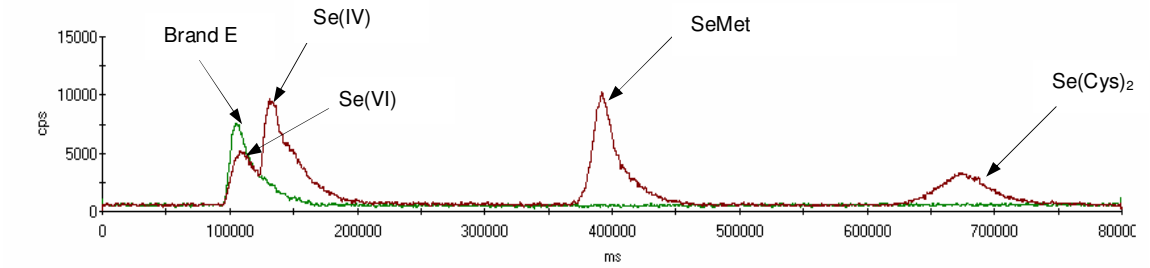

Figure 53. CX-HPLC-ICP-MS chromatograms of Brand E and 50.0 ng/mL of Se(selenomethionine), Se(IV) and 25.0 ng/mL of Se(selenocystine), Se(VI) parameters given in Table 34, CX-MP2.

In the figure above, retention time of the signal obtained from Brand E is matching with the retention time of Se(VI). Anion exchange chromatography having high resolution for Se(IV) and Se(VI) was also used to make a quantitative determination of selenium species. For this aim, extraction solution of Brand E and a mixed Se standard were injected to AE-HPLC-ICP-MS system with AE-SP1, and the chromatograms shown in Figure 54 were obtained.

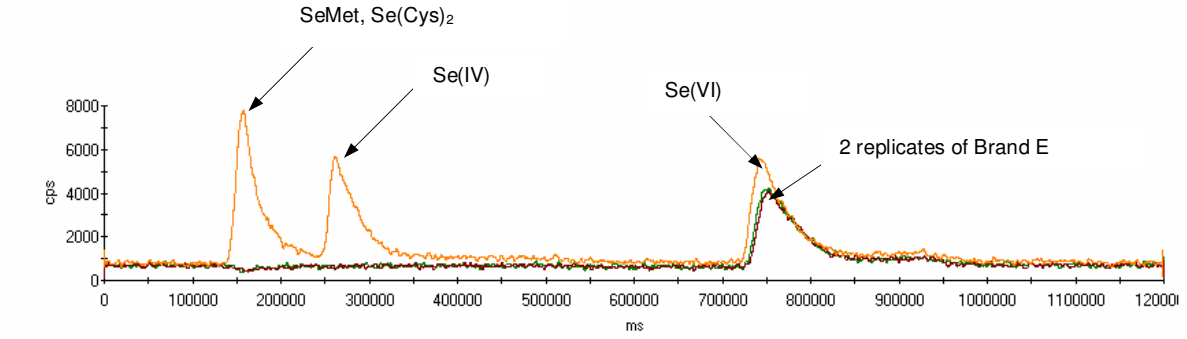

Figure 54. AE-HPLC-ICP-MS chromatograms Brand E and mixed standard solution containing 50.0 ng/mL of Se(VI), Se(IV) and 25.0 ng/mL of Se(selenocystine), Se(selenomethionine) using the parameters given in Table 29, AE-SP1.

Retention times of Se(VI) in 2 replicate measurments are exactly the same with the standard. Se(VI) was found to be present in Brand E. 25.2 ± 1.3 µg selenium per tablet in the form of Se(VI) were found. On the label of this brand, sample was reported to contain selenium in the form of selenate, and selenate was detected in both CX-HPLC-ICP-MS and AE-HPLC-ICP-MS studies. The results found compatible with the

label information.

A.3.8.1.6. Brand F

On the label of Brand F, it is stated that amount of selenium in the form of selenomethionine per tablet is 100 µg. Selenium amount was determined using standard addition method and total selenium per tablet was found to be 4.5 ± 0.2 µg. Brand F appears to contain very low amount of selenium as about 20 fold lower than the reported value. Extraction solution of Brand F was injected to CX-HPLC-ICP-MS with CX-MP2 in order to find out the chemical form of selenium. One of the chromatogram of replicate samples can be seen in Figure 55.

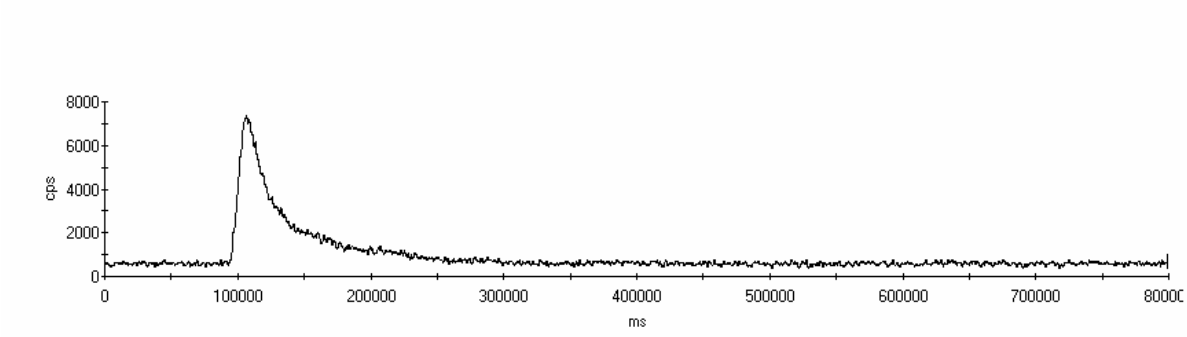

Figure 55. CX-HPLC-ICP-MS chromatogram of Brand F using the parameters given in Table 34, CX-MP2.

As seen in Figure 55, there is no signal in the retention time of selenomethinone while there is a signal in the retention time of inorganic selenium just like the Brand D. In order to make sure whether there is any shift in the selenomethionine signal to the lower retention times or not, selenomethionine was spiked to extraction solution of Brand F, and this solution was injected to CX-HPLC-ICP-MS system. Chromatograms for both Brand F and selenomethionine spiked Brand F are shown in Figure 56.

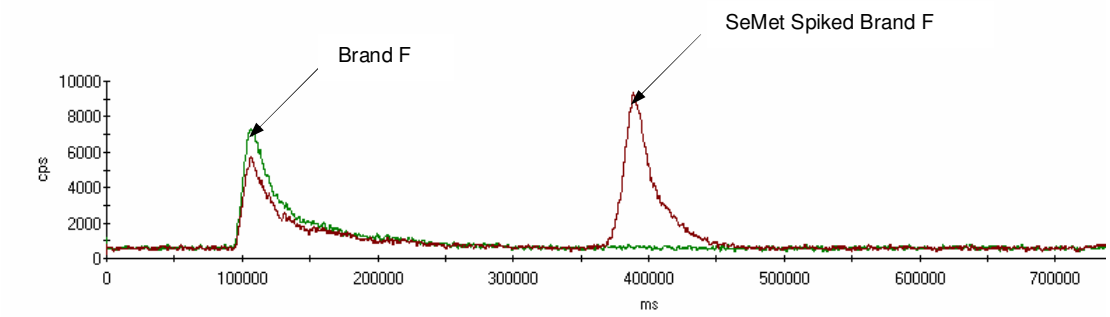

Figure 56. CX-HPLC-ICP-MS chromatograms of Brand F and selenomethionine spiked Brand F using the parameters given in Table 34, CX-MP2.

It is evident in Figure 56 that there is no shift in the retention time of selenomethionine in the matrix of Brand F and thus there is only one signal in the chromatogram of Brand F. This shows that selenomethionine is not present in Brand F unlike it is stated on the bottle of this vitamin. Extraction solution of this brand was injected to AE-HPLC-ICP-MS to make a quantitative measurement of inorganic selenium species. In Figure 57, the chromatograms obtained by Brand F and a selenium mixed standard containing 100.0 ng/mL of Se(VI), Se(IV) and 50.0 ng/mL of Se(selenocystine), Se(selenomethionine) can be seen.

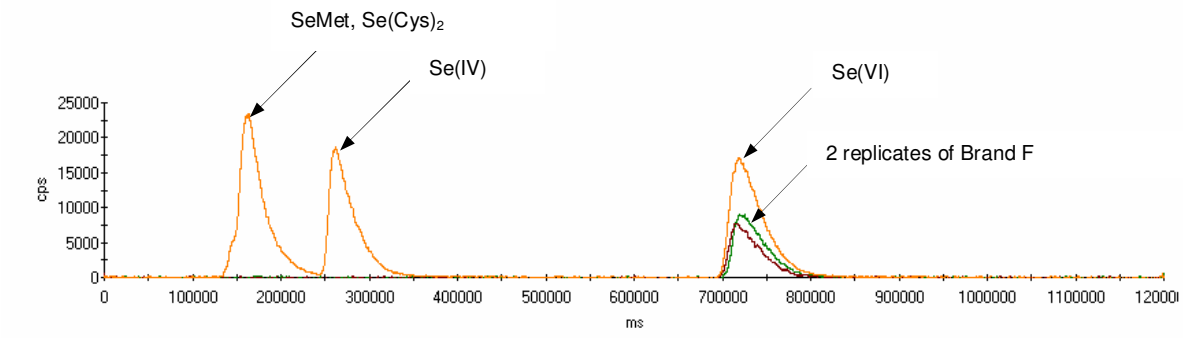

Figure 57. AE-HPLC-ICP-MS chromatograms of Brand F and 100.0 ng/mL of Se(VI), Se(IV) and 50.0 ng/mL of Se(selenocystine), Se(selenomethionine) using the parameters given in Table 29, AE-SP1.

It is clear in Figure 57 that retention time of the signals obtained from Brand F is matching with the retention time of Se(VI). Therefore, Brand F appears to have only inorganic selenium, Se(VI), despite the label claim. There is also no matrix effect to cause any shift in Se(VI) signals. Amount of Se(VI) was found as 4.3 ± 1.0 µg. This selenium supplement brand was not found to have near label Se values based on total

selenium. In addition, the chemical form of Brand F is dramatically different within the supplement.

Total amount of selenium for 6 different brands obtained using ICP-MS measurement after nitric acid digestion and amounts of Se(IV), Se(VI), Se(selenomethionine) and Se(selenocystine) obtained using CX-HPLC-ICP-MS with CX-MP2 and AE-HPLC-ICP-MS with AE-SP1 measurement after Extraction Method 1 are given in Table 40.

Table 40. Composite assay for total selenium and selenium species.

Sample	Results in µg Se/Tablet, N=3, Mean ± S.D.					
	Value given on label	Total Selenium (N=2)	Se(IV)	Se(VI)	Se(SeMet)	$Se(Se(Cys)_2)$
Brand A	100 (SeMet)	109.1 ± 1.5	N.D.	N.D.	114.1 ± 8.2	N.D.
Brand B	50 (SeMet)	54.4 ± 5.4	N.D.	N.D.	50.4 ± 2.6	N.D.
Brand C	25 (SeMet)	23.7 ± 1.1	N.D.	N.D.	23.9 ± 0.4 (Std. Add.)	N.D.
Brand D	25 (SeMet)	32.8 ± 1.6	N.D.	29.5 ± 2.0	N.D.	N.D.
Brand E	25 (Se(VI))	26.0 ± 0.8	N.D.	25.2 ± 1.3	N.D.	N.D.
Brand F	100 (SeMet)	4.5 ± 0.2	N.D.	4.3 ± 1.0	N.D.	N.D.

N.D.=Not Detected

A.3.9. Speciation of Selenium in Chicken Samples

Chicken breast samples were taken from Bursa and Kayseri. It was claimed that all these samples contain high amount of selenium because chickens were fed with selenium enriched diets. Standard addition and direct calibration methods were applied to one of the samples to decide which one of these methods is proper for further studies. The chromatogram of a mixed standard solution using the parameters given in Table 33, CX-MP1, is given in Figure 58. Chromatogram of extraction solution of Sample A can also be seen in Figure 59.

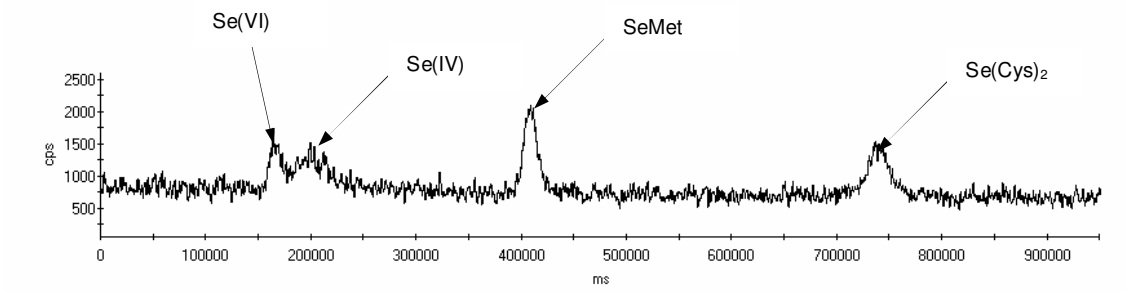

Figure 58. Chromatograms of mixed standard solutions in Signals obtained using 5.0 ng/mL of Se(VI), Se(IV), Se(selenocystine) and Se(selenomethionine) species injected to cation exchange-HPLC-ICP-MS system by using the parameters given in Table 33, CX-MP1.

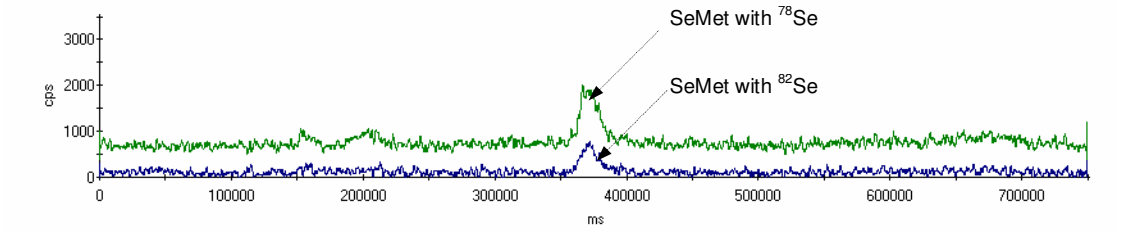

Figure 59. Cation Exchange-HPLC-ICP-MS chromatograms of sample A from Kayseri, Turkey using the parameters given in Table 33, CX-MP1.

As seen in Figure 59, there is a selenomethinone signal with the retention time of 370 s. In addition, there are two very small signals at earlier elution time. These are most probably some inorganic selenium species, but signal/noise ratio for these species are not high enough to make quantitative measurement. Hence, concentrations corresponding to these signals were not calculated. As seen in Figure 59, ^{78}Se and ^{82}Se signals have the same retention times as expected. This shows that signals did belong to selenium and there were no spectral intereference to affect the analysis. Although ^{78}Se has a higher baseline when compared with ^{82}Se due to $^{40}Ar^{38}Ar^{+}$ interference, baseline of ^{78}Se chromatogram is constant. Hence, there is no problem in using ^{78}Se in the calculations. In all calculations, both ^{78}Se and ^{82}Se signals were taken into consideration to verify the results.

In order to decide whether we have any matrix interference in the sample, standard addition method was applied to a selected sample called Sample A. In Figure 60, chromatograms of Sample A obtained by the addition of different amount of selenomethionine are shown.

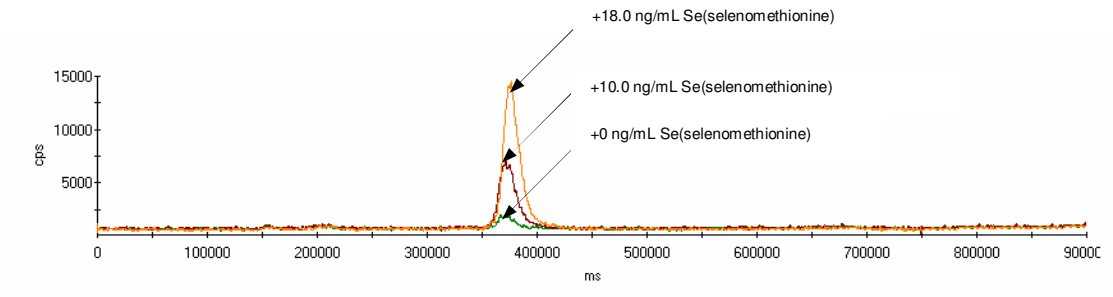

Figure 60. Cation Exchange-HPLC-ICP-MS chromatograms of Sample A (+0, +10, +18 ng/mL of Se(selenomethionine)) from Kayseri in Turkey using the parameters given in Table 33, CX-MP1.

As seen in Figure 60, retention times of all the signals are very close to each other and peak symmetries are sufficiently good to make qualitative and quantitative measurements.

Concentration of Se(selenomethionine) was calculated using direct calibration method as well. Concentration of Se(selenomethionine) in ng/g (dry mass) was calculated as 234 ± 21 using direct calibration method and 227 ± 20 using standard addition method. As it is seen, there is no significantly difference in the results obtained by applying two methods. This indicates that there was no significant interference effect due to sample matrix and standard addition method was not used. Hence, direct calibration method was applied to all samples for further experiments of this study.

Chromatogram of Sample C from Bursa can be seen in Figure 61.

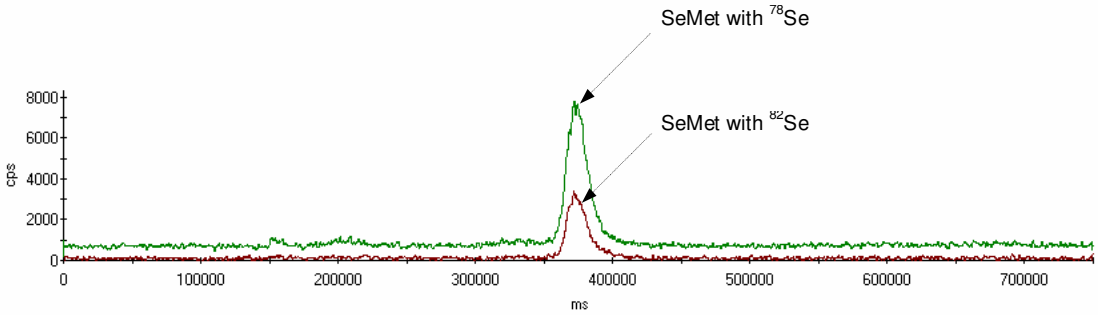

Figure 61. Cation Exchange-HPLC-ICP-MS chromatogram of Sample C from Bursa in Turkey using the parameters given in Table 33, CX-MP1.

In the chromatogram of Sample C, a symmetric selenomethionine signal was observed. Concentration of Se(selenomethionine) was calculated as 1174 ± 81 ng/g in dry mass. Selenomethionine in Sample C was found as about 4 times more than the Sample A and B. Other selenium species of interest were not observed. Same signals at the same retention times were observed in both chromatograms of selenium isotopes, ^{78}Se and ^{82}Se. This shows that signals belong to selenium and there were no spectral intereference to affect the analysis. Similar observation was observed for Sample D.

Concentrations of selenium species in four chicken breast samples taken form Bursa and Kayseri are given in Table 41.

Table 41. Concentration of selenium species in 4 chicken breast samples after extraction procedure.

Sample	Se found in sample, Mean ± S.D., N=2, ng/g (dry mass)				
	Total Se	Se(IV)	Se(VI)	Se(SeMet)	Se(Se(Cys)$_2$)
Sample A from Kayseri	508 ± 18	N.D.	N.D.	234 ± 21	N.D.
Sample B from Kayseri	475 ± 19	N.D.	N.D.	223 ± 20	N.D.
Sample C from Bursa	2224 ± 112	N.D.	N.D.	1174 ± 81	N.D.
Sample D from Bursa	1788 ± 93	N.D.	N.D.	1077 ± 89	N.D.

N.D.: Not detected

In Table 41, selenomethionine concentrations in Sample C and D taken from Bursa were higher than Sample A and B taken from Kayseri. Although there are very small signals with the same retention times of Se(IV) and Se(VI), these signals are not enough to make quantitative measurements. Hence, in all samples, these species were not detected. There is no selenocystine signal in the extraction solution of all samples.

A.3.10. Selenium Speciation in Control, Inorganic and Organic Selenium Fed Chicken Breast Samples

Chicken breast samples were taken from chickens fed Se enriched diet were freeze-dried using the procedure given in “Experimental” part.

Extraction process was applied for lyophilized chicken breast samples to extract selenium content. This extraction process is different than the process given in “Experimental” part. Shaker and sonicator were used as extraction devices. In the extraction step, 200.0 mg of sample was weighed and put into a 15 mL centrifugation tube and then sample was suspended in 10.0 mL of 30.0 mM of Tris HCl buffered to 7.2. 20.0 mg of protease XIV were also added to centrifuge tube as the enzyme. Samples were shaken for 24 h in shaker at room temperature (24-27 ^{o}C). After shaking, samples were sonicated 60 min at room temperature. After extraction process, samples were filtered using 10.0 KDa ultrafiltration membranes (Filter Code: YM10 Dia: 63.5 mm). In the ultrafiltration cell, argon was used as purging gas. Filtered solutions were injected to Cation Exchange-HPLC-ICP-MS system under conditions given in Table 35, CX-MP3. The quality control was performed by systematic control of the column and spike recoveries throughout the study.

Sample codes of 75 chicks in control group, inorganic selenium fed group and organic selenium fed group are given in Table 42.

Table 42. Sample codes of 75 chicks in control group, inorganic selenium fed group and organic selenium fed group.

CONTROL GROUP	1D – 1a	1D – 2a	1D – 3a	1D – 4a	1D – 5a
	1D – 1b	1D – 2b	1D – 3b	1D – 4b	1D – 5b
	1D – 1c	1D – 2c	1D – 3c	1D – 4c	1D – 5c
	1D – 1d	1D – 2d	1D – 3d	1D – 4d	1D – 5d
	1D – 1e	1D – 2e	1D – 3e	1D – 4e	1D – 5e
INORGANIC Se GROUP	2D – 1a	2D – 2a	2D – 3a	2D – 4a	2D – 5a
	2D – 1b	2D – 2b	2D – 3b	2D – 4b	2D – 5b
	2D – 1c	2D – 2c	2D – 3c	2D – 4c	2D – 5c
	2D – 1d	2D – 2d	2D – 3d	2D – 4d	2D – 5d
	2D – 1e	2D – 2e	2D – 3e	2D – 4e	2D – 5e
ORGANIC Se GROUP	3D – 1a	3D – 2a	3D – 3a	3D – 4a	3D – 5a
	3D – 1b	3D – 2b	3D – 3b	3D – 4b	3D – 5b
	3D – 1c	3D – 2c	3D – 3c	3D – 4c	3D – 5c
	3D – 1d	3D – 2d	3D – 3d	3D – 4d	3D – 5d
	3D – 1e	3D – 2e	3D – 3e	3D – 4e	3D – 5e

A.3.10.1. Control Group Chicken Sample

Chromatogram of mixed selenium standard containing 50.0 ng/mL of Se(IV), Se(VI) and 25.0 ng/mL of Se(selenomethionine), Se(selenocystine) using the parameters given in Table 35 can be seen in Figure 62.

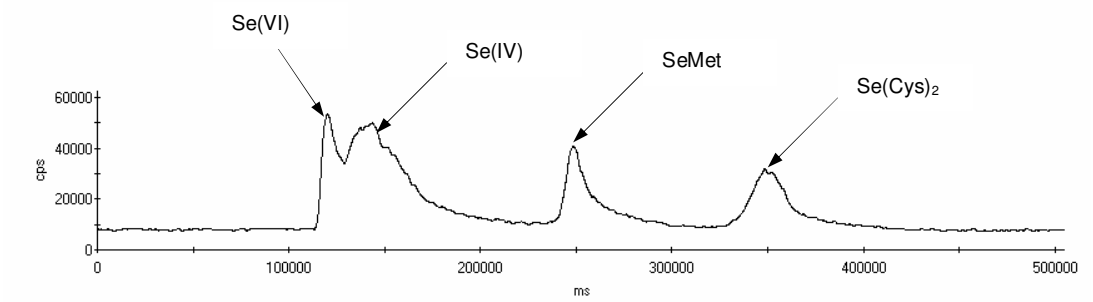

Figure 62. Chromatogram of mixed selenium standard including 50.0 ng/mL of Se(IV), Se(VI) and 25.0 ng/mL of Se(selenomethionine), Se(selenocystine) using the parameters given in Table 35, CX-MP3.

Extraction solutions of 25 chicken breast samples in control group were injected to CX-HPLC-ICP-MS system to make speciation analysis of selenomethionine and selenocystine. Chromatograms of 1D-1a, 1D-1b, 1D-1c, 1D-1d and 1D-1e samples are shown in Figure 63.

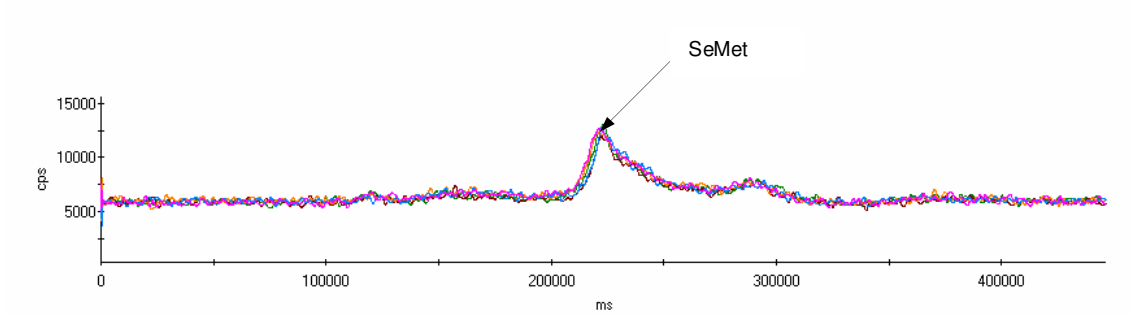

Figure 63. CX-HPLC-ICP-MS ^{78}Se chromatograms of 1D-1a, aD-1b, 1D-1c, 1D-1d and 1D-1e samples extracts in control group using the parameters given in Table 35, CX-MP3.

In addition to ^{78}Se chromatograms, other selenium isotopes, ^{74}Se, ^{76}Se, ^{77}Se and ^{82}Se, were also monitored to make sure that any signal in the chromatogram is due to selenium. ^{82}Se chromatograms of the same 5 chicken samples in control group can be seen in Figure 64.

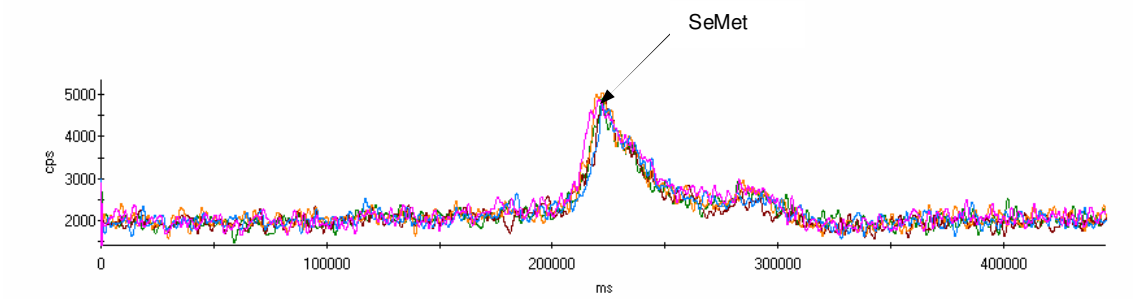

Figure 64. CX-HPLC-ICP-MS ^{82}Se chromatograms of 1D-1a, aD-1b, 1D-1c, 1D-1d and 1D-1e sample extracts in control group using the parameters given in Table 35, CX-MP3.

It is clear in both Figure 63 and Figure 64, there are no signals in the retention times of both inorganic species and selenocystine. There is only one species having the retention time at 220 s in the chicken samples for the quantitative measurements. In addition to this signal, there is another very small signal having the retention time of 285 s. Peak height of this signal is very close to noise level. Retention time of signal at 220 seconds is not matching with selenomethionine signal shown in Figure 62 at 250 s. There is a shift in selenomethionine signal of sample 1D-1a to lower retention time. In order to make sure whether this signal is due to selenomethionine, a spiking experiment was performed. For this aim, selenomethionine was spiked to one of the chicken breast extract solutions. In addition, same amount of selenomethionine was spiked to a more diluted sample to observe the shift to higher retention time. All the chromatograms can be seen in Figure 65.

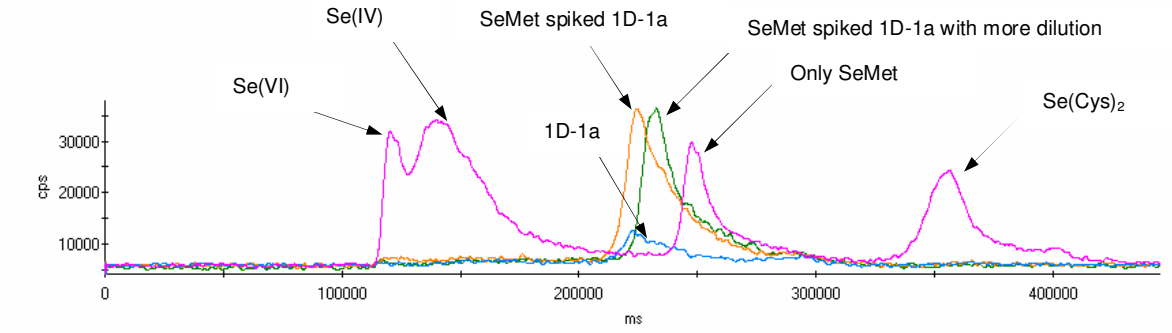

Figure 65. CX-HPLC-ICP-MS chromatograms of 1D-1a extract, selenmethionine spiked 1D-1a in different dilutions and mixed selenium standard using the parameters given in Table 35.

It is shown in Figure 65 that retention times of all species obtained from sample and selenomethionine spiked sample are same. Retention time of selenomethionine spiked more diluted samples was getting closer to the retention time of selenomethinone signal in standard solution. It means that shift in the retention time is most probably because of high content of matrix. By dilution, this shifting is lowered. Hence, it can be seen that the signal having the retention time of 220 s is due to selenomethionine. These differences in the retention times of standard selenium solution and real sample extracts have been also observed in literature. Moreno et al. studied stability of total selenium and selenium species in lyophilized oysters and in their enzymatic extracts [161]. In this study, there were differences in the retention times of selenomethionine obtained from 10.0 ppb of Se as Se species using cationic exchange chromatography (using 4 mM pyridine, pH=2.8, 1.0 mL/min), the liquid extract after enzymatic hydrolysis and ultrafiltration with 10.0 KDa cut-off filters and the solid residue after enzymatic hydrolysis and ultrafiltration with 10.0 KDa cut-off filters. There was about 100 seconds shift to lower retention time in real samples [161]. In addition, the similar shifts in selenomethionine and some other selenium peaks were observed in the study published by Kotrebai et al; in this study, HPLC was used for the separation of selenium species using perfluorinated carboxylic acid ion-pairing agents, and inductively coupled plasma and electrospray ionization mass spectrometric detection was used for detection [137]. Trifluoroacetic acid (0.1%), pentafluoropropanoic acid (0.1%) or heptafluorobutanoic acid (0.1%; HFBA) were used as ion-pairing agents in methanol–water (1:99, v/v) solutions as mobile phases. Using reversed-phase HPLC–ICP-MS with 0.1% HFBA as mobile phase more than 20 selenium compounds were separated in 70 minutes in an isocratic elution mode. The retention time of selenomethionine was found as 12.96 minutes using the selenium standard mixture with 0.1% HFBA as ion-pairing agent while this value was about 11 min in the yeast and garlic extracts using the same mobile phase. Indeed, there was a shift in the signal of Gama-glutamyl-Se-methylselenocysteine when the chromatograms of standard solution and yeast and garlic extracts are compaired due to matrix content [137].

Extraction solution of two samples in control group was also injected to AE-HPLC-ICP-MS with AE-SP1 system to prove that there is no inorganic selenium species in the control group samples. Chromatograms can be seen in Figure 66.

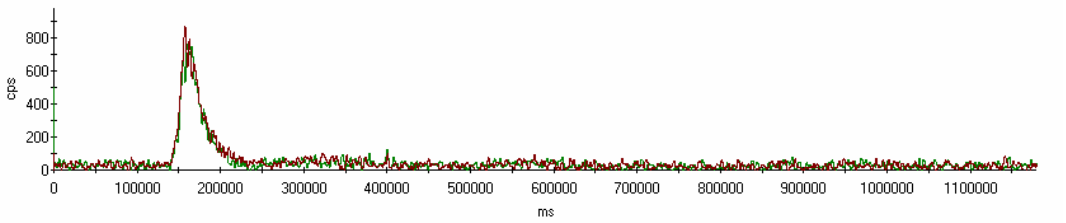

Figure 66. AE-HPLC-ICP-MS chromatograms of extraction solutions of control group samples using the parameters given in Table 29, AE-SP1.

As seen in Figure 66, there is no inorganic selenium signal in the chromatogram of control group samples using AE-HPLC-ICP-MS method. Organometallic selenium species, selenomethionine, was not retained in column and was eluted in dead time.

Previous chicken breast studies from Kayseri and Bursa shows that there is no need to use standard addition method in the calculation of selenomethionine content. Slopes of both external calibration method and standard addition method were found to be very close to each other. Hence, external calibration method was applied to make quantitative measurement of selenium species. In the calculation, peak area of signal, ICPS, was used. All the results for control group samples can be seen in Table 43.

Table 43. Concentration of Se(IV), Se(VI), Se(selenomethionine) and Se(selenocystine) in the control group samples.

Sample No	Se found in sample, Mean ± S.D., ng/g (dry mass)				
	Total Se	Se(IV)	Se(VI)	Se(selenomethionine)	Se(selenocystine)
1D – 1a	694 ± 112	N.D.	N.D.	493 ± 42	N.D.
1D – 1b	766 ± 77	N.D.	N.D.	475 ± 40	N.D.
1D – 1c	657 ± 101	N.D.	N.D.	503 ± 43	N.D.
1D – 1d	673 ± 21	N.D.	N.D.	517 ± 44	N.D.
1D – 1e	611 ± 84	N.D.	N.D.	480 ± 41	N.D.
1D – 2a	530 ± 120	N.D.	N.D.	400 ± 31	N.D.
1D – 2b	704 ± 29	N.D.	N.D.	484 ± 38	N.D.
1D – 2c	694 ± 139	N.D.	N.D.	456 ± 36	N.D.
1D – 2d	663 ± 42	N.D.	N.D.	492 ± 39	N.D.
1D – 2e	588 ± 7	N.D.	N.D.	459 ± 36	N.D.
1D – 3a	672 ± 157	N.D.	N.D.	518 ± 41	N.D.
1D – 3b	743 ± 77	N.D.	N.D.	529 ± 42	N.D.
1D – 3c	541 ± 61	N.D.	N.D.	496 ± 39	N.D.
1D – 3d	616 ± 15	N.D.	N.D.	513 ± 40	N.D.
1D – 3e	577 ± 154	N.D.	N.D.	486 ± 38	N.D.
1D – 4a	680 ± 71	N.D.	N.D.	435 ± 37	N.D.
1D – 4b	666 ± 66	N.D.	N.D.	460 ± 39	N.D.
1D – 4c	683 ± 38	N.D.	N.D.	443 ± 38	N.D.
1D – 4d	689 ± 46	N.D.	N.D.	446 ± 38	N.D.
1D – 4e	597 ± 69	N.D.	N.D.	419 ± 35	N.D.
1D – 5a	687 ± 50	N.D.	N.D.	517 ± 41	N.D.
1D – 5b	710 ± 144	N.D.	N.D.	509 ± 40	N.D.
1D – 5c	759 ± 36	N.D.	N.D.	500 ± 39	N.D.
1D – 5d	954 ± 38	N.D.	N.D.	545 ± 42	N.D.
1D – 5e	731 ± 5	N.D.	N.D.	488 ± 38	N.D.

N.D.: Not detected

Mass balance of the system was done by the sum of the determined species in comparison with the total selenium concentration. As given before, determination of total selenium was validated by the analysis of DOLT-4 and 1566b Oyster Tissue CRMs. Selenomethione was the only species quantified using CX-HPLC-ICP-MS method. Percent recovery for the determined species, selenomethionine, was changing from 57% to 92%. Average recovery was found as 72 ± 8. There was one signal that was not quantified in the chromatogram.

A.3.10.2. Inorganic Selenium Fed Group Chicken Samples

Inorganic selenium fed chicken samples were extracted using the same method described above. Shaker and sonicator were used as extraction devices. Extraction solutions were injected to CX-HPLC-ICP-MS system with CX-MP3 to make speciation analysis of selenomethionine and selenocystine. Chromatograms of 2D-1a, 2D-1b, 2D-1c, 2D-1d and 2D-1e samples in inorganic selenium fed group are shown in Figure 67.

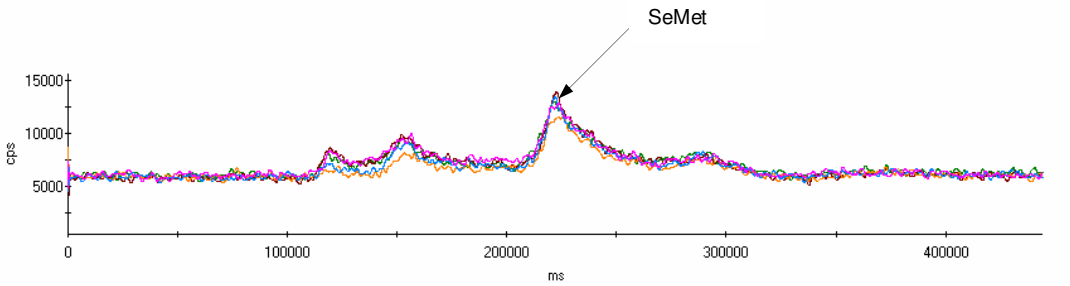

Figure 67. CX-HPLC-ICP-MS ^{78}Se chromatograms of 2D-1a, 2D-1b, 2D-1c, 2D-1d and 2D-1e samples extracts in inorganic selenium fed group using the parameters given in Table 35, CX-MP3.

^{74}Se, ^{76}Se, ^{77}Se and ^{82}Se isotopes were also monitored to assure that the signal in the chromatogram of ^{78}Se is not due to any interferences. Chromatograms of 2D-1a, 2D-1b, 2D-1c, 2D-1d and 2D-1e samples in inorganic selenium fed chicken group by monitoring ^{82}Se isotope are shown in Figure 68.

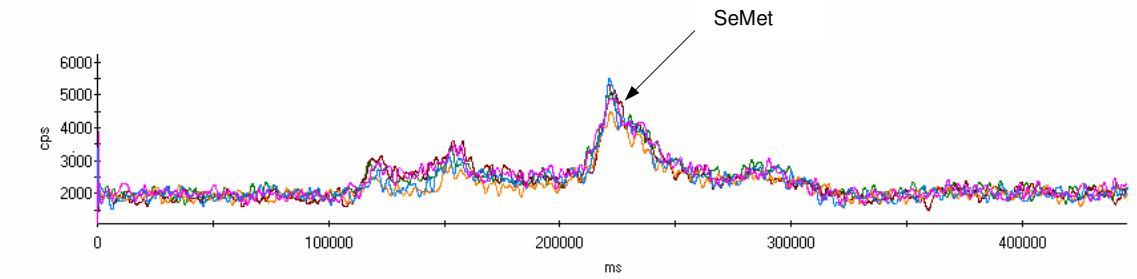

Figure 68. CX-HPLC-ICP-MS ^{82}Se chromatograms of 2D-1a, 2D-1b, 2D-1c, 2D-1d and 2D-1e samples extracts in inorganic selenium fed group using the parameters given in Table 35, CX-MP3.

As seen in Figure 67 and Figure 68, in addition to the selenomethionine signal, there are 3 signals having the retention times of 120, 155 and 285 s, respectively. The retention times of signals at 120 and 155 seconds are very close to inorganic selenium species in standard samples. Hence, extraction solutions were also injected to AE-HPLC-ICP-MS system to find out whether these signals are due to inorganic selenium species. The chromatograms of 2D-1a, 2D-1b, 2D-1c, 2D-1d and 2D-1e samples in inorganic selenium fed group using AE-HPLC-ICP-MS system with AE-SP1 are shown in Figure 69.

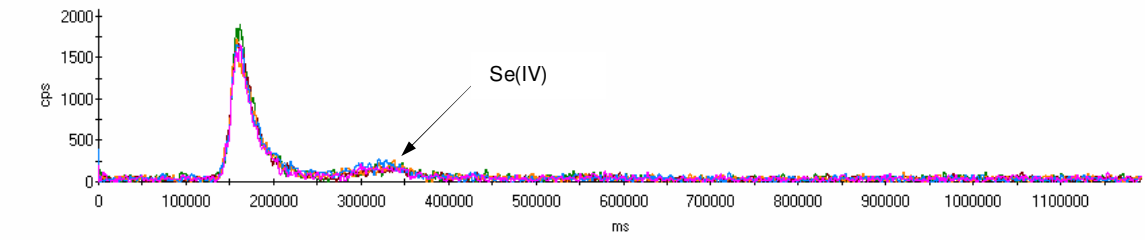

Figure 69. AE-HPLC-ICP-MS chromatograms of 2D-1a, 2D-1b, 2D-1c, 2D-1d and 2D-1e sample extraction solutions using the parameters given in Table 29, AE-SP1.

As seen in Figure 69, Se(IV) was observed in inorganic selenium fed group samples. The signals of Se(VI) was not observed in the chromatograms. It means that signal having the retention times of 155, 285 s do not belong to inorganic selenium species and they are eluted in the dead time. Se(IV) signals can be easily seen in Figure 70.

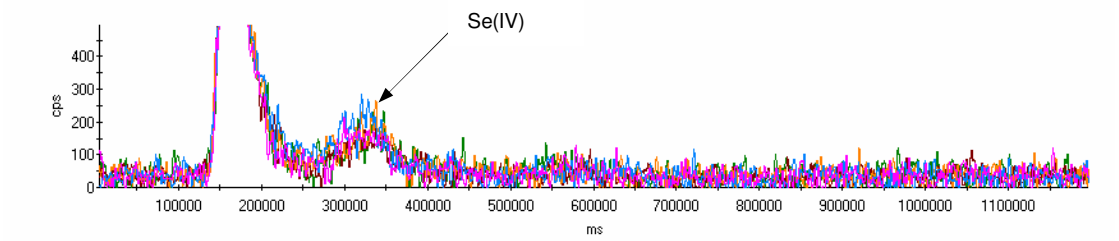

Figure 70. Se(IV) signals of 2D-1a, 2D-1b, 2D-1c, 2D-1d and 2D-1e using the parameters given in Table 29, AE-SP1.

Se(IV) was also spiked to one of the inorganic selenium fed chicken to assure that the signal having the retention time of 325 s is due to Se(IV). AE-HPLC-ICP-MS chromatogram of one of the chicken (2D-4e) in selenium fed group and Se(IV) spiked chicken (2D-4e) are shown in Figure 71.

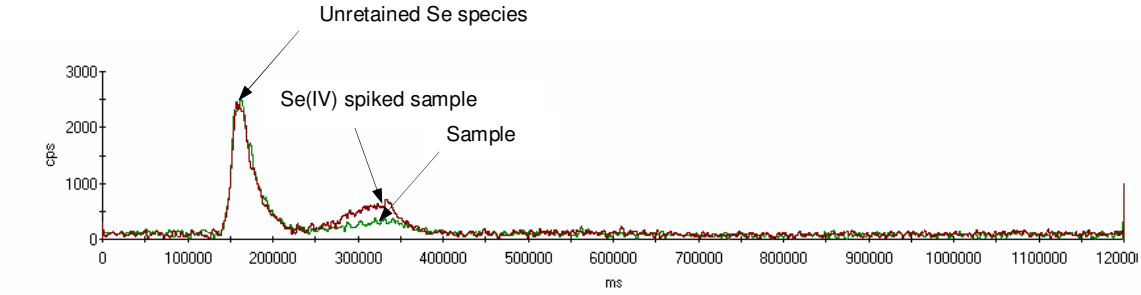

Figure 71. AE-HPLC-ICP-MS chromatograms of the chicken (2D-4e) in selenium fed group and Se(IV) spiked chicken (2D-4e) using the parameters given in Table 29, AE-SP1.

In Figure 71, retention times of the signals are exactly the same for both Se(IV) spiked and un-spiked samples. Hence, it can be concluded that signal having the retention time of 325 s is due to Se(IV). Similar experiments were performed for Se(VI) to show that there is no Se(VI) in the samples. For this aim, Se(VI) was spiked at low concentration to the extraction solution of chicken sample (2D-4e). Chromatograms can be seen in Figure 72.

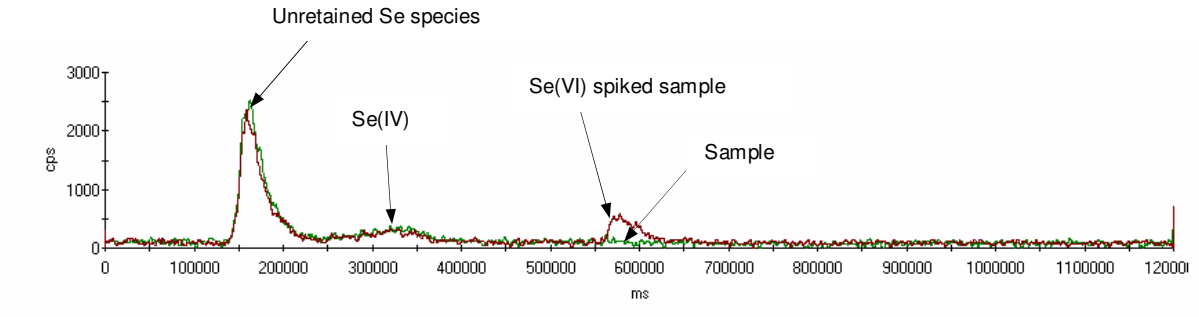

Figure 72. AE-HPLC-ICP-MS chromatograms of the chicken (2D-4e) in selenium fed group and Se(VI) spiked chicken (2D-4e) using the parameters given in Table 29, AE-SP1.

It was observed that there is one additional signal in addition to Se(IV) and unretained selenium species in the chromatogram of Se(VI) spiked sample. This signal has the retention time of 570 s. In the chromatogram of mixed standard solution, signal of Se(VI) is at 720 s. In Figure 72, the signal of spiked Se(VI) shifts to a lower retention time just like the selenomethionine in cation exchange column studies, but there was no Se(VI) signal observed in inorganic selenium fed chicken samples. In the calculation of Se(IV) concentration, external calibration method was applied and peak area of signal was used to draw linear calibration plot. All the results for inorganic selenium fed group samples are given in Table 44.

Table 44. Concentration of Se(IV), Se(VI), Se(selenomethionine) and Se(selenocystine) in inorganic selenium fed group samples.

Sample No	Se found in sample, Mean ± S.D., ng/g (dry mass)				
	Total Se	Se(IV)	Se(VI)	Se(selenomethionine)	Se(selenocystine)
2D – 1a	1073 ± 111	204 ± 42	N.D.	476 ± 41	N.D.
2D – 1b	1487 ± 154	191 ± 39	N.D.	517 ± 45	N.D.
2D – 1c	781 ± 81	130 ± 27	N.D.	461 ± 40	N.D.
2D – 1d	1107 ± 114	186 ± 38	N.D.	528 ± 45	N.D.
2D – 1e	1152 ± 119	177 ± 36	N.D.	521 ± 45	N.D.
2D – 2a	1006 ± 104	147 ± 30	N.D.	521 ± 45	N.D.
2D – 2b	1056 ± 109	211 ± 43	N.D.	514 ± 41	N.D.
2D – 2c	1098 ± 114	158 ± 33	N.D.	552 ± 48	N.D.
2D – 2d	884 ± 91	201 ± 41	N.D.	501 ± 43	N.D.
2D – 2e	976 ± 101	147 ± 30	N.D.	499 ± 43	N.D.
2D – 3a	921 ± 95	132 ± 27	N.D.	477 ± 48	N.D.
2D – 3b	1224 ± 127	140 ± 28	N.D.	488 ± 50	N.D.
2D – 3c	640 ± 66	166 ± 34	N.D.	443 ± 45	N.D.
2D – 3d	1448 ± 150	201 ± 41	N.D.	570 ± 58	N.D.
2D – 3e	928 ± 96	145 ± 30	N.D.	449 ± 46	N.D.
2D – 4a	1167 ± 121	101 ± 21	N.D.	484 ± 49	N.D.
2D – 4b	1361 ± 141	146 ± 30	N.D.	498 ± 51	N.D.
2D – 4c	1322 ± 137	174 ± 35	N.D.	516 ± 52	N.D.
2D – 4d	1198 ± 124	259 ± 53	N.D.	472 ± 48	N.D.
2D – 4e	1097 ± 113	196 ± 40	N.D.	514 ± 52	N.D.
2D – 5a	1128 ± 117	145 ± 29	N.D.	480 ± 49	N.D.
2D – 5b	1170 ± 121	137 ± 28	N.D.	437 ± 44	N.D.
2D – 5c	1050 ± 109	138 ± 28	N.D.	518 ± 53	N.D.
2D – 5d	944 ± 98	105 ± 21	N.D.	501 ± 51	N.D.
2D – 5e	890 ± 92	110 ± 22	N.D.	465 ± 47	N.D.

N.D.: Not detected

Mass balance of the samples in the inorganic selenium fed group was also done by the sum of the determined species in the chromatogram in comparison with the total selenium concentration. Selenomethionine and Se(VI) were the species quantified using anion and cation exchange HPLC-ICP-MS methods. There were two undetermined species in the chromatograms. Percent recovery for the determined species, selenomethionine and Se(VI), varied from 47% to 95%. Average recovery was found as 62 ± 11 because of two undetermined species. Average value found for inorganic selenium fed group was lower than value found for control group. This is due to one additional undetermined species in inorganic selenium fed group when compared with the control group.

A.3.10.3. Organic Selenium Fed Group Chicken Samples

Samples in organic selenium fed group were extracted and extraction solutions of 25 chicken breast were injected to CX-HPLC-ICP-MS system with CX-MP3 to find the concentration of selenomethionine and selenocystine. Shaker and sonicator were used as extraction devices. Chromatograms of 3D-1a, 3D-1b, 3D-1c, 3D-1d and 3d-1e samples are shown in Figure 73.

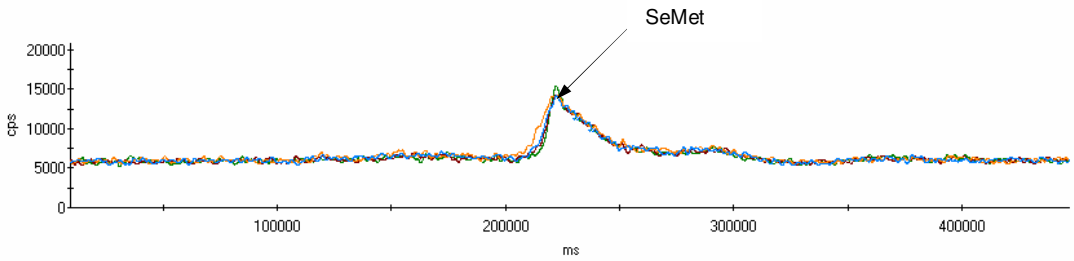

Figure 73. CX-HPLC-ICP-MS ^{78}Se chromatograms of 3D-1a, 3D-1b, 3D-1c, 3D-1d and 3d-1e samples in organic selenium fed group using the parameters given in Table 35, CX-MP3.

^{82}Se chromatograms of the 3D-1a, 3D-1b, 3D-1c, 3D-1d and 3d-1e samples in organic selenium fed group are shown in Figure 74.

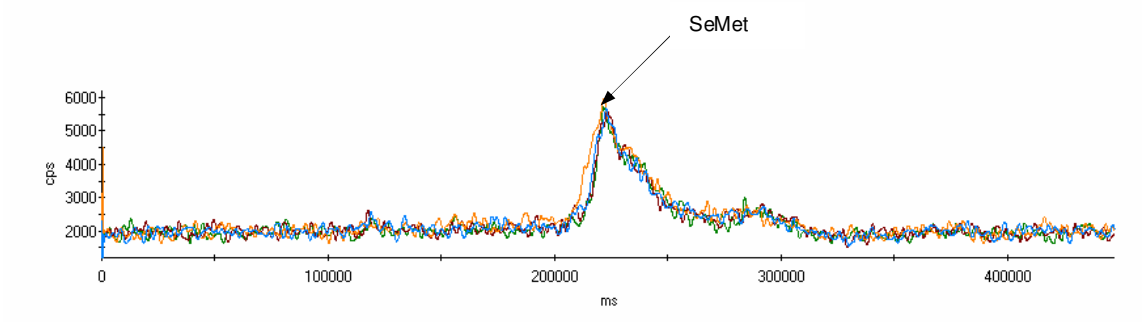

Figure 74. CX-HPLC-ICP-MS ^{82}Se chromatograms of 5 chicken samples extracts in organic selenium fed group using the parameters given in Table 35, CX-MP3.

According to data shown by Figure 73 and Figure 74, there is only one species in the chromatograms of organic selenium fed group to be quantified. In addition, a small signal in the retention time of 285 s was observed. This signal is most probably coming from selenocysteine. Lobinski et al. detected SeCys and SeMet in all chicken samples and accounted these values for the vast majority (> 90%) of the selenium present [192]. Hence, we have just two signals in the chromatograms and one of them can be identified as selenomethionine. The other one might be selenocysteine. At the retention time of inorganic selenium species, there were no signals observed. In order to prove there is no inorganic selenium species, same extraction solutions were injected to AE-HPLC-ICP-MS system. Chromatograms obtained can be seen in Figure 75.

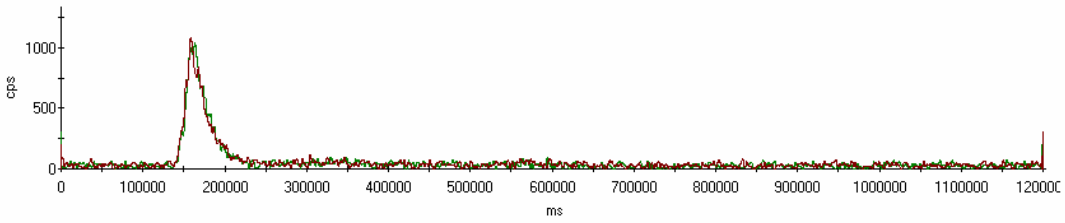

Figure 75. AE-HPLC-ICP-MS chromatograms of extraction solutions of organic selenium fed group samples using the parameters given in Table 29, AE-SP1.

There is no signal observed in the retention times of Se(IV) and Se(VI) in the chromatograms of organic selenium fed group samples using AE-HPLC-ICP-MS method. Organometalic selenium species were not retained in column and eluted in dead time under the conditions used here. In the determination of selenomethionine, ICPS values of the signals were used. All the results for inorganic selenium fed group samples are given in Table 45.

Table 45. Concentration of Se(IV), Se(VI), Se(selenomethionine) and Se(selenocystine) in organic selenium fed group samples.

Sample No	Se found in sample, Mean ± S.D., ng/g (dry mass)				
	Total Se	Se(IV)	Se(VI)	Se(selenomethionine)	Se(selenocystine)
3D – 1a	742 ± 105	N.D.	N.D.	633 ± 50	N.D.
3D – 1b	914 ± 82	N.D.	N.D.	723 ± 57	N.D.
3D – 1c	798 ± 105	N.D.	N.D.	635 ± 50	N.D.
3D – 1d	847 ± 101	N.D.	N.D.	675 ± 53	N.D.
3D – 1e	825 ± 85	N.D.	N.D.	664 ± 52	N.D.
3D – 2a	781 ± 66	N.D.	N.D.	574 ± 49	N.D.
3D – 2b	818 ± 9	N.D.	N.D.	605 ± 52	N.D.
3D – 2c	828 ± 108	N.D.	N.D.	679 ± 58	N.D.
3D – 2d	806 ± 42	N.D.	N.D.	648 ± 55	N.D.
3D – 2e	840 ± 87	N.D.	N.D.	678 ± 59	N.D.
3D – 3a	825 ± 34	N.D.	N.D.	531 ± 42	N.D.
3D – 3b	875 ± 81	N.D.	N.D.	547 ± 45	N.D.
3D – 3c	702 ± 96	N.D.	N.D.	469 ± 37	N.D.
3D – 3d	770 ± 5	N.D.	N.D.	513 ± 40	N.D.
3D – 3e	754 ± 225	N.D.	N.D.	444 ± 35	N.D.
3D – 4a	1149 ± 119	N.D.	N.D.	657 ± 56	N.D.
3D – 4b	1032 ± 107	N.D.	N.D.	664 ± 57	N.D.
3D – 4c	1092 ± 113	N.D.	N.D.	594 ± 51	N.D.
3D – 4d	872 ± 90	N.D.	N.D.	616 ± 53	N.D.
3D – 4e	958 ± 99	N.D.	N.D.	611 ± 52	N.D.
3D – 5a	1211 ± 125	N.D.	N.D.	801 ± 68	N.D.
3D – 5b	843 ± 87	N.D.	N.D.	662 ± 57	N.D.
3D – 5c	807 ± 83	N.D.	N.D.	714 ± 61	N.D.
3D – 5d	914 ± 94	N.D.	N.D.	673 ± 57	N.D.
3D – 5e	1167 ± 121	N.D.	N.D.	817 ± 70	N.D.

N.D.: Not detected

By considering the sum of the determined species in the chromatograms of organometallic selenium fed group and the total selenium concentration, mass balance of the samples was also done. Selenomethionine was only species quantified using cation exchange HPLC-ICP-MS methods. There was one undetermined species in the chromatograms. Percent recovery for the determined species, selenomethionine, varied from 54% to 88%. Average recovery was found as 72 ± 9 because of the undetermined species. Average value found for organic selenium fed group was found to be very close to value found for control group because in both sample groups there was only one undefined species.

A.3.11. Statistical Analysis of Chicken Results

A.3.11.1. Statistical Analysis for Total Selenium in Control and Experiment Groups

Different statistical analyses were carried out for total selenium in control and experiment groups. The results are given in Table 46. The number of significant figures here are not realistic but was kept for the sake of computations.

Table 46. Descriptive statistics and confidence intervals for the mean of total selenium measurements in control and experiment groups.

Descriptives								
					95% Confidence Interval for Mean			
	N	Mean, Se, ng/g (dry mass)	Std. Deviation	Std. Error	Lower Bound	Upper Bound	Minimum	Maximum
Control	25	675.4000	85.27602	17.05520	640.1998	710.6002	530.00	954.00
Inorganic Se Fed Group	25	1084.3200	197.85363	39.57073	1002.6500	1165.9900	640.0	1487.0
Organic Se Fed Group	25	886.8000	138.79811	27.75962	829.5070	944.0930	702.00	1211.00

As it is seen in Table 46, mean of total selenium in control, inorganic selenium and organic selenium groups are 675 ± 85, 1084 ± 198 and 887 ± 139 ng/g in dry mass, respectively. The selenium concentration in inorganic selenium group is the highest one as compared to control and organic selenium groups.

To test the normality assumption which is necessary for one-way Analysis of Variance (ANOVA), Shapiro-Wilks test is applied to control and two experiment groups.

Table 47. Tests of Normality.

	Shapiro-Wilk		
	Statistic	df	Sig.
Control Group	.901	25	.020
Inorganic Se Fed Group	.983	25	.944
Organic Se Fed Group	.861	25	.003

As it is seen from Table 47, since p>0.05 normality assumption is not satisfied in control and organic fed groups, one-way (ANOVA) is not conducted to test equality of group means.

Kruskal-Wallis Test carried out to find which of these three group means are different from each other.

Table 48. Kruskal-Wallis Test for total selenium in control and experiment groups.

	N	Mean Rank			
Control Group	25	15.16		Chi-Square	47.691
Inorganic Se Fed Group	25	57.28		Df	2
Organic Se Fed Group	25	41.56		Asymp. Sig.	**.000**
Total	75				

Since <0.05, all of the three group means are different from each other (Reject $H_o: \mu_1 = \mu_2 = \mu_3$).

In addition, Mann-Whitney Test was also carried out to make sure whether there is a difference between groups or not.

Table 49. Mann-Whitney Test for the mean of total selenium measurements in control and experiment groups.

	N	Mean Rank	Sum of Ranks			
Control Group	25	13.96	349.00		Mann-Whitney U	24.000
Inorganic Se Fed Group	25	37.04	926.00		Wilcoxon W	349.000
Total	50				Z	-5.598
					Asymp. Sig. (2-tailed)	**.000**
Control Group	25	14.20	355.00		Mann-Whitney U	30.000
Organic Se Fed Group	25	36.80	920.00		Wilcoxon W	355.000
Total	50				Z	-5.482
					Asymp. Sig. (2-tailed)	**.000**
Inorganic Se Fed Group	25	33.24	831.00		Mann-Whitney U	119.000
Organic Se Fed Group	25	17.76	444.00		Wilcoxon W	444.000
Total	50				Z	-3.755
					Asymp. Sig. (2-tailed)	**.000**

Since <0.05, means of control, inorganic selenium fed group and organic selenium fed group are different from each other (Reject $H_o: \mu_1 = \mu_2$, $H_o: \mu_1 = \mu_3$ and $H_o: \mu_2 = \mu_3$).

A.3.11.2. Statistical Analysis for Se(selenomethionine) in Control and Experiment Groups

For the Se(selenomethionine) in control and experiment groups, different statistical analyses were carried out.

Table 50. Descriptive statistics and confidence intervals for the mean of Se(selenomethionine) measurements in control and experiment groups.

Descriptives

	N	Mean, Se, ng/g (dry mass)	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
0	25	482,4760	35,49028	7,09806	467,8263	497,1257	399,90	545,00
1	25	496,1200	32,55166	6,51033	482,6833	509,5567	437,20	569,70
2	25	633,0760	88,87401	17,77480	596,3906	669,7614	443,80	817,00
Total	75	537,2240	89,44940	10,32873	516,6436	557,8044	399,90	817,00

0= Control Group, 1= Inorganic Se Fed Group, 2= Organic Se Fed Group

As seen in Table 50, Mean $\pm$ S.D of Se(selenomethionine) in control, inorganic selenium fed and organic selenium fed groups are 482 ± 35, 496 ± 33 and 633 ± 89 ng/g in dry mass, respectively. It is clear that concentration of Se(selenomethionine) in organic selenium fed group is the highest one as compared to control and inorganic selenium fed groups.

To test the normality assumption which is necessary for one-way Analysis of Variance (ANOVA), Shapiro-Wilks test is applied to control and two experiment groups. Since p>0.05 normality assumption is satisfied with 5% level of significance, ANOVA is conducted to test equality of group means, i.e., $H_o: \mu_1 = \mu_2 = \mu_3$ v.s ., H_1:at least one group mean is different, and the results are given in Table 51.

Table 51. ANOVA test for Se(selenomethionine) in control and experiment groups.

ANOVA

VAR00001

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	346862,206	2	173431,103	50,920	,000
Within Groups	245226,251	72	3405,920		
Total	592088,457	74			

As seen from Table 51 with 5% level of significance group means are different. In addition, pairwise comparisons tests were carried out to find which of these three group means are different from each other. Here, Tukey HSD, Scheffee, LSD and Bonferroni tests are used for this purpose.

Table 52. Pairwise Comparison results of total selenium in control and experiment groups.

Multiple Comparisons

Dependent Variable:VAR00001

	(I) VAR00002	(J) VAR00002	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	0	1	-13,64400	16,50677	,688	-53,1468	25,8588
0:Control Group		2	-150,60000*	16,50677	,000	-190,1028	-111,0972
1: Inor. Se Fed Group	1	0	13,64400	16,50677	,688	-25,8588	53,1468
		2	-136,95600*	16,50677	,000	-176,4588	-97,4532
2: Org. Se Fed Group	2	0	150,60000*	16,50677	,000	111,0972	190,1028
		1	136,95600*	16,50677	,000	97,4532	176,4588
Scheffe	0	1	-13,64400	16,50677	,712	-54,9037	27,6157
		2	-150,60000*	16,50677	,000	-191,8597	-109,3403
	1	0	13,64400	16,50677	,712	-27,6157	54,9037
		2	-136,95600*	16,50677	,000	-178,2157	-95,6963
	2	0	150,60000*	16,50677	,000	109,3403	191,8597
		1	136,95600*	16,50677	,000	95,6963	178,2157
LSD	0	1	-13,64400	16,50677	,411	-46,5497	19,2617
		2	-150,60000*	16,50677	,000	-183,5057	-117,6943
	1	0	13,64400	16,50677	,411	-19,2617	46,5497
		2	-136,95600*	16,50677	,000	-169,8617	-104,0503
	2	0	150,60000*	16,50677	,000	117,6943	183,5057
		1	136,95600*	16,50677	,000	104,0503	169,8617
Bonferroni	0	1	-13,64400	16,50677	1,000	-54,1054	26,8174
		2	-150,60000*	16,50677	,000	-191,0614	-110,1386
	1	0	13,64400	16,50677	1,000	-26,8174	54,1054
		2	-136,95600*	16,50677	,000	-177,4174	-96,4946
	2	0	150,60000*	16,50677	,000	110,1386	191,0614
		1	136,95600*	16,50677	,000	96,4946	177,4174

*. The mean difference is significant at the 0.05 level.

It is clear in Table 52 that mean values of control and inorganic selenium fed groups are not different. Tukey HSD, Scheffe, LSD and Bonferroni show that mean value of Se(selenomethionine) in organic selenium fed group is clearly different than control and inorganic selenium fed groups.

PART A

CHAPTER 4

A.4. Conclusion

In this study, speciation analysis of four selenium species, Se(IV), Se(VI), selenomethionine and selenocystine, was performed in selenium supplement tablets, egg samples and chicken breast samples. AE-HPLC, CX-HPLC, C18-HPLC and C8-HPLC systems were tried to find the best separation conditions. For the all samples, AE-HPLC system was used to make quantitative measurements of Se(IV) and Se(VI) while CX-HPLC system was used to resolve and separate selenomethionine and selenocystine from inorganic species. In the detection step, ICP-MS instrument was used.

In egg sample studies, there was a difference in the slopes of external calibration and standard addition methods in the determination of total selenium. Hence, standard addition method was applied. CX-HPLC-ICP-MS method was used for the determination of organic selenium species. Se(IV), Se(VI) and selenocystine could not detected in any of the samples. Selenomethionine was the only species detected in all egg samples. There was a shift in the retention time of selenomethionine due to high content of matrix using CX-HPLC-ICP-MS system. A spiking experiment was performed to show that this shift is due to matrix. There were very small signals observed in the retention times of inorganic selenium species, but signal/noise ratio is not high enough to make any quantitative analysis. Total selenium and selenomethionine concentration in egg samples produced by chicken fed by selenium enriched diet were found to be higher than control sample.

In selenium supplement tablet studies, six brands of selenium supplement tablet were analyzed for their selenium contents. Speciation information was compared with

the value given on the tablets and the total levels of selenium measured with ICP-MS. Results found agree reasonably well with the selenium content reported on the bottle labels of Brand A, Brand B, Brand C and Brand E. The same selenium species with the type of selenium species given on the label of supplement tablets were found for Brand A, Brand B, Brand C and Brand E. Brand D has a dramatically different profile for the actual chemical form of selenium within the supplement tablets. It is written on the label of Brand D that selenomethionine is present in this supplement tablet, but instead Se(VI) was found to be present; no selenomethionine was detected using CX-HPLC-ICP-MS or AE-HPLC-ICP-MS. It was also found that Brand F appears to contain very low amount of selenium, about 20 fold lower than the value given on the bottle labels. In addition, chemical form of selenium is dramatically different within this supplement. Only inorganic selenium, Se(VI), was observed in Brand F despite the label claims of content being only selenomethionine. Spiking experiment was performed and it was found that there is no matrix effect to cause shift in selenomethionine signals.

In chicken breast study, chickens from Bursa and Kayseri were used to optimize the all parameters for the determination of selenium species. Enzymatic hydrolysis was used instead of an acidic one in order to protect selenium species from uniformation. CX-HPLC-ICP-MS system was used to determine the concentration of selenomethionine and selenocystine while AE-HPLC-ICP-MS was used for inorganic selenium species, Se(IV), Se(VI). Selenomethionine was the only species observed in Bursa and Kayseri samples. Direct calibration and standard addition methods were applied for the determination of selenomethionine. There was no difference observed for the slopes calibration plots by both methods. Hence, external calibration method was used for the further chicken breast samples.

In inorganic and organic selenium fed chicken study, statistical analysis was used for both selenomethionine and total selenium in all groups. Using Kruskal-Wallis test, it was found that there is no difference in all of the three group means ($p<0.05$). Total amount of selenium in inorganic selenium fed group was found to be higher than the control and organic selenium fed groups. Se(VI) and selenocystine could not detected in any of the samples. For selenomethionine results, ANOVA test was conducted to test the equality of group means and it was found that at least one group means different with 5% level of significance. Selenomethionine concentration in organic selenium fed group was found to be higher than the control and inorganic

selenium fed group. There is no statistical difference in the group means of control and inorganic selenium fed groups. Spiking experiments showed that shift in selenomethionine signal to lower retention time was due to matrix. In addition to selenomethionine signal, there were 2 additional signals observed in CX-HPLC-ICP-MS chromatograms of selenium fed group samples. Retention times of these signals were very close to inorganic selenium species and dead time. Hence, AE-HPLC-ICP-MS was applied to analyze inorganic fed group samples for their inorganic selenium contents. It was observed that Se(IV) was only determined as inorganic selenium species in the inorganic selenium fed group.

PART B

DETERMINATION OF ARSENOBETAINE IN DORM-2, DORM-3 AND DOLT-4 CRMs USING CATION EXCHANGE-HPLC-ICP-MS AND CATION EXCHANGE-HPLC-ES-MS SYSTEMS

CHAPTER 1

B.1. INTRODUCTION

Arsenic is known as a toxic trace element. According to World Health Organization (WHO), maximum arsenic level that can be allowed in drinking water is 0.01 mg/L [201]; however, the toxicity of arsenic species depends on the chemical forms of arsenic. Toxicity of inorganic arsenic species, As(III) and As(V), is much higher than organic arsenic species like arsenobetaine and arsenosugars [202]. Arsenic is present in inorganic forms in water and air while it is in organoarsenic forms like arsenobetaine, dimethylarsenic acid, arsenocholine and arsenosugars in seafood [203]. While the high number of As species are present in environmental samples, many papers deal with mostly arsenobetaine due to high abundance of this species in marine fish [204].

Speciation analyses of arsenic are very popular in literature. First and most important part in speciation analysis is the extraction step. Extraction conditions should be efficient to release the species of interest from the matrix. Different extraction approaches can be applied to obtain high extraction efficiency. The most commonly used methods in literature for the extraction of arsenic species are mixing/shaking, sonication, and pressurized extraction systems [205]. Dufailly et al. used microwave assisted extraction with 10.0 mL of methanol-water mixture (8 : 2, v/v) for the extraction of arsenic species in fish samples including some CRMs [206]. Mato-Fernández et al.

developed a pressurized liquid extraction procedure with methanol/water mixture for extracting arsenical species from marine biological material (mussel and fish) and standard reference materials (GBW-08751, BCR-278R and DORM-2) [207]. Sánchez-Rodas et al. used a new extraction procedure with a methanol-water mixture using microwave oven and ultrasonic probe to extract the arsenic species in the chicken without uniformation. Using this extraction method, extraction efficiencies was obtained from 80 to 100% [208]. Accelerated solvent extraction, sonication with water, sonication with formic acid were applied to marine certified reference materials (DORM-2, Dolt 2 and Tort 2) to release most of the arsenic species from matrix to the solution [209].

After the extraction step, species should be separated from each other using chromatographic techniques. Reverse phase chromatography [210], capillary electrophoresis [211, 212] and ion-exchange chromatography [213, 214] have been used for the separation of different arsenic species from each other. In literature, ion-exchange chromatography has been preferred chromatographic technique due to high separation ability for arsenic species. Isocratic and gradient elution procedures can be applied in the ion-exchange system. Sloth et al. used gradient elution anion or cation exchange HPLC system to separate arsenic acid, arsenous acid, monomethylarsonic acid, dimethylarsinic acid, arsenobetaine, trimethylarsine oxide, dimethylarsinoylacetic acid, trimethylarsoniopropionate and dimethylarsinoylethanol in the urine samples including certified reference materials (NIES No. 18 and NIST SRM2670a) [215]. Simon et al. used cation-exchange chromatography to separate arsenobetaine, arsenocholine, trimethylarsine oxide and tetramethylarsonium ion in biological matrices and a certified reference fish tissue (DORM-2) within 20 min [216]. Anion and cation exchange chromatographic technique were used for the determination of arsenobetaine, dimethylarsinic acid, arsenite and trimethylarsine oxide in muscle of 16 freshwater fish (9 different species belonging to 4 different families) by Slejkovec et al. [217].

In the speciation analysis of arsenic, the last step is the detection. There are many different techniques in literature for the determination of arsenobetaine in different matrices. Hydride generation-atomic absorption spectrophotometry (HG-AAS) [218], hydride generation-atomic fluorescence spectrometry (HG-AFS) [216], inductively coupled plasma mass spectrometry (ICP-MS) [202, 209], capillary electrophoresis with inductively coupled plasma-mass spectrometric and electrospray time of flight mass

spectrometry [219], high-performance liquid chromatography-ultrasonic nebulizer-high power nitrogen-microwave-induced plasma mass spectrometry [220], multidimensional liquid chromatography combined with inductively coupled plasma, electrospray and electrospray high-field asymmetric waveform ion mobility spectrometry with mass spectrometry [221] are some of the systems for the determination of arsenobetaine.

Isotope dilution experiments were performed to obtain high accuracy and precision when compared with traditional method such as external and standard addition methods. It is known that isotope dilution mass spectrometry has been used in literature because this method provides the results with unchallenged precision and accuracy [222]. As an example, when the isotope dilution was used for butyltin measurement, the precision of results is increased roughly by one order of magnitude when compared with standard additions [223, 224]. In this study, AsB-D_3 was used as isotope dilution reagents in HPLC-ES-MS study.

The purpose of the current study is to quantify the arsenobetaine content of three marine-tissues CRMs, DORM-2, DORM-3 and DOLT-4, produced by National Research Council of Canada so as to provide a more comprehensive set of speciation values. Water soluble arsenic species were determined using Cation Exchange-HPLC-ICP-MS and Isotope Dilution-Cation Exchange-HPLC-ES-MS systems.

B.1.1. Information about DORM-2, DORM-3 and DOLT-4

DORM-2 (Dogfish Muscle Certified Reference Material for Trace Metals) was certified by National Research Council of Canada, Institute for National Measurement Standards. All of the results are based on the at least two independent methods of analysis. The uncertainties show the 95 percent tolerance limits for an individual sub-sample of 0.250 g or greater [225].

DORM-3 (Fish Protein Certified Reference Material for Trace Metals) is another CRM certified by National Research Council of Canada, Institute for National Measurement Standards. DORM-3 was prepared from a fish protein homogenate. Bones and the majority of the oil was removed by an enzyme hydrolysis procedure. The protein hydrolysate was spray dried, sieved to pass a 297 µm screen, blended and bottled. A minimum dose of 25 kGy gamma irradiation was used in the sterilization of the material at the Canadian Irradiation Centre, Laval, Québec. The expanded

uncertainty in the certified value given in Table 53 is equal to U = ku_c where u_c is the combined standard uncertainty calculated according to the ISO Guide and k is the coverage factor [226].

DOLT-4 (Dogfish Liver Certified Reference Material for Trace Metals) was prepared from dogfish by National Research Council of Canada, Institute for National Measurement Standards. Cold vapor atomic absorption spectrometry, inductively coupled plasma mass spectrometry, electrothermal vaporization atomic absorption spectrometry, isotope dilution gas chromatography mass spectrometry, hydride generation atomic absorption spectrometry are some of the methods that have been used in the certification of this CRM. In the calculation of expanded uncertainty in the certified value given in Table 53, U = ku_c as a formula was used [227]. Certified values of the elements for DORM-2, DORM-3 and DOLT-4 are given in Table 53.

Table 53. Trace elements in DORM-2, DORM-3 and DOLT-4.

	Certified Values, mg/kg		
Element	DORM-2	DORM-3	DOLT-4
Aluminum	10.9 ± 1.7		
Arsenic	18.0 ± 1.1	6.88 ± 0.30	9.66 ± 0.62
Cadmium	0.043 ± 0.008	0.290 ± 0.020	24.3 ± 0.8
Cobalt	0.182 ± 0.031		
Chromium	34.7 ± 5.5	1.89 ± 0.17	
Copper	2.34 ± 0.16	15.5 ± 0.63	31.2 ± 1.1
Iron	142 ± 10	347 ± 20	1833 ± 75
Lead	0.065 ± 0.007	0.395 ± 0.050	0.16 ± 0.04
Mangnese	3.66 ± 0.34		
Mercury	4.64 ± 0.26	0.382 ± 0.060	2.58 ± 0.22
Nickel	19.4 ± 3.1	1.28 ± 0.24	0.97 ± 0.11
Selenium	1.40 ± 0.09		8.3 ± 1.3
Silver	0.041 ± 0.013		0.93 ± 0.07
Thallium	0.004		
Tin	0.023	0.066 ± 0.012	
Zinc	25.6 ± 2.3	51.3 ± 3.1	116 ± 6
Methylmercury (as Hg)	4.47 ± 0.32	0.355 ± 0.056	1.33 ± 0.12
Arsenobetaine (as As)	16.4 ± 1.1		
Tetramethylarsonium (as As)	0.248 ± 0.054		

PART B

CHAPTER 2

B.2. EXPERIMENTAL

B.2.1. Apparatus

ICP-MS measurements were performed using ELAN 6000 (PE-SCIEX, Thornhill, Ontario, Canada) equipped with a Ryton spray chamber. All ICP-MS parameters were daily optimized to find the best sensitivity for arsenic using the software program. In the HPLC studies, a Dionex BioLC, model LCM (Dionex Corp., Sunnyvale, California, USA), fitted with 50.0 µL of loop was used. Cation exchange separation was achieved using Supelcosil SCX (250 x 4.6 mm x 5 µm), Supelquard SCX Guard Column. Chromatographic signals were processed using in-house software in Excel.

ES-MS measurements were performed using LTQ-Orbitrap Mass Spectrometer. All ES-MS parameters were optimized to find best sensitivity for arsenic using soft-ware program. In the HPLC studies, Agilent 1100 Series model fitted with 15.0 µL of loop was applied. Cation exchange separation was achieved using Supelcosil LC-SCX HPLC Column (250 x 3.0 mm x 5 µm).

Sonication experiments for all CRMs were done using a Branson 3510 sonication instrument. In the extraction procedure, a sonication probe (Branson Sonifier 450) and a shaker (Wrist Action Shaker, Model 75, Burrell Corporation, Pittsburgh, PA, U.S.A) were also used. Extraction solutions were separated from residue using a IEC HN-SII (IEC Lab systems, Heedham Hts, MA, USA) centrifuge instrument. In the filtration procedure, 25.0 mm Syringe Filters (0.2 micron) was applied.

B.2.2. Standards and Reagents

Analytical reagent grade chemicals were used throughout this study. In all dilutions, extraction and standard preparation procedures, de-ionized water purified to 18 MΩ.cm resistively using a NANOpure water purification system (Barnstead/Thermolyne, Dubuque, Iowa, USA) was used.

Arsenobetaine was obtained from Fluka. 1000 mg/L of Arsenobetaine-D_3 solution was prepared by dissolving appropriate amount of solid pure AsB-D_3. Purification of AsB-D_3 was controlled using Cation Exchange-HPLC-ICP-MS system. The standardization of AsB-D_3 was done using AsB. 50.0 ng/mL of AsB-D_3 was prepared and injected to CX-HPLC-ICP-MS system to control purification of AsB-D_3. There was no other peak observed except for AsB-D_3 in the chromatogram. All working solutions were prepared on the day of the analysis using appropriate dilutions from stock solutions.

In the extraction, formic acid (UN 1779, Anachemia, Canada Inc.), ammonium hydroxide (Anachemia, Canada Inc.), ammonium acetate (Ac 592, Anachemia, Canada Inc.) were used. In order to clean the cation exchange column, methyl alcohol (MXC488-1, EMD Chemicals) was used.

B.2.3. Cation Exchange-HPLC-ICP-MS System

In the first part of this study, ICP-MS parameters were optimized to find high sensitivity for As. In the optimization, 25.0 ng/mL of As was used. It is known that arsenic has only one isotope, ^{75}As. Hence, during the analysis this isotope was monitored. The main problem for arsenic analysis in ICP-MS is the spectral interference caused by ^{40}Ar-$^{35}Cl^+$, but this interference problem could be eliminated or reduced using chromatographic separation method combined with ICP-MS. In the case of cation exchange or C-8 or C-18, chloride is eluted in dead time and does not affect the arsenic signal.

All the parameters optimized can be seen in Table 54. Throughout the all studies, cones of ICP-MS system were periodically cleaned to eliminate clogging possibility.

Table 54. Optimization results of ICP-MS parameters.

Optimization Parameter	**Result**
Instrument	ELAN 6000 (PE-SCIEX), Thornhill, ON, Canada)
RF Power	1250 W
Sample Flow Rate	1.0 mL/min
Nebulizer Gas Flow	1.1 L/min
Lens Voltage	8.0 V

Cation exchange (CX)-HPLC system was used throughout this study to separate arsenobetaine from other possible As species in the samples. CX-HPLC separations were carried out using Supelcosil SCX (250 x 4.6 mm x 5 µm) column and Supelquard SCX Guard Column. All parameters of CX-HPLC system used in the separation of arsenobetaine can be seen in Table 55.

Table 55. Parameters of CX-HPLC system used in the isoratic separation of arsenobetaine.

Parameter	**Result**
Column	Supelcosil SCX (250 x 4.6 mm x 5 µm), Supelquard SCX Guard Column
Mobile Phase	20.0 mM of Ammonium Formate (pH=2.8)
Flow Rate	1.0 mL/min
Loop Volume	50 µL

The chromatogram of 50.0 ng/mL of As(AsB) obtained using optimum CX-HPLC and ICP-MS parameters can be seen in Figure 76.

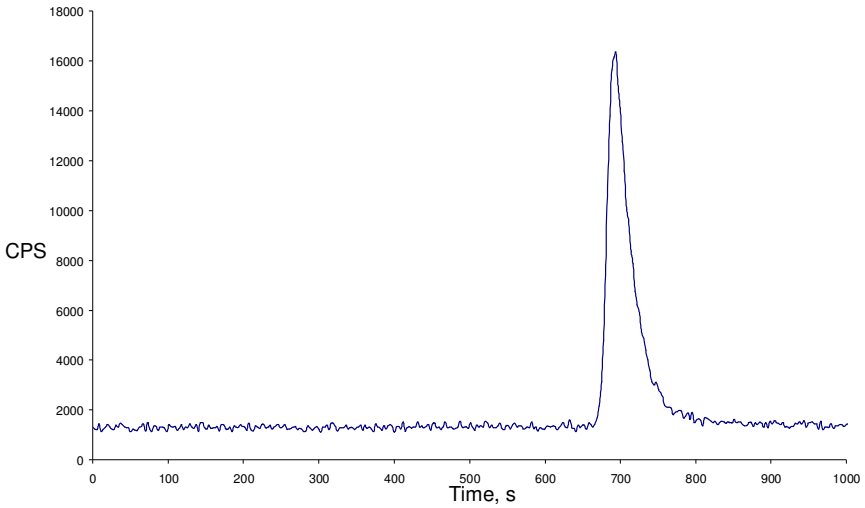

Figure 76. The chromatogram of 50.0 ng/mL of As(AsB) obtained using optimum CX-HPLC and ICP-MS parameters.

In order to find out both the dead time of CX-HPLC-ICP-MS system and separation efficiency of cation exchange column, a mixed standard solution containing 25.0 ng/mL of As(V) and As(AsB) was prepared. Both chromatograms for AsB and mixed solution can be seen Figure 77.

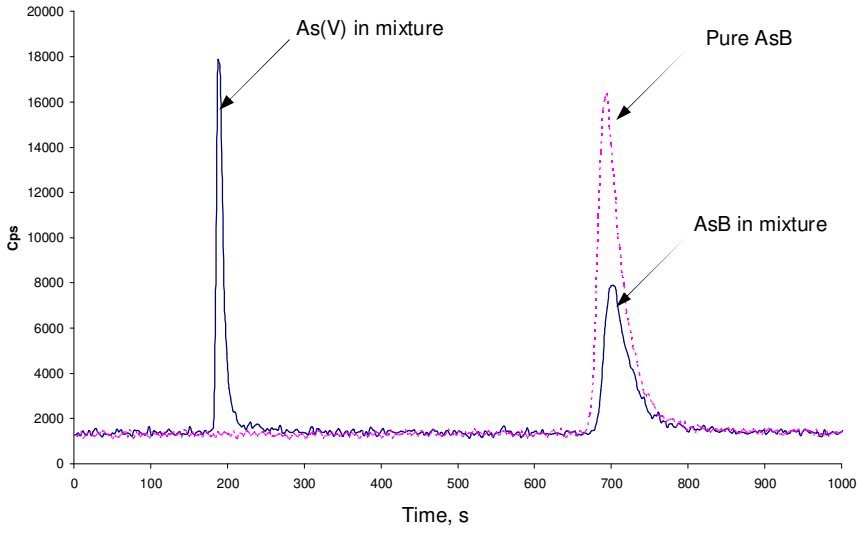

Figure 77. Chromatograms for 50.0 ng/mL of As(AsB) and mix solutions containing 25.0 ng/mL of As (V) and As(AsB).

As seen in Figure 77, retention time of AsB in pure solution and As in mixture are exactly same.

B.2.3.1. Analytical Performance of CX-HPLC-ICP-MS System

Different concentration of As(AsB) standards were prepared in a Class 100 clean room and injected to CX-HPLC-ICP-MS system using the optimum parameters. Chromatograms of standard As(AsB) are shown in Figure 78.

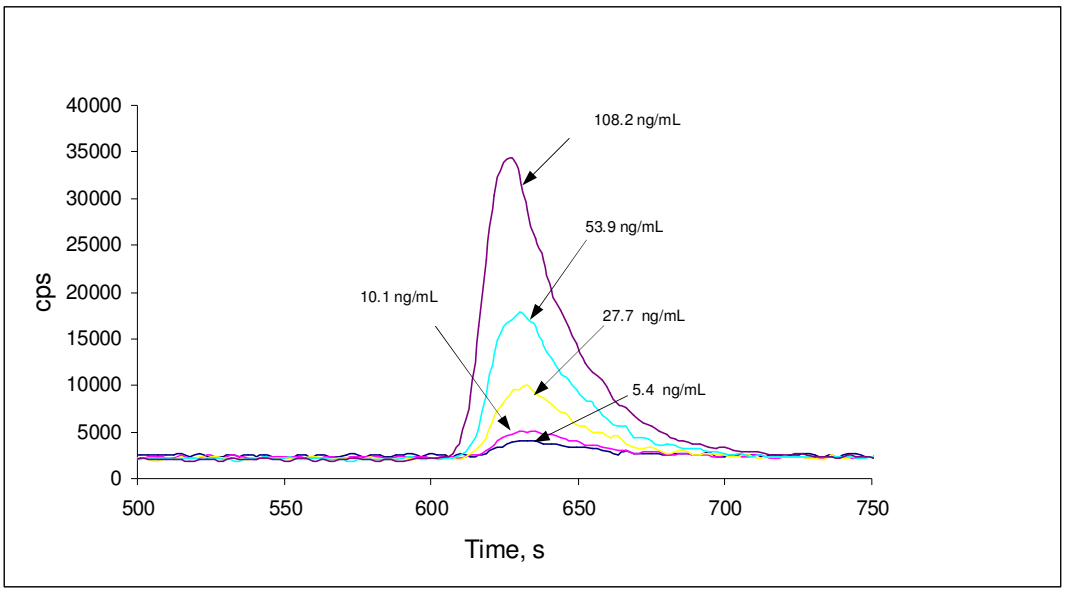

Figure 78. CX-HPLC-ICP-MS chromatograms of standard As(AsB) in different concentrations.

As seen in the figures, retention times of all standards in different concentration were found to be the same. Signals are good regarding not only intensities but also peak shapes. The peak area (ICPS) of each signal shown in figures above was measured, and the calibration plot for As(AsB) was obtained using ICPS as peak areas.

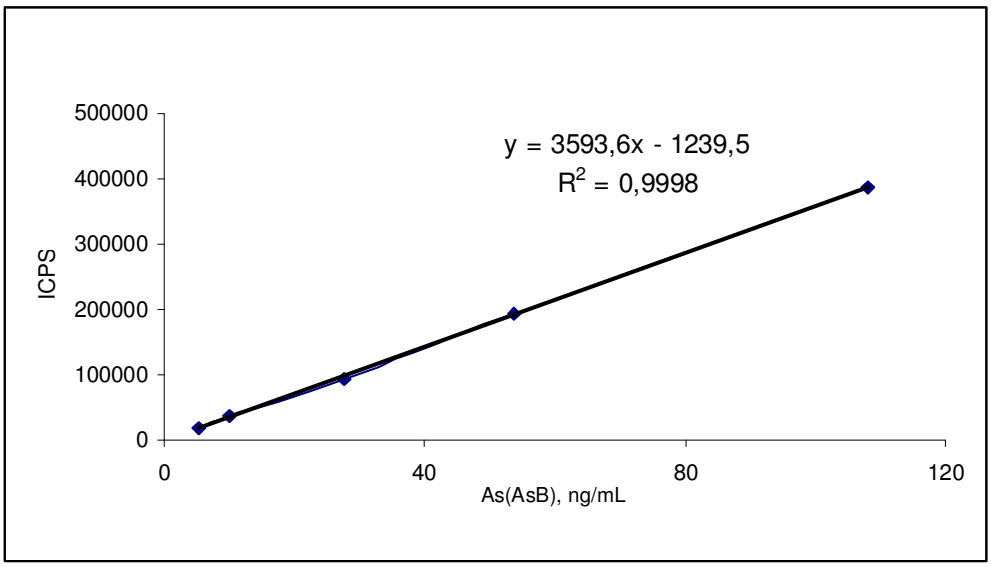

Figure 79. Linear calibration plot of As(AsB) obtained using CX-HPLC-ICP-MS system.

Limit of detection and limit of quantitation values of the system were calculated using the consecutive measurements of 5.4 ng/mL of As(AsB) (N=5). The signals obtained from 5.4 ng/mL of As(AsB) are shown in Figure 80.

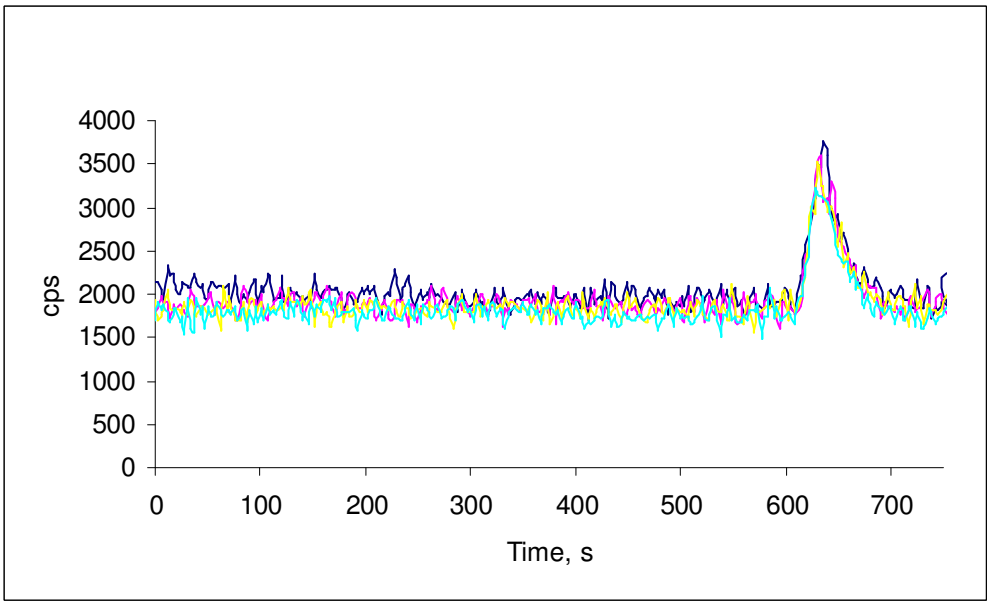

Figure 80. CX-HPLC-ICP-MS signals of 5.4 ng/mL of As(AsB), N=5.

In the calculation of LOD and LOQ values, peak areas of signals were used. The following formulas were used to calculate LOD and LOQ values.

LOD= 3xStandard Deviation/Slope

LOQ= 10xStandard Deviation/Slope

The analytical figures of merit of CX-HPLC-ICP-MS system are given in Table 56.

Table 56. Figures of merit of CX-HPLC-ICP-MS system.

	Result
LOD	0.63 ng/mL
LOQ	2.09 ng/mL
Calibration Plot Equation	y= 3593.6x – 1239.5
R^2	0.9998

As seen in table above, CX-HPLC-ICP-MS system is sufficiently sensitive to determine the As(AsB) in many matrix. R^2 value of calibration plot was found to be very close to 1.0.

B.2.4. ES-MS Studies

In the first part of HPLC-ES-MS studies, ES-MS parameters were optimized to reach a high sensitivity for AsB and its isotope, AsB-D_3. All of the optimizations were done by using software of the instrument. All the parameters optimized are shown in Table 57. As mentioned in HPLC-ICP-MS part of this section, AsB-D_3 was used as isotope dilution reagent to combine the internal calibration capacity of standard addition technique and the precision of isotope dilution technique. Indeed, in normal conditions, normal standard addition with AsB would be enough to calculate AsB content in extract using HPLC-ICP-MS. However, this "non-isotopic" standard addition are facing 2 issues with ES-MS; a) Lower reproducibility between runs of ES-MS signals due to instrument loses sensitiviy with time or can be affected by artefacts b) Calibration curve of AsB was

not linear in the wide range especially to quantify low amounts of AsB contrary to ICP-MS. This isotope spike, AsB-D_3, was used as an internal correction as it is similarly affected by the lack of linearity of the signal. In brief, the advantage of AsB-D_3 spiking was, first, to bring the precision of isotope dilution that is immune to chromatographic or instrumental variability and, second, to correct internally for the lack of linearity of the ES-MS signals observed for low concentrated compounds.

Table 57. Parameters of ES-MS system used in AsB determination in CRMs.

ESI Source	
Spray Voltage (kV)	3.04
Spray Current (µA)	0.02
Sheath Gas Flow Rate	25.00
Aux Gas Flow Rate	20.00
Sweep Gas Flow Rate	0.03
Capillary Voltage (V)	13.07
Capillary Temp (°C)	324.95
Tube Lens (V)	70.06

All the parameters seen in Table 57 were controlled before starting the measurements for sensitivity check. In the case of decrease in the sensitivity by 30%, optimization of the parameters was repeated to reach at least the previous sensitivity.

Cation exchange (CX)-HPLC system was used throughout this study for the separation of AsB from the other possible arsenic species in the samples. CX-HPLC separations were carried out using Supelcosil LC-SCX HPLC Column (250 x 3.0 mm x 5 µm). All the parameters of CX-HPLC system used in the separation of arsenobetaine isotopes can be seen in Table 58.

Table 58. Parameters of CX-HPLC system used in the separation.

Parameter	
Instrument	Agilent 1100 Series
Column	Supelcosil LC-SCX HPLC Column (250 x 3.0 mm x 5 µm)
Solvent Program	a) 0-6.3 min: -0.10 M ammonium acetate in 30% MeOH, 0-100% -20.0 mM ammonium formate in 30% MeOH, pH:2.8, 100-0% b) 6.3-10.0 min: -0.10 M ammonium acetate in 30% MeOH, 100-0% -20.0 mM of ammonium formate in 30% MeOH, pH:2.8, 0-100%
Flow Rate	0.20 mL/min
Loop Volume	15.0 µL

B.2.5. Extraction Studies

The most crucial step in the speciation studies is the extraction procedure. In this step, all species of interest should be taken from matrix to the solution without any uniformation. Different analytical approaches can be used to find an effective extraction procedure. Efficiency of extraction procedures depends on the sample matrix where the element is to be determined. In general, the following extraction methods have been used in literature for different elements such as Se and As [228];

a) Solvent extraction

b) Basic hydrolysis

c) Enzymatic hydrolysis

d) Supercritical fluid extraction,

e) Accelerated solvent extraction,

f) Solid phase extraction,

g) Derivatization

h) Fast extraction methods.

In the extraction procedure, the main purpose is to obtain high extraction efficiency. Extraction conditions should be chemically mild but efficient to release the species from the matrix [228].

In the extraction part of this study, the main purpose was to obtaine high extraction efficiency for AsB. It is known that AsB is a stable compound and conversion of AsB to other As species needs a strong acid medium. Hence, conversion possibility was very low for AsB. Extraction studies were started using de-ionized water. Different extraction methods were applied to find the best extraction method. All the extraction procedures can be seen as follow.

B.2.5.1. Extraction Procedures

For the extraction procedure, the following steps were applied. The parameters of choice were given in Table 59.

In the extraction, DORM-2 was used as sample to find the best extraction procedure. About 0.250 g of DORM-2 was accurately weighed into a 50 mL falcon type centrifuge tube. A measured volume of extracting solvent was added. The contents were mixed by plain sonication or a sonication probe or a shaker or any combination of these for the described period at room temperature (24-27 ^{0}C). Samples were centrifuged at 4500 rpm for 5.0 min. Then, supernatant was filtered using 25 mm of Syringe Filters (0.2 µm). If multiple extractions were applied, the procedure was repeated by using the residue instead of the initially weighed sample. The resulting solutions obtained by combining the centrifugates were analyzed.

In the further studies, all extraction solution would be analyzed in ES-MS system. In the HPLC-ES-MS system, isotope dilution method would be applied. Deuterated AsB, AsB-D_3, was used as isotope of AsB in the isotope dilution study. Hence, deuterated AsB was selected in standard addition method expect for Extraction Method 1, 2 and 4. Stock solution of AsB-D_3 was prepared from its solid in clean room.

Table 59. Extraction method used for Dorm-2.

Extraction Method	Number of Extractions	Standard used	Extraction Device	Extracting Solvent	Volume of Extracting Solvent, mL	Extraction period, min	Centrifuging Period
1a	1	AsB	Sonication	H_2O	10.0	20.0	5.0 min
1b	1	AsB	Sonication Probe	H_2O	10.0	4.0	5.0 min
2	3	AsB	Sonication	H_2O	$(10.0)^3$	$(20.0)^3$	5.0 min after each step
3	1	AsB-D_3	Sonication	H_2O	10.0	20.0	5.0 min
4	2	AsB	Sonication	H_2O	$(10.0)^2$	$(20.0)^2$	5.0 min after each step
5	2	AsB-D_3	a) Sonication b)Sonication Probe	H_2O	$(10.0)^2$	a) 20.0 b) 5.0	5.0 min after each step
6	3	AsB-D_3	Sonication	H_2O	$(8.0)^3$	$(20.0)^3$	5.0 min after each step
7	3	AsB-D_3	Shaker	20%MeOH aqueous	$(10.0)^3$	$(30.0)^3$	5.0 min after each step
8	2	AsB-D_3	Shaker Shaker	H_2O	a)20.0 b)10.0	a)15.5x60 b)30	5.0 min after each step
9	3	AsB-D_3	Shaker	H_2O	$(10.0)^3$	$(30.0)^3$	5.0 min after each step

All the external calibration or standard addition plots were constructed by at least three points. Direct extraction or standard additions were used as mentioned. Unless described otherwise the result was a sum of all the consecutive extraction steps. In the standard addition applications, the standard analyte was spiked before the extraction step. The results are given in Table 60.

Table 60. Results for Dorm-2 analysis using different extraction and calibration procedures.

Extraction Method	Calibration	Stage used if multiple extraction were used	As(AsB), N=2, mg/kg
1a	DC, AsB	1	13.15 ± 0.53
1a	SA, AsB	1	14.18 ± 0.44
1b	DC, AsB	1	13.69 ± 0.48
2	DC, AsB	1+2	15.48 ± 0.52
3	SA, AsB-D_3	1	13.23 ± 1.28
4	SA, AsB-D_3	1+2	14.12 ± 0.91
5	SA, AsB-D_3	1+2	14.68 ± 0.95
6	SA, AsB-D_3	1+2+3	13.89 ± 0.82
7	SA, AsB-D_3	1+2+3	14.75 ± 1.37
8	SA, AsB-D_3	1+2	15.20 ± 0.98
9	**SA, AsB-D_3 (4 points)**	**1+2+3**	**15.39 ± 0.45**

DC: Direct Calibration, SA: Standard Addition

Relatively better results were obtained using Extraction 2, 8 and 9 regarding the As(arsenobetaine) concentration. In extraction 2, sonication device was used. In this instrument, temperature of extraction solution was getting higher with time. The system had to be cooled to eliminate the any possible decomposition of arsenobetaine. In extraction method 8, standard deviation of the result is higher than the results obtained from Extraction 2 and 9. Another reason to select the Extraction 9 as optimum one among others is that number of the samples to be extracted in the shaker is higher than

sonication instrument. Hence, “Extraction Method 9” was selected as optimum extraction method among the others. R^2 of calibration graph of standard addition method was found to be at least 0.997 using this method. It was decided that, 3rd extraction step would be applied for the all CRM samples in order to make sure there is no AsB remaining in the residue. Reproducibility of the results was found to be lower than 3.0%. Retention times of the all signal in each standard addition point were fount to be very close to each other. Hence, we decided to use this method for DORM-2, DORM-3 and DOLT-4 CRMs.

PART B

CHAPTER 3

B.3. RESULTS and DISCUSSION

B.3.1. CX-HPLC-ICP-MS Studies

As mentioned before, the chromatographic separation conditions given in Table 55 were used in the extraction study to find the best extratction procedure. Reproducibility of the retention time of arsenobetaine detoriated with time. While retention time of arsenobetaine was 640 s at the beginning of extraction study using 20.0 mM ammonium formate (pH=2.8), aresenobetaine signal shifted to lower retention times, 550 s. For this reason, gradient elution with 100.0 mM ammonium acetate and 20.0 mM of ammonium formate (pH=2.8) was applied. All the experimental parameters for chromatographic system can be seen in Table 61.

Table 61. Experimental parameters for chromatographic system.

Parameter	
Column	Supelcosil SCX (250 x 4.6 mm x 5 µm), Supelquard SCX Guard Column
Solvent Program	a) 0-2.0 min, 100.0 mM ammonium acetate, 100% b) 2.0-4.0 min, 100.0 mM ammonium acetate, 100-0%; 20.0 mM of ammonium formate (pH=2.8), 0-100% c) 4.0-6.0 min, 100.0 mM ammonium acetate, 0-100%; 20.0 mM ammonium formate (pH=2.8), 100-0% d) 6.0-8.0 min, 100.0 mM ammonium acetate, 100%
Flow Rate	1.0 mL/min
Loop Volume	50.0 µL

B.3.1.1. Analytical Performance of the System

Different concentration of As(AsB) standards were prepared in water from the main stock solution and injected to CX-HPLC-ICP-MS system using the parameters shown in Table 61. HPLC-ICP-MS chromatogram of 5.5 ng/mL As(AsB) can be seen in Figure 81. As seen in this figure, retention time of arsenobetaine was found as 320 s using parameters given in Table 61 while this value was 550 s in the other chromatographic separation system using the parameters given in Table 55. Linear calibration plot obtained using 2.0-500 ng/mL of As(AsB) is shown in Figure 82.

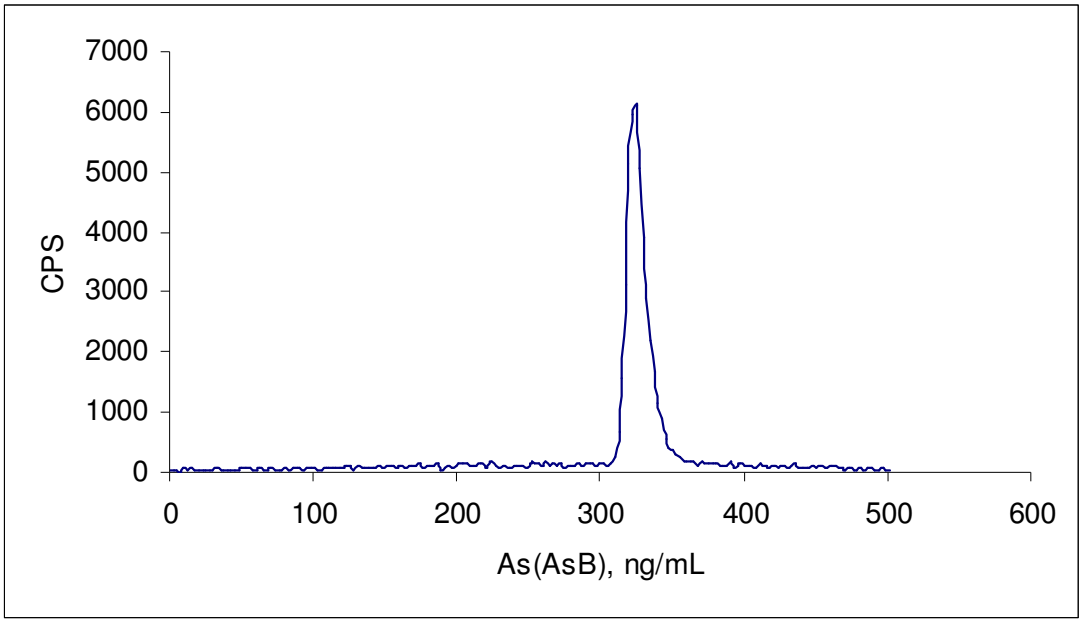

Figure 81. HPLC-ICP-MS chromatogram of 5.5 ng/mL As(AsB).

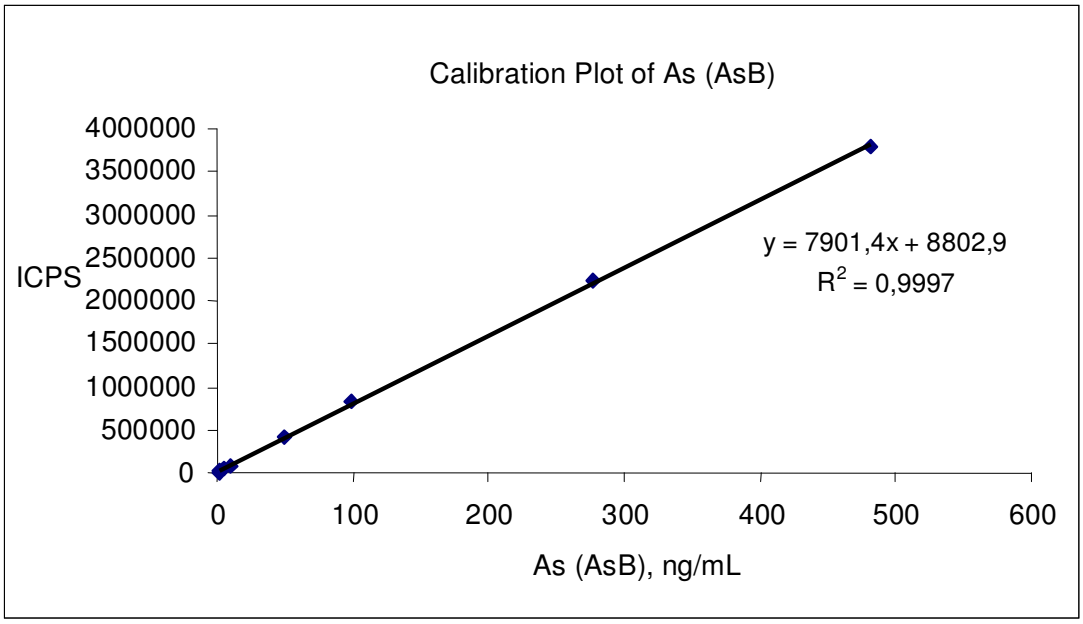

Figure 82. Calibration plot obtained using 2.0-500 ng/mL As(AsB).

As seen in Figure 82, linear range for As(AsB) was obtained between 2.0-500 ng/mL. Limit of detection and limit of quantitation values of the system were calculated using replicate measurements of 2.0 ng/mL of As(AsB) (N=5). In the calculation of LOD and LOQ values, peak areas of the signals were used. The formulas given before were applied to calculate LOD and LOQ values. The analytical figures of merit for CX-HPLC-ICP-MS system are given in Table 62.

Table 62. Analytical figures of merit of CX-HPLC-ICP-MS system (N=5).

Parameter	**Result**
LOD	0.29 ng/mL
LOQ	0.97 ng/mL
Calibration Plot Equation	y= 7901.4x +8802.9
R^2	0.9997

As seen in the table above, linearity of the calibration plot is very good and this system is sufficiently sensitive for the determination of arsenic using HPLC-ICP-MS.

Recovery of arsenic species from the column were also investigated. For this aim, extraction solution of DORM-2 was injected to HPLC-ICP-MS under the optimum conditions given in Table 61. After the column, eluent was collected throughout the elution. The same experiment was also performed without column. Extraction solution of DORM-2 was also injected to HPLC using the same loop, and eluent was collected before the column. Both solution with and without column were injected to ICP-MS, and it was observed that there is no difference in the results obtained from with and without column experiments (lower than 2%, N=2). This proves that the column recovery is about 100% and this chromatographic method is proper for quantitative determination of AsB.

B.3.1.2. DORM-2, DORM-3 and DOLT-4 Studies

DORM-2, DORM-3 and DOLT-4 CRMs were analyzed for their As(AsB) content after finishing all of the optimizations for HPLC and ICP-MS systems. In the all

extractions, AsB-D_3 was added samples as standard addition reagent because ES-MS system would be used for the analyses of all extraction solutions after CX-HPLC-ICP-MS measurements.

In the extraction of DORM-2, DORM-3 and DOLT-4, “Extraction Method 9” was used. Five replicates were prepared for the analysis of each CRM. Four points standard addition method was applied to samples to eliminate any possible interference coming from matrix. Either AsB or AsB-D_3 was added as mentioned. As(AsB) determination of DORM-2, DORM-3 and DOLT-4 were done using CX-HPLC-ICP-MS system using the parameters in Table 54 for ICP-MS and Table 61 for HPLC system.

B.3.1.2.1. Determination of As(AsB) in DORM-2, DORM-3 and DOLT-4

For the analysis of all samples in this section, ICP-MS and HPLC parameters as given in Table 54 and Table 61, respectively, were used.

HPLC-ICP-MS chromatograms for one of the replicates of DORM-2 using the Extraction Method 9 can be seen in Figure 83; standard addition method was used spiking with AsB-D_3.

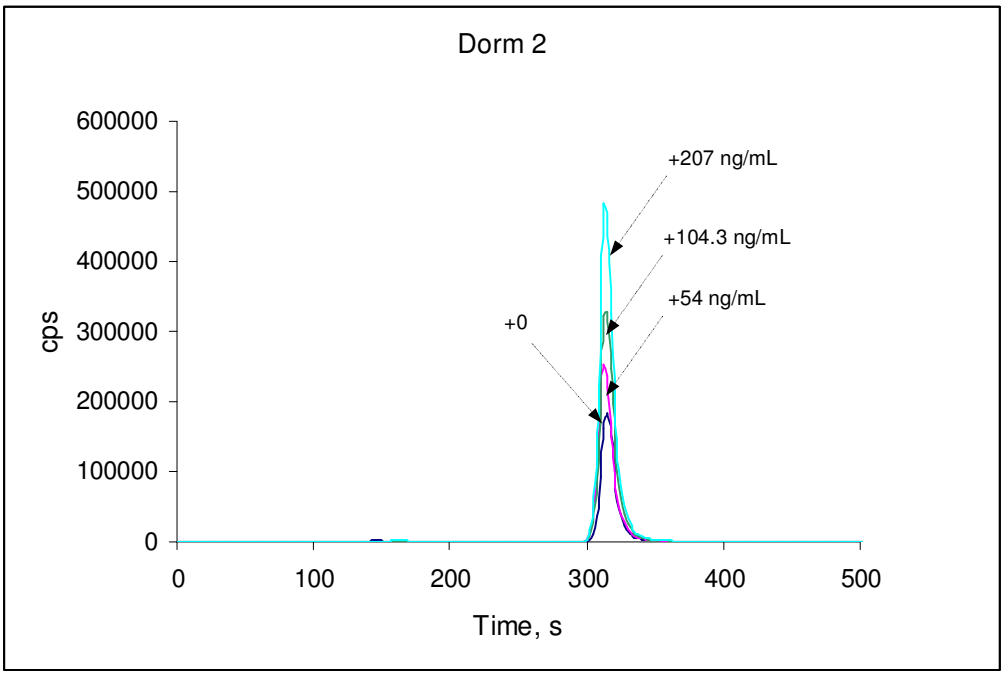

Figure 83. CX-HPLC-ICP-MS chromatograms of DORM-2, Extraction Method 9 (AsB-D_3 as standard addition reagent, 3 step extraction) using shaker.

Concentration of As(AsB) in extraction solution of DORM-2 obtained using CX-HPLC-ICP-MS can be seen in Table 63.

Table 63. Concentration of As(AsB) in extract solution of DORM-2 obtained using CX-HPLC-ICP-MS system.

Sample	As(AsB), mg/kg	Line Equation for Standard Addition	R^2
DORM-2, Parallel 1	15.88	y = 6918.6x + 908319	0.9978
DORM-2, Parallel 2	15.39	y = 7103.7x + 916197	0.9996
DORM-2, Parallel 3	14.66	y = 7306.2x + 900390	0.9993
DORM-2, Parallel 4	15.07	y = 7088x + 894715	0.9994
DORM-2, Parallel 5	14.71	y = 7438.2x +899639	0.9967
Mean ± standard deviation	15.14 ± 0.50		

The chromatograms obtained from DORM-3 using the Extraction Method 9 can be seen in Figure 84.

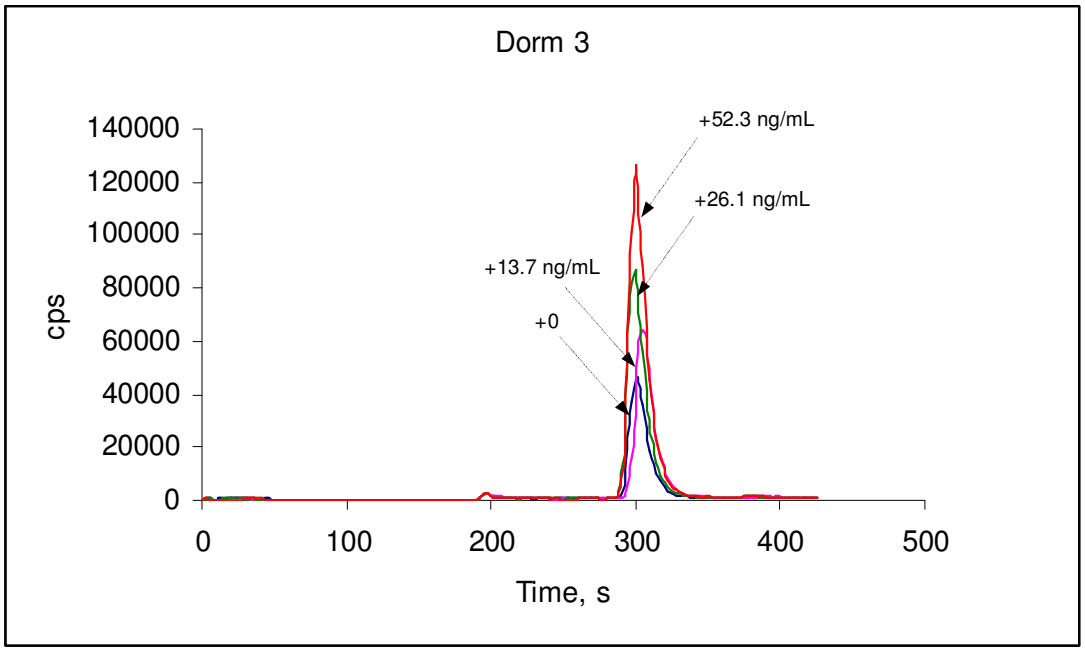

Figure 84. CX-HPLC-ICP-MS chromatograms of DORM-3 using Extraction Method 9 (AsB-D_3 as standard addition reagent, 3 step extraction).

Similar procedure was also applied to DORM-3; results are given in Table **64**.

Table 64. Results for As(AsB) in DORM-3 obtained using CX-HPLC-ICP-MS system and Extraction Method 9.

Sample	As(AsB), mg/kg	Line Equation for Standard Addition	R^2
DORM-3, Parallel 1	3.77	y = 7575.2x + 240194	1.0000
DORM-3, Parallel 2	3.65	y = 9606.5x + 298296	0.9985
DORM-3, Parallel 3	3.88	y = 9363.7x + 303874	0.9995
DORM-3, Parallel 4	3.77	y = 9439.9x + 298155	0.9987
DORM-3, Parallel 5	3.81	y = 9133.8x + 292329	0.9996
Mean ± standard deviation	3.78 ± 0.08		

Standard addition chromatograms of one of the replicate of DOLT-4 are shown in Figure 85.

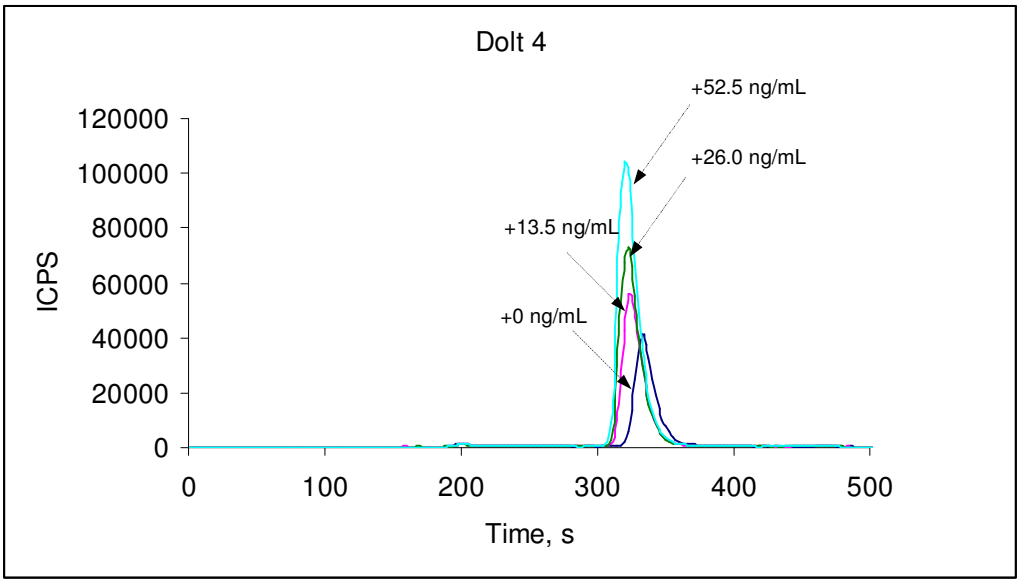

Figure 85. CX-HPLC-ICP-MS chromatograms of DOLT-4 using Extraction Method 9 (AsB-D_3 as standard addition reagent, 3 step extraction) using shaker.

As shown in Figure 85, peak shapes of all the signals are sufficiently good for quantitative measurement. There was a very small shift in the retention time of +0 ng/mL of standard addition point relative to other signals. Results for As(AsB) in DOLT-4 obtained by using CX-HPLC-ICP-MS can be seen in Table 65.

Table 65. Results for As(AsB) in DOLT-4 obtained using CX-HPLC-ICP-MS system and Extraction Method 9.

Sample	**As(AsB), mg/kg**	**Line Equation for Standard Addition**	**R^2**
DOLT-4, Parallel 1	4.06	y = 8857.9x + 299313	0.9970
DOLT-4, Parallel 2	3.91	y = 8508.7x + 277251	0.9944
DOLT-4, Parallel 3	4.11	y = 8468.2x + 291731	0.9989
DOLT-4, Parallel 4	4.63	y = 8184.5x + 319819	0.9961
DOLT-4, Parallel 5	4.76	y = 7844.4x + 311584	0.9978
Mean ± standard deviation	4.29 ± 0.38		

In order to find out whether AsB and AsB-D_3 will have the same behavior, another standard addition analysis was performed by using AsB in spiking. In the extraction of DORM-3, extraction method 9 was carried out. Two replicates were prepared for the analysis. Four point standard addition method was applied to DORM-3. As(AsB) determination of DORM-3 was done using CX-HPLC-ICP-MS system using the parameters in Table 54 for ICP-MS and Table 61 for HPLC system.

The chromatograms obtained from DORM-3 using AsB in standard addition can be seen in Figure 86.

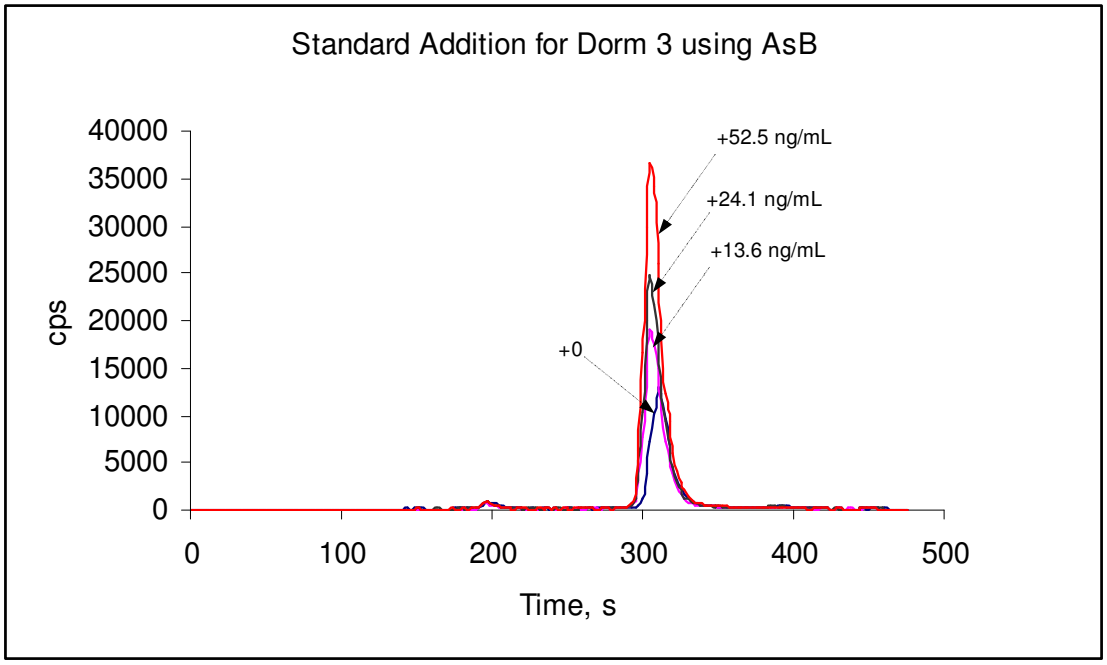

Figure 86. CX-HPLC-ICP-MS chromatograms of DORM-3, Extraction Method 9 (AsB, standard addition, 3 step extraction) using shaker.

Concentration of As(AsB) in extraction solution of DORM-3 obtained using CX-HPLC-ICP-MS can be seen in Table 66.

Table 66. Concentration of As(AsB) in extraction solution of DORM-3 obtained using CX-HPLC-ICP-MS system.

Sample	**As(AsB), mg/kg**	**Line Equation for Standard Addition**	**R^2**
DORM-3, Parallel 1	3.86	y = 2505.5x + 80095	0.9992
DORM-3, Parallel 2	3.75	y = 2438.5x + 76844	0.9982
Mean ± standard deviation	3.81 ± 0.08		

As it is seen in Table 66, the results found by using AsB in standard addition method were found to be very close those obtained using AsB-D_3. Hence, it can be concluded that AsB-D_3 and AsB have similar behaviors in HPLC-ICP-MS system.

B.3.2. CX-HPLC-ES-MS Studies

As(arsenobetain) concentration in DORM-2, DORM-3 and DOLT-4 were determined using Isotope Dilution-CX-HPLC-ES-MS system in this part of study.

B.3.2.1. DORM-2, DORM-3 and DOLT-4 Studies using HPLC-ES-MS System

HPLC-ICP-MS method has been widely used to determine arsenobetaine in marine matrices. It is well known that arsenic in arsenobetaine is monitored by ICP-MS studies. Hence, separation should be very efficient for arsenobetaine from other possible arsenic species in the medium to eliminate any possible interferences. In the case of having the same retention time with the other arsenic compounds, it is impossible to determine it accurately by using an elemental detector, ICP-MS. Using the ES-MS instrument, qualitative and quantitative measurement of arsenobetaine can be done easily and accurately because a mass resolution is possible as well as the time resolution. Arsenobetaine contents of DORM-2, DORM-3 and DOLT-4 were determined after finishing all the optimizations of HPLC and ES-MS systems. In all extractions, AsB-D_3 was added to samples as a spiking chemical. As mentioned before, the same extraction solutions used in HPLC-ICP-MS system were also analyzed in HPLC-ES-MS system. In the extraction of DORM-2, DORM-3 and DOLT-4, Extraction Method 9 that is the optimum one was applied. For each CRM, at least 12 replicates using the different spiking amounts of AsB-D_3 were prepared for the analysis. In this extraction method, three consecutive extraction steps were used as mentioned before. As(AsB) determination of DORM-2, DORM-3 and DOLT-4 were done using CX-HPLC-ES-MS system using the parameters in Table 57 for ES-MS and Table 58 for CX-HPLC system. Product ion mass spectra of *m*/*z* 179 and 182 were monitored for arsenobetaine and deuterated arsenobetaine, respectively, in ES-MS detection.

B.3.2.1.1. DORM-2

We had totally 12 replicates for DORM-2 spiked at different concentrations. The chromatograms of AsB and AsB-D_3 obtained from one of the replicate of DORM-2 can be seen in Figure 87 and Figure 88.

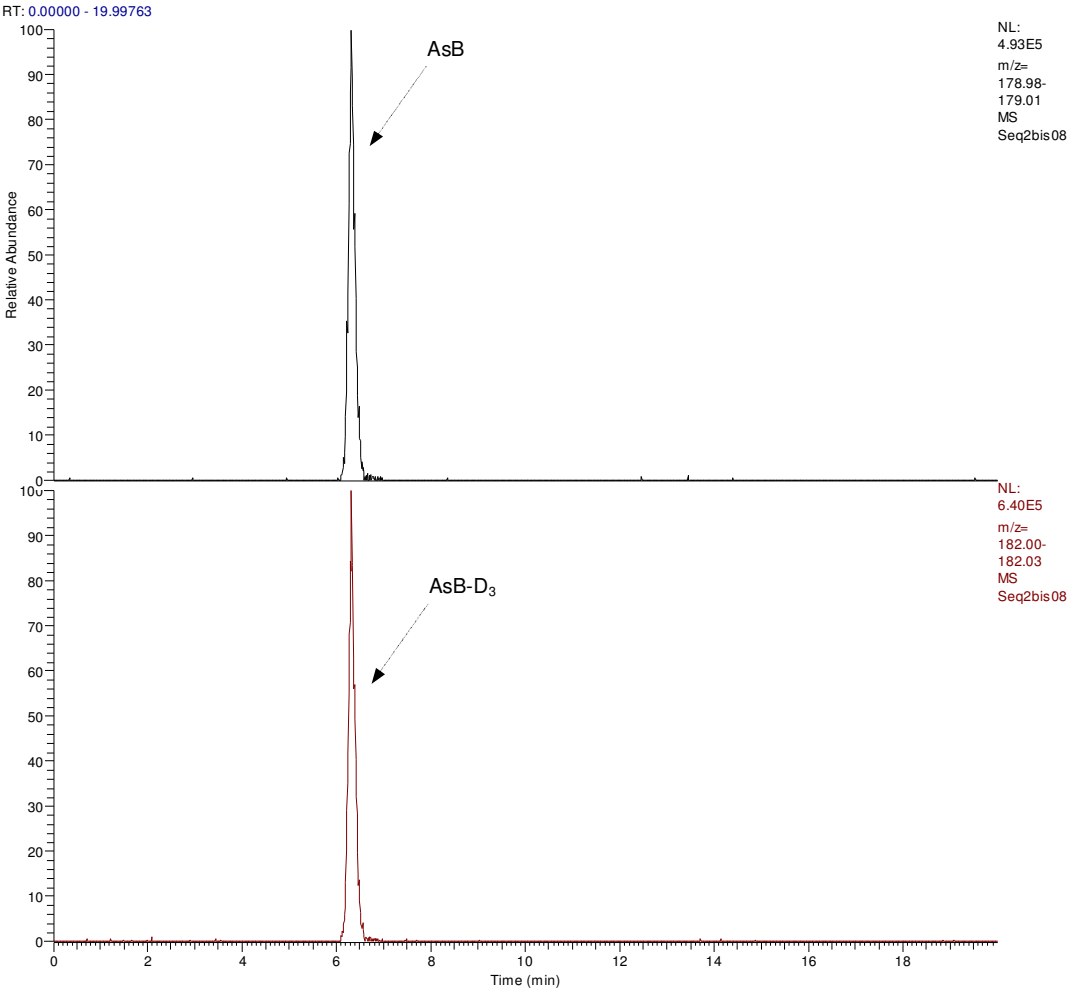

Figure 87. CX-HPLC-ES-MS chromatograms of AsB (179 dalton) and AsB-D_3 (182 dalton) in one of the replicate of DORM-2.

Peak widths of the signals were found to be lower than 0.5 min and the peak shapes are good enough to perform qualitative and quantitative measurements. Like the DORM-3 and DOLT-4 chromatograms, there is no shift in the retention times of AsB

and AsB-D_3 by the effect of high content of matrix. Both species have the exactly same retention times, 6.3 min. It was stated that in the case of even slight differences in chromatographic retention times of the native analyte and enriched spike there may be potential errors due to the eluting matrix components that can significantly change thus altering the signal response (ion suppression), which, in turn, tampers with the accuracy of internal standard method [222]. As it is clear in Figure 87 this is not the case for DORM-2.

Total ion chromatogram of DORM-2 can be seen in the following figure.

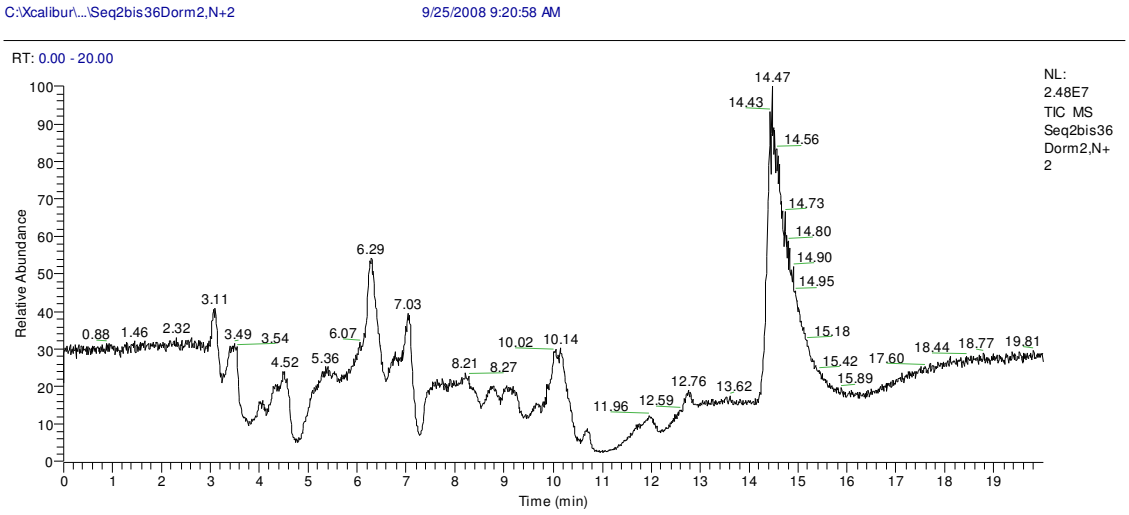

Figure 88. CX-HPLC-ES-MS total ion chromatogram of one of the replicates of DORM-2.

As seen in the figure, in the retention time of AsB and AsB-D_3 chromatogram is not so crowded to cause interference effects on the analyte signals. Start and end points of signals to measure the peak areas of the signals were decided automatically by software program of instrument. Ratio of AsB/AsB-D_3 was measured and used in the calculations. This ratio was put into the lab-made formula prepared in Excel. Results for As(AsB) in DORM-2 obtained using CX-HPLC-ES-MS can be seen in Table 67.

Table 67. Concentration of As(AsB) in extraction solution of DORM-2 obtained using CX-HPLC-ES-MS system.

Sample	As(AsB), mg/kg
DORM-2-Replicate 1	14.90
DORM-2- Replicate 2	14.85
DORM-2- Replicate 3	14.99
DORM-2- Replicate 4	14.82
DORM-2- Replicate 5	15.01
DORM-2- Replicate 6	14.72
DORM-2- Replicate 7	14.62
DORM-2- Replicate 8	14.74
DORM-2- Replicate 9	14.85
DORM-2- Replicate 10	14.89
DORM-2- Replicate 11	14.89
DORM-2- Replicate 12	14.94
Mean ± standard deviation	14.85 ± 0.11

Reproducibility of the DORM-2 results was found to be the best among the other CRMs. %RSD value was found to be 0.76. This shows that results are very close to each other in all spiking measurements. HPLC-ES-MS results for DORM-2 were also in good agreement with the results found using HPLC-ICP-MS system. In HPLC-ICP-MS, average value for As(AsB) was 15.14 ± 0.50 while the mean value was found to be 14.85 ± 0.11 using HPLC-ES-MS methods. Reproducibility of the HPLC-ES-MS results was better than the results found by HPLC-ICP-MS system due to isotope dilution measurement in HPLC-ES-MS system.

B.3.2.1.2. DORM-3

12 replicates using different spiking concentrations of AsB-D_3 were prepared for the analysis of DORM-3. Deuterated AsB was spiked to sample to make quantitative measurement of AsB in DORM-3 as CRM. The chromatograms obtained from one of the replicates of DORM-3 can be seen in Figure 89 and Figure 90.

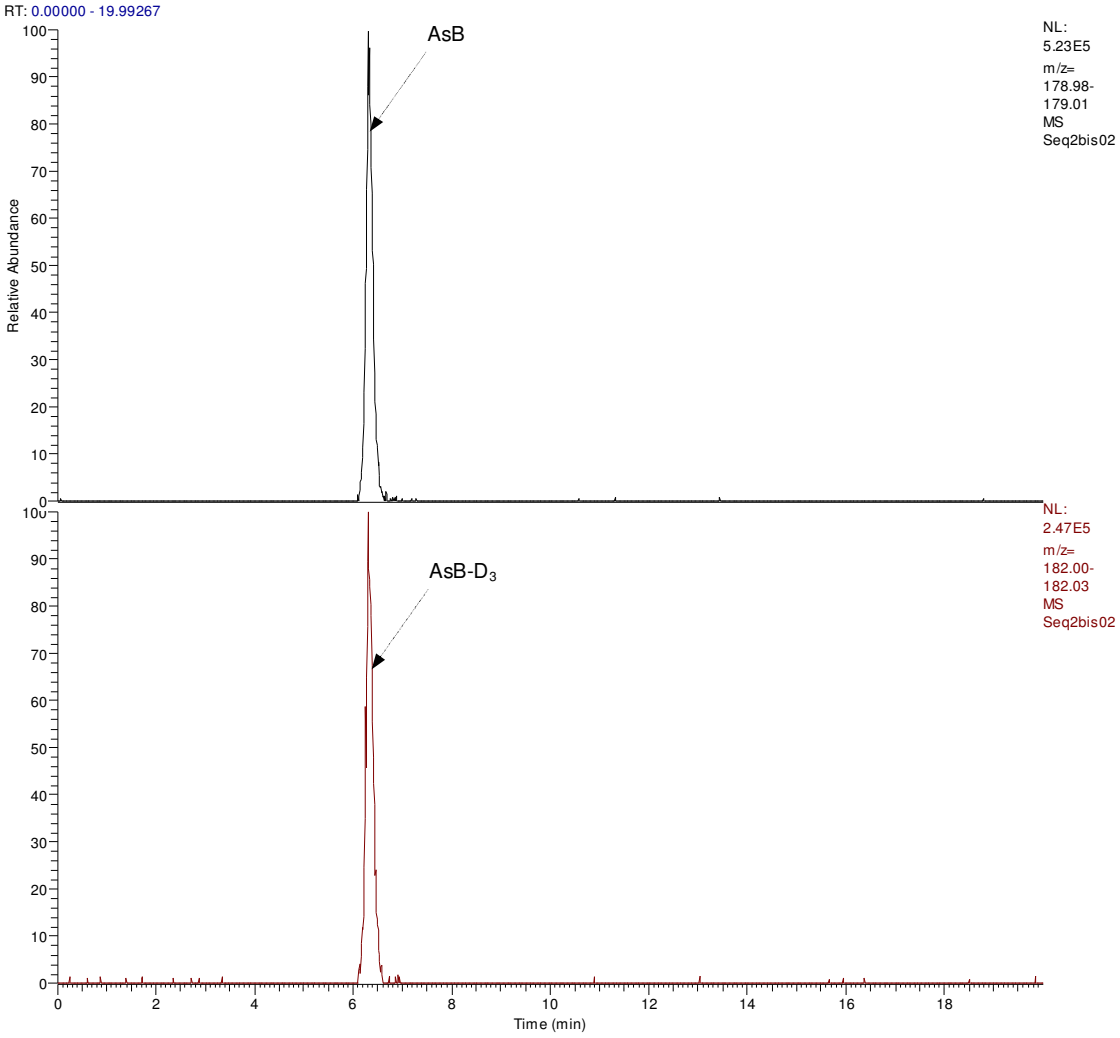

Figure 89. CX-HPLC-ES-MS chromatograms of AsB (179 dalton) and AsB-D_3 (182 dalton) in one of the replicate of DORM-3.

Retention times of AsB and AsB-D_3 are matching and the features of both signals are good regarding peak shapes and sharpness like in DORM-2. 178.98-179.01 mass range was monitored for AsB while 182.00-182.03 was scanned for AsB-D_3. Chromatographic separation was continued for 20 min. In both chromatograms, there are no other signals to have only interference effects on analyte signals. Total ion chromatogram of DORM-3 extract can be seen in Figure 90.

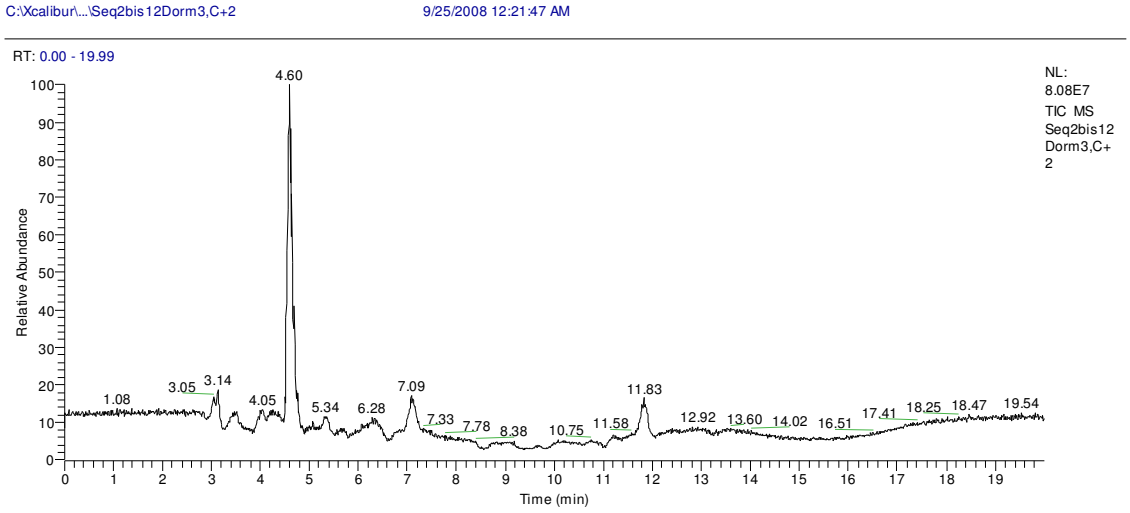

Figure 90. CX-HPLC-ES-MS total ion chromatogram of one of the replicates of DORM-3.

Peak areas of the signals were calculated automatically by software of instrument. Concentration of As(AsB) in DORM-3 obtained using CX-HPLC-ES-MS can be seen in Table 68.

Table 68. Concentration of As(AsB) in extraction solution of DORM-3 obtained using CX-HPLC-ES-MS system.

Sample	As(AsB), mg/kg
DORM-3- Replicate 1	3.69
DORM-3- Replicate 2	3.68
DORM-3- Replicate 3	3.48
DORM-3- Replicate 4	3.67
DORM-3- Replicate 5	3.56
DORM-3- Replicate 6	3.51
DORM-3- Replicate 7	3.66
DORM-3- Replicate 8	3.77
DORM-3- Replicate 9	3.60
DORM-3- Replicate 10	3.63
DORM-3- Replicate 11	3.69
DORM-3- Replicate 12	3.58
Mean ± standard deviation	3.63± 0.08

As seen in the table, reproducibility of the results were found to very close to each other. %RSD was found as 2.30. HPLC-ES-MS results were in good agreement with the results found using HPLC-ICP-MS. In HPLC-ICP-MS system, average value for As(AsB) was 3.78 ± 0.08 while this value was found to be 3.63 ± 0.08 using HPLC-ES-MS methods. In both systems, RSD values were lower than 3.0%.

B.3.2.1.3. DOLT-4

15 replicates using the different spiking amounts of AsB-D_3 were prepared for the analysis of DOLT-4. The chromatograms obtained from one of the replicate of DOLT-4 can be seen in Figure 91, Figure 92 and Figure 93.

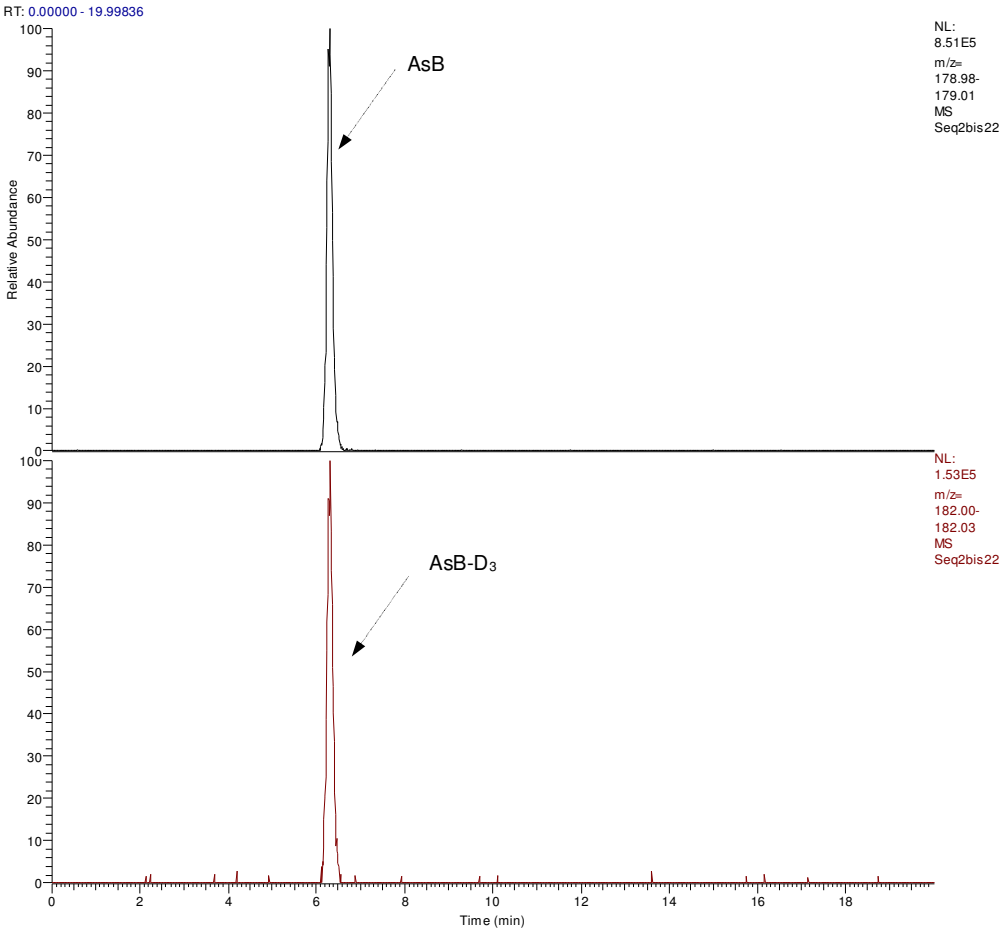

Figure 91. CX-HPLC-ES-MS chromatograms of AsB (179 dalton) and AsB-D_3 (182 dalton) in one of the replicates of DOLT-4.

It is clear in Figure 91 that retention times of both AsB and AsB-D_3 are exactly same, 6.3 min, and the peak shapes at both chromatograms are very sharp with no tailing. Chromatograms were continued until 20 minutes to clean up the column.

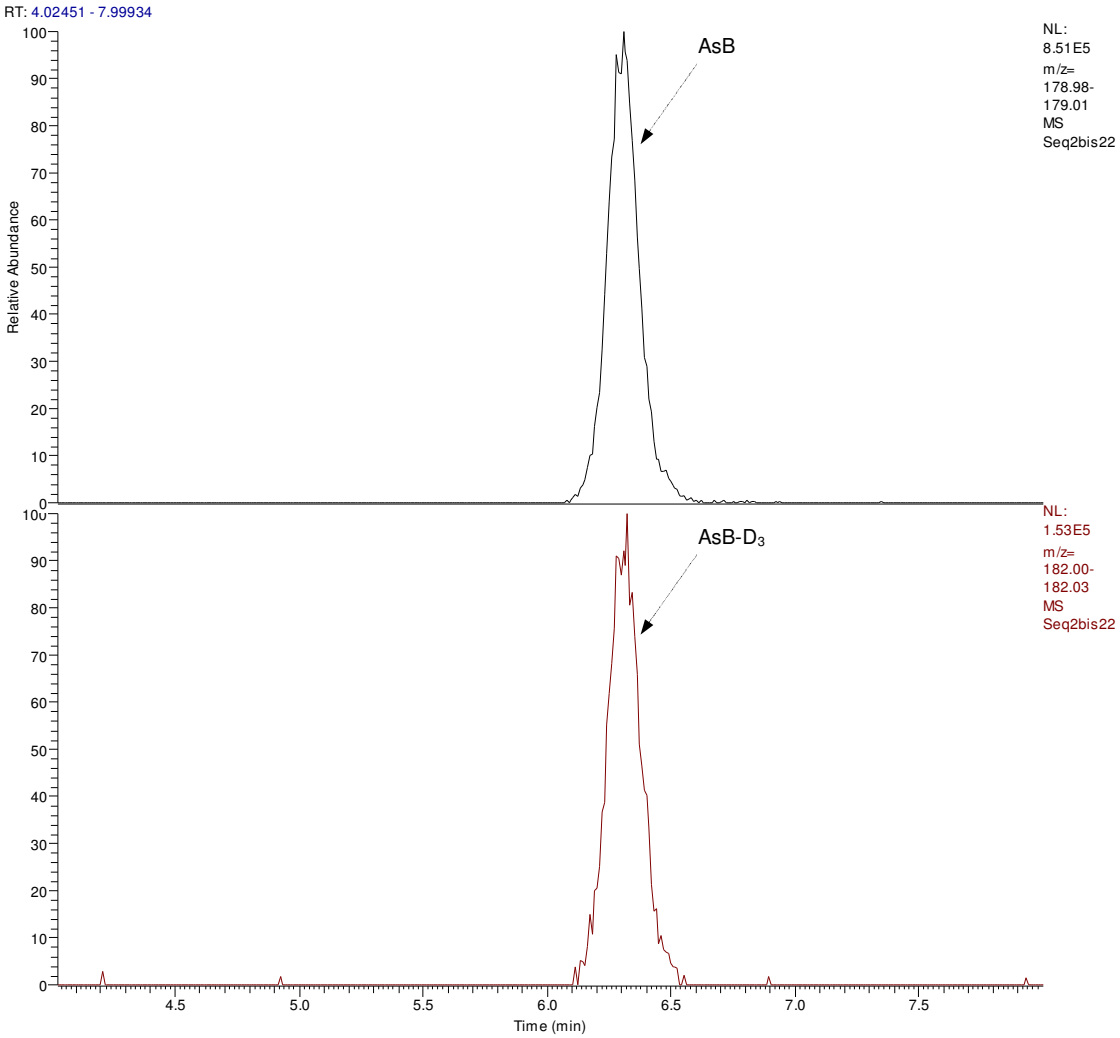

Figure 92. CX-HPLC-ES-MS chromatograms of AsB (179 dalton) and AsB-D_3 (182 dalton) in one of the replicate of DOLT-4.

In Figure 92, it is shown that there are no other signals having the m/z ratios close to 179 and 182 at the retention times of AsB and AsB-D_3; this expanded figure was included to have a better judgment regarding peak shapes. Both isotopes have the same retention times, 6.3 min. Total ion chromatogram of sample can be seen in Figure 93.

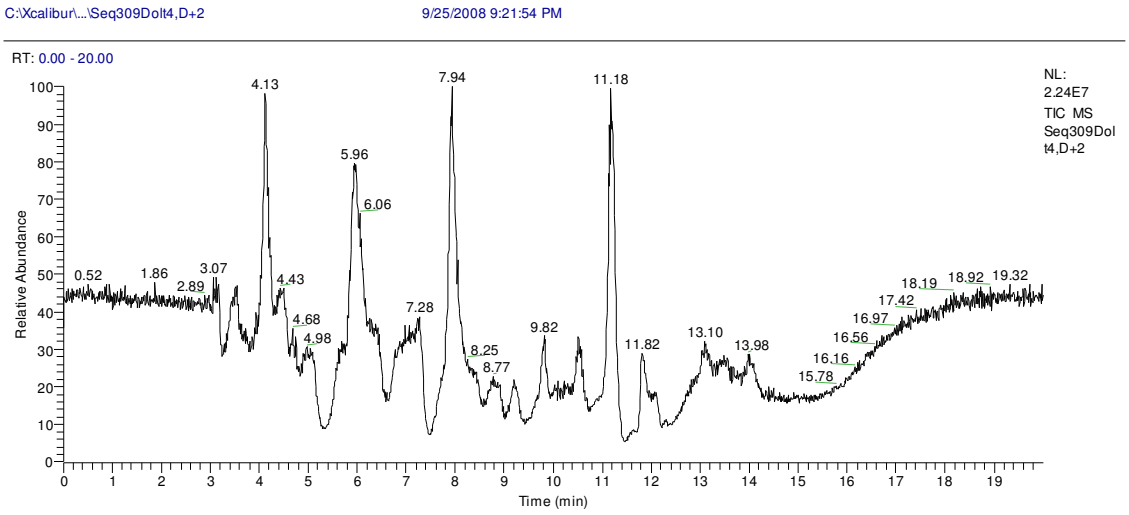

Figure 93. CX-HPLC-ES-MS total ion chromatogram of one of the replicates of DOLT-4.

Concentration of As(AsB) in DOLT-4 obtained using CX-HPLC-ES-MS can be seen in Table 69.

Table 69. Concentration of As(AsB) in extraction solution of DOLT-4 obtained using CX-HPLC-ES-MS system.

Sample	As(AsB), mg/kg
DOLT-4- Replicate 1	4.44
DOLT-4- Replicate 2	4.29
DOLT-4- Replicate 3	4.28
DOLT-4- Replicate 4	4.40
DOLT-4- Replicate 5	4.52
DOLT-4- Replicate 6	4.32
DOLT-4- Replicate 7	4.39
DOLT-4- Replicate 8	4.28
DOLT-4- Replicate 9	4.32
DOLT-4- Replicate 10	4.52
DOLT-4- Replicate 11	4.39
DOLT-4- Replicate 12	4.37
DOLT-4- Replicate 13	4.37
DOLT-4- Replicate 14	4.38
DOLT-4- Replicate 15	4.42
Mean ± standard deviation	4.38 ± 0.08

As seen in the table, results of the all replicates were found to be very close to each other. %RSD was found as 1.73. These results were in good agreement with the results found using HPLC-ICP-MS. In HPLC-ICP-MS system, average value for As(AsB) was 4.29 ± 0.38 while this value was found to be 4.38 ± 0.08 using HPLC-ES-MS methods.

PART B

CHAPTER 4

B.4. Conclusion

Cation Exchange-HPLC-ICP-MS with standard addition method and Cation Exchange-ES-MS systems with isotope dilution method were used to determine arsenobetaine content of DORM-2, DORM-3 and DOLT-4 as CRMs. All the system parameters in extraction, separation and detection steps were optimized to reach not only a good extraction from matrix but also a good separation and detection.

In HPLC-ICP-MS method, four points standard addition method was applied to DORM-2, DORM-3 and DOLT-4 CRMs. In all the standard addition experiments, AsB-D_3 was used as the standard addition reagent. AsB was also used as standard addition reagent to make sure whether there is a difference in the results obtained using AsB and AsB-D_3 in the extraction. Chemical behaviors of AsB and AsB-D_3 in ICP-MS system were also studied and it was observed that there is no difference in the chemical behavior of these two isotopes in ICP-MS. AsB contents of DORM-2, DORM-3 and DOLT-4 were found to be 15.14 ± 0.50, 3.78 ± 0.08 and 4.29 ± 0.38, respectively, using HPLC-ICP-MS. If the total amount of arsenic in these CRM samples were taken into consideration, concentration of arsenic in the form of AsB per total arsenic concentration for DORM-2, DORM-3 and DOLT-4 were found to be 83.9%, 54.9% and 44.4%, respectively. It is clear that AsB per total As concentration of DORM-2 was found to be significantly higher than values obtained for DORM-3 and DOLT-4. Reproducibility of the DORM-3 results were found to be the best one among other CRMs. %RSD values for DORM-2, DORM-3 and DOLT-4 were found as 3.30%, 2.12% and 8.86%, respectively.

In the HPLC-ES-MS system, AsB content of DORM-2, DORM-3 and DOLT-4 were determined using isotope dilution analysis by spiking of AsB-D_3. Deuterated

arsenobetaine was added to samples before the extraction step. In ES-MS detection, protonated molecular ions of two isotopes having *m*/*z* 179 and 182 were monitored for arsenobetaine and deuterated arsenobetaine, respectively. In the isotope dilution analysis, different spiking concentrations of AsB-D_3 for DORM-2, DORM-3 and DOLT-4 were used. For the DORM-2, precision of the results was found to be sufficiently low; %RSD was found as 0.74. %RSD value was found as 2.30 for DORM-3. In HPLC-ICP-MS system, average value for As(AsB) was 3.78 ± 0.08 while this value was found to be 3.63 ± 0.08 using HPLC-ES-MS method. Two results are in good agreement with each other. For the DOLT-4, results of the all replicates were found to be very close to each other; %RSD was found as 1.74. Average AsB concentration was found to be 4.38 ± 0.08. HPLC-ES-MS results were in good agreement with the results HPLC-ICP-MS like DORM-2 and DORM-3. Reproducibility of the results obtained from DORM-2 was found to be the best among all the CRMs tested, DORM-3 and DOLT-4. Higher precision of the results obtained by HPLC-ES-MS than HPLC-ICP-MS was the main advantages of isotope dilution analysis using deuterated arsenobetaine. One single spking of AsB-D_3 was enough to calculate the AsB concentration in CRM samples while four points standard addition were used in HPLC-ICP-MS system.

PART C

DETERMINATION OF THIOLS IN YEAST USING HPLC-ICP-MS AND HPLC-ES-MS SYSTEMS

CHAPTER 1

C.1. INTRODUCTION

Determination of thiol compounds in different matrices has been an important analytical issue. It is known that biological thiols (cysteine, homocysteine and glutathione) play an important role in metabolism and cellular homeostasis [229]. In literature, mostly the thiols and their disulfides having a low molecular mass have been analyzed in different matrices because of their important role in metabolism and in the antioxidant defense network [230]. Among the others, homocysteine, glutathione, cysteine, selenocysteine and cysteinyl-glycine have been analyzed most frequently.

C.1.1. Thiols and Their Body Functions

Homocysteine (HCys) is one of the thiol amino acids that is naturally occurring; it is formed by methylation of methionine. It was first identified in 1960s as a urinary metabolite in institutionalized patients with homocystinuria. It was also found that concentration of circulating homocysteine in men is about 25% higher than premenopausal women. There are many factors including age, gender, renal function, nutrition, disease states, and medications for the regulation of HCys level in human plasma [231]. In addition, there are mainly two ways defined in literature for the metabolization of homocysteine as remethylation and transulfuration [232]. In addition, it is known that most of the HCys (80%) is present in plasma as either conjugated to proteins through disulfide bonding or in the form of disulfide HCy-Cys. Elevate

concentration of HCys in the plasma is the precursor for some diseases including cardiovascular and cerebrovascular diseases in men [233].

Glutathione (GSH) is a major antioxidant and detoxifier with many essential metabolic functions in humans [230]. GSH is distributed in the nature in the form of isotripeptide (g-L-glutamyl-L-cysteinyl- L-glycine). It can be in oxidized and reduced forms. Oxidation of the -SH group of the reduced glutathione is very easy to form the disulphide bond to form the oxidized glutathione (GSSG) and protein-bound glutathione. Hence, glutathione is generally present in the environment in the form of GSH, GSSG and PSSG [234]. It was stated that GSH and its disulfide, glutathione disulfide (GSSG) are very crucial compound for human health. They have many roles in many physiological and pathological processes [235]. Enhanced tissue level of GSH resulting from dietary glutathione suggests its role as nutrients [230]. In addition, GSH protects the cell against oxidative and nitrosative stress. It can be easily converted to the reduced type by glutathione reductase [235]. In literature, there are many methods for the determination of GSH in high concentrations. Chemical and enzymatic methods have been used for this aim in literature. It is also known that determination of GSSG is more difficult than free GSH. Scientists have determined the concentration of GSSG in different matrices after reduction of GSSG to GSH. Enzymatic, chemical and electrolytic reduction has mostly been used for reduction of GSSG [236]. Decrease in the GSH concentration in the body is the precursor for the many diseases in different organs including lung that is especially vulnerable to oxidative attack [237].

Cysteine (Cys) with a thiol group is one of the naturally occurring amino acids. It has many important roles in several biological processes. This amino acid takes an important role in the protein synthesis with methionine [238]. In addition, it has a critical role in protein structure by virtue of its ability to form disulfide bonds with other cysteine residues [239]. There is a relationship between the level of cysteine and glutathione in the body. For example, according to a study on mosquito GSH deficiency by aging was linked to Cys decrease [240]. In addition, it is known that cysteine is involved in the transport/storage of NO [233].

Selenocysteine (SeCys) is also one of the amino acid. It is present in several enzymes including glutathione peroxidases, tetraiodothyronine 5' deiodinases, thioredoxin reductases, formate dehydrogenases, glycine reductases and some hydrogenases. The structure of the selenocysteine is very similar to cysteine with the

exception of an atom of selenium taking the place of the usual sulfur. This amino acid has a specialized tRNA that are initially charged with serine, but the resulting Ser-tRNA(Sec) is not used for translation [241, 242].

Cysteinylglycine (CysGly) is one of the sulfhydryldipeptide and it is generated from the extracellular glutathione, a nonprotein thiol. This compound is known as very reactive. It causes the reduction of ferric iron to ferrous iron. In addition, it was proved that cysteinylglycine causes lipid peroxidation in human plasma LDL lipoproteins and oxidative damage on DNA bases [243, 244, 245]. It is also known that the most abundant thiol in the plasma after cysteine is CysGly [233].

C.1.2. Reduction of Disulfide to Free Thiols

Disulfide bridges have a very important role in preservation of the structure of single and multi-chain proteins. Localization of the formation or reduction of a disulfide bond, stabilization of disulfide species are some of the main issues studied regarding disulfide bonds. The studies on disulfide bonds are crucial tools to obtain information about protein structure, thermodynamics and folding [246]. Hence, determination of disulfides in natural matrices is important to understand the body functions of these species. In order to analyze polypeptide chains, breaking of the disulfide bonds are crucial. It was stated that formation of the disulfide bridge is a very important step in the process of folding of a protein. Hence, analyses of free thiols and disulfides have been undertaken by many scientists to take valuable information about the structure and function of proteins [247]. In literature, there are many studies to reduce oxidized thiols to free thiols. In the reduction process, different chemicals have been applied to obtain a high reduction efficiency. In general, after the reduction of disulfides, some derivatization reagents have been used to make the analysis easier. Reduced thiols (dithiothreitol, 2-mercaptoethanol), strong reductants ($NaBH_4$) and phosphine derivatives (tris(2-carboxyethyl)phosphine (TCEP), tri*n*- butylphosphine (TBP), triphenylphosphine) have been widely used for the reduction of disulfide in different matrices [248]. There are also some studies in literature for the simultaneous determination of derivatized thiols and corresponding disulfides in biological samples [249, 250]. In general, quantitative determination of thiols has been done separately.

TCEP has been used in the reduction of disulfide by many scientists. This chemical is one of the phosphine derivatives. It is known that phosphine derivatives have many advantages as a reducing agent for thiols. These types of chemicals do not interfere with thiol-reactive labels. Hence, we do not have to remove excess amount of reagents from reaction medium. In addition, they are very stable in a wide pH range (1.5–8.5). As another advantage of these chemicals, no gas is produced after the reaction [251, 252, 248]. Pelletier and Lucy used TCEP for the on-line reduction of disulfides to their corresponding thiols. In their study, simultaneous detection of thiols and disulfides was achieved [248]. Sack et al. applied TCEP as reducing agent for the quantification of total GSH and other low molecular weight thiols by precolumn derivatization with OPA; they obtained very low detection limits for thiols using TCEP as reducing agent [253]. In addition, Gray used water soluble TCEP at pH 3 for the reduction of disulfide [254]. In this study, it was observed that although the rate of reduction of disulfide bonds varied widely using TCEP, most peptides did not show a strongly preferred route in the reduction step [254]. In another study, TCEP was used to find out the disulfide structure of recombinant human AGRP protein. Reduction of disulfides was achieved using TCEP under acidic conditions; and than free thiols were alkylated using *N*-ethylmaleimide or fluorescein-5-maleimide [255]. In this study, AGRP protein was partially reduced with TCEP that is prepared in 1.0 M of sodium acetate at pH 4.6. Disulfide bonds initially reduced were directly alkylated with NEM using the same buffer in the reduction medium [255]. Seiwert and Karst applied the tris(2-carboxyethyl)phosphine for the reduction of the disulfide-bound thiols for the determination of cysteine, glutathione, cysteinylglycine, *N*-acetylcysteine, homocysteine, and their disulfides in urine samples [256]. Reinbold et al. also used tris(2-carboxyethyl)phosphine for the determination of total glutathione and total cysteine in wheat flour by a stable isotope dilution assay using high-performance liquid chromatography/tandem mass spectrometry [257].

2-mercaptoethanol and $NaBH_4$ have also been widely used in the reduction of disulfide. Chwatko and Bald used 2-mercaptoethanol to convert protein-bound cysteine to free cysteine; the total cysteine in human plasma was determined by using high-performance liquid chromatography and ultraviolet detection after pre-column derivatization [229]. Kusmierek and Bald used $NaBH_4$ for the determination reduced and oxidized forms. In that study, reduced and total glutathione and cysteine in citrus

fruit juices were determined after the reduction of oxidized thiols to free thiols [230]. Raspi et al. used sulfite ion in the reduction of disulfide to overcome the reaction between reducing agents that have been used in literature (mercaptoethanol) and PHMB as derivatization agent; in this study, evaluation of the number of sulfhydryl and disulfide groups per protein molecule, in native or denatured (reduced or not) form was done using radiochromatographic method [258]. Some enzymes like enzyme glutathione reductase was applied to convert GSSG to its free thiol, GSH, in the presence of the cofactor NADPH [259].

Free thiols have been mostly used for the reduction of disulfide bonds to its free thiols. It is now clear that there are two basic steps in the reaction of disulfide and free thiols throughout the reduction step. In the disulfide structure, W represents rest part of thiol after -S.

W-S-S-W + R-SH ↔ W-S-S-R + W-SH (First Step)
W-S-S-R + R-SH ↔ R-S-S-R + W-SH (Second Step)

Similar reactions take place between dithiothreitol (DTT) and disulfides during the reduction of disulfides. In first part of the reaction system, DTT like a monothiol reacts with disulfides to give a mixed disulfide. This step has an equilibrium constant near unity. In the second step, free -SH end of formed disulfide reacts with the disulfide group in the chain to give not only cyclic disulfide named 4,5-dihydroxy-l,P-dithiane but also cysteine. It was reported that the equilibrium constant of the second reaction step is $1.3x10^4$; due to this high equilibrium constant in the reaction, DTT can be easily used for the reduction of disulfides without producing any byproducts such as mixed disulfides [260]. In 1971, Meienhofer et al. used DTT for the reduction of disulfides in proteins and peptides without side reactions in the reaction medium [261]. Before the detection step, reduced thiols were alkylated with alkyl chlorides and then S-alkylated derivatives were isolated after evaporation of ammonia that is used in the preparation of DTT [261]. Bramanti used DTT for the reduction of denatured proteins; in that study, human serum albumin, bovine serum albumin, a- lactalbumin (a-Lac) from bovine milk, and lysozyme from chicken egg (Lys) were denatured with urea and reduced with DTT [247]. El-Zohri et al. applied DTT for the elimination of possible oxidation during the sample preparation and analysis; DTT as an anti-oxidant was added at the very

beginning for the determination of glutathione and phytochelatins in plant tissues [262]. Andersson et al. analyzed the total, free (non-protein-bound), and reduced forms of homocysteine, cysteine, glutathione, cysteinylglycine, and y-glutamylcysteine in human plasma using isocratic reversed-phase ion-pair high-performance liquid-chromatographic method [263]. Samples were treated with DTT as reducing agent for disulfides. After the postcolumn derivatization with 4,4'-dithiodipyridine thiols were determined using colorimetric method at 324 nm. LOD for homocysteine was found to be lower than 50 nmol/L plasma [263]. In another study, DTT was used to reduce the disulfide bonds between homocysteine and other thiols in the total plasma homocysteine determination by Huang et al [264]. Cystine and homocystine were prepared as acidic mixture and reduced under the optimum parameters of DTT concentration, reduction temperature, and reaction time. 1.0 M of DTT was used at 70 ^{0}C of reaction medium temperature and throughout 1.0 hour as reaction time for the reduction of disulfides. It was concluded that more than 96% of the disulfides of cystine and homocystine were reduced to their thiols in the reaction medium [264]. Hong et al. applied DTT for the reduction of the disulfide bonds of immunoglobulin G (IgG) to characterize the structure of IgG [265]. Gel electrophoresis was applied to separate the fragments [265].

C.1.3. Derivatization of Thiols

It is known that there are many proteins containing thiol groups and/or disulfide bonds. These groups are considered as important functional groups for biological systems. Selective reaction of the thiol groups where sulfhydryl groups take part with different chemicals were commonly applied in the quantitative determination [258]. In literature, there are many different types of derivatization methods including different chemicals to analyze the thiols in different matrices. After the derivatization of thiols with a chemical, it is possible to determine them in very low concentrations. Analyzing period of time including sample preparation and determination should be very short to protect analytes against decomposition. Some of the methods developed for the determination of thiols are time-consuming, so analytes can decompose and not easily analyzed.

1,3,5,7-tetramethyl-8-phenyl-(2-maleimide)-difluoroboradiaza-s-indacene (TMPAB-o-M) was applied to clinical and biological samples for the determination of

reduced glutathione in the presence of relatively high levels of cysteine by Guo et al [266]. Spectrofluorimetric determination of the GSH was done after derivatization step; it was observed that fluorescence is restored with a 350-fold intensity increase after reaction with thiol [266]. Benkova et al. found a new derivatization reagent named N-(2-acridonyl)-maleimide (MIAC) for the determination of thiol groups [267]. It is claimed that the reaction between MIAC and aminothiols is specific, very fast and highly fluorescent products are produced; LODs for homocysteine, cysteine and glutathione were found to be 1.2, 1.4 and 2.0 pmol, respectively [267].

Reinbold et al. applied high-performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) for the simultaneous quantitation of total glutathione and total cysteine in wheat flour [268]. Three different chemicals, *N*-ethylmaleimide (NEMI), iodoacetic acid (IAA) and 4-vinylpyridine (distilled; 4-VP) were applied to find the best derivatization procedure for glutathione and cysteine [268]. Niskijima et al. figured out a new method to simultaneously measure the amounts of GSH and Cys in biological samples. In this method, GSH and Cys were firstly alkylated with iodoacetic acid and then derivatization of free amino groups was done using 1-dimethylaminonaphathlene 5-sulfonyl chloride (dansyl chloride). Finally, GSH and Cys were analyzed using HPLC with fluorescence detection [269]. Hammermeister et al. has also used the dansyl chloride to confirm the identity of dansylated derivatives of cysteine and glutathione, and their respective dimers, cystine and glutathione disulfide using high performance liquid chromatography/electrospray ionization-mass spectrometry [270]. Schofield and Chen analyzed wheat flour for reduced and oxidized glutathione. In theirs method, they extracted the thiols form matrix using 5% (w/v) perchloric acid not only to prevent sulphydryl-disulphide (SH/SS) interchange reactions but also to separate PCA-extractable peptides from proteins. Iodoacetic acid was used for the alkylation of thiols and then dinitrophenylation of free amino groups was done with 1-fluoro-2,4-dinitrobenzene [271]. Kusmierek and Bald applied a different chemical in the derivatization step for the determination of different species of glutathione and cysteine in fruit juices. Derivatization of thiols was done using 2-chloro-1-methylquinolinium tetrafluoroborate. After the derivatization step, thiols were separated form each others via chromatographic system and then analyzed with UV-absorbance detection [230].

P-hydroxymercuribenzoate (PHMB) is the most commonly used derivatization agent in literature. In the case of utilization of mercurial probes (organic RHg^+ and inorganic mercury Hg^{2+}) for the derivatization of –SH groups, affinity and specificity of the reaction is very high [272]. PHMB has been chosen as the derivation reagent for 3 reasons:

(1) PHMB interacts with –SH groups at room temperature in a short time (<90s) with high affinity and specificity; (2) The thiol-PHMB complexes are shown stable for 12 h if kept at room temperature, or 3 months if stored at -20°C; (3) The thiol-PHMB complexes maintain the solubility of the non-complexed peptides. Hence, this chemical has been used for the sensitive determination of different types of thiols including cysteine, glutathione, homocysteine, Cys-Gly and other volatile thiols. In the determination of thiols, researchers have mostly used UV or fluorescence detectors. It is known that these compounds have neither a strong UV absorption nor fluorescence. Hence, derivatization of thiol groups before detection system is crucial so as to improve the sensitivity. Bramanti et al. developed a sensitive, specific method for the low-molecular-mass thiols such as cysteine, cysteinylglycine, glutathione, and homocysteine [273]. They applied their method for the determination of glutathione in blood after the validation of method where PHMB was used as derivatization agent. After the derivatization step, derivatized thiols were reacted with bromine to give Hg(II). In the detection step, Hg(II) was coverted to Hg^0 via sodium borohydride reduction and determined using atomic fluorescence spectrometry in an Ar/H_2 miniaturized flame [273]. Bramanti and D'Ulivo also used mercury probe derivatization coupled with liquid chromatography-atomic fluorescence spectrometry for the determination of hydrogen sulfide and volatile thiols in air samples [274]. Nitrosothiols were also derivatized using PHMB. Bramanti and Jacovozzi et al. used PHMB for the determination of *S*-nitrosoglutathione and other nitrosothiols using chemical vapor generation atomic fluorescence detection [275]. In this study, GSNO and other RSNOs (CysNO, HCysNO and CysGlyNO) in human plasma were determined. The reaction GSNO-PHMB by UV measurements at 334 nm was studied in detail and characterization of the products was also done using Electrospray Ionization Mass Spectrometry and Reversed Phase Chromatography (RPLC) coupled on-line and sequentially with a UV–visible diode array detector (DAD) followed by a cold vapor generation atomic fluorescence spectrometer [275].

In the derivatization, there are some possible reactions between –SH/-SeH containing species and mercury compounds of the type Hg^{2+} or RHg^{+} (R = methyl, phenyl, etc.) [274]:

1. $RSH + HO–Hg–C_6H_4–COOH \rightarrow RS–Hg–C_6H_4–COOH + H_2O$
2. $RS^{-} + HO–Hg–C_6H_4–COO^{-} \rightarrow RS–Hg–C_6H_4–COO^{-} + OH^{-}$

Reaction 1 (above) occurs in acidic media while Reaction 2 takes place in alkaline media.

C.1.4. Chromatographic Methods for the Separation of Thiols

In the speciation analysis of thiols, separation of species from each other is the next step after the sample preparation including reduction if needed and derivatization. Capillary electrophoresis, gas chromatography and high performance liquid chromatography have been widely used in literature for the separation of different thiol species from each other. All these methods can be utilized for the separation of different thiol species in different matrices.

Because of the high separation power of capillary electrophoresis, not only the discrimination against the complicated cell matrix but positive identification of the analytes based on retention times as well can be achieved for different thiols using this technique [276]. Capillary electrophoresis technology also allows a decrease in the analysis time and reduction of the costs. Hence, there are many studies where this method was applied. Zinellu et al. applied high-throughput capillary electrophoresis method for plasma cysteinylglycine measurement. In this study, analysis time was reduced about 50% using a rapid capillary electrophoresis method for the selective quantification of plasma cysteinylglycine [277]. In another study, capillary electrophoresis was used for the separation of homocysteine, glutathione, cysteinylglycine, and cystationine. This method was successfully applied for the plasma samples to analyze 6-iodoacetamidofluorescein derivatives [278]. Davey et al. applied the high-performance capillary electrophoresis for the simultaneous analysis of the oxidised and reduced forms of the major cellular hydrophillic antioxidants, ascorbic acid (vitamin C) and glutathione (γ-L-glutamyl-L-cysteinylglycine) [279].

Another chromatographic method for the separation of thiols is gas chromatography. GC instruments may be combined with different detectors. Gas chromatography mass spectrometry is one of the combinations and specially applied to homocysteine determination [280]. Gas chromatography has been applied for separation of thiols in petroleum products. Zhao and Xia used GC equipped with a flame photometric detector (FPD) and the normalization method in order to analyze the enriched thiol sample for the composition and structure of thiols in gasoline from several refineries in China [281]. Gas chromatography coupled to ICP–MS is another method for the determination of thiols. Remy et al. applied this method for determination of total homocysteine in human serum. In this method, analytes were first reduced with sodium borohydride, and then converted to their *N*-trifluoroacetyl-*O*-isopropyl derivatives. After the derivatization step, sample was injected to gas chromatograph equipped with an HP-5 capillary column. Double-focusing inductively coupled plasma mass spectrometer (DF-ICP–MS) was used in the detection step [282]. ICP–MS could be used for the determination of *S*-amino acid, but monitoring of the major sulfur isotope (^{32}S, 95% abundance) is impossible because of polyatomic interferences generated by O_2^+ in quadrupole based ICP-MS system. At this point, DF-ICP–MS allows separation of the ^{32}S isotope from such isobaric interference because of the higher resolving power. Remy et al. coupled gas chromatography to double focusing ICP–MS for the determination of sulfur amino acids, their method was based on formation of their *N*-trifluoroacetyl-*O*-isopropyl derivatives in real serum samples. Detection limits for cysteine, homocyteine and methionine were found as 1.9, 0.68 and 0.60 µmol L^{-1}, respectively [282]. Mestres developed a method for the determination of eleven sulphur compounds in white and red wines [283]. In order to obtain high sensitivity for analytes, several parameters including temperature, time, ionic strength, headspace volume and the volume of headspace injected were optimized. All analytes were separated from each other under the optimum conditions. In order to concentrate the analytes, a cryogenic trap was applied, and then these analytes were chromatographically separated and analyzed using GC temperature programming on a poly(ethylene glycol) capillary column with FPD detection at 394 nm [283].

The most popular method for the separation of different thiols from each others in different sample matrices is HPLC. Different separation modes of HPLC such as reversed-phase, size-exclusion and ion-exchange (anion and cation exchange) have

been used in literature for thiol speciation. The combination of HPLC with MS detector is very popular in literature because of low detection limits. In addition to that combination, HPLC has been combined with different detection techniques like fluorescence [284], mass spectrometry [285, 250, 286] and electrochemical detection [287, 288, 289]. HPLC methods using either fluorescence or tandem mass spectrometry detection gave better sensitivity in tissues than those obtained using selected ion monitoring mass spectrometry for the determination of different types of thiols [290].

Jiang et al. used high-performance liquid chromatography for separation and electrospray tandem mass spectrometric for determination of cysteine, total homocysteine, S-adenosylmethionine, S-adenosylhomocysteine, cystathionine, methionine, glutathione and cysteinylglycine in plasma with N-(2-mercaptopropionyl)-glycine as internal standard [286]. Separation time for eight aminothiols was 20 minutes [286]. Houze et al. described an ion-exchange HPLC method with electrochemical detection for rapid quantification of glutathione, homocysteine, cysteinylglycine, and methionine in blood samples. This method was applied to patients with chronic renal failure or non-insulin-dependent diabetes for the determination of reference values [291]. Melnyk et al. developed a method for the determination of most common monothiols and disulfides present in plasma or tissue extracts [292]. In the developed method, reversed phase ion-pairing high performance liquid chromatography with coulometric electrochemical detection system was applied to simultaneously quantify free oxidized and reduced aminothiols. In addition, determination of total aminothiols was achived after chemical reduction. The limit of detection of this method is quite low, 5 fmol/mL for monothiols and 50 fmol/mL for dithiols [292].

C.1.5. Determination of Thiols

In the speciation of thiols, the last step is the detection step. Qualitative and quantitative detection of thiol species are done in this step.

Fluorescence is one of the most popular detection systems for the thiol compounds. It is known that many thiols have no functional groups to give fluorescence signal. Hence, some derivatization reagents have been applied to thiols to get compound having fluorescence properties. Fluorescein isothiocyanate [293], 5-(Bromomethyl) fluorescein [294], *p*-hydroxymercurybenzoate [275] and fluorescein-5-

maleimide [295] are some of the chemicals that have been used for thiol determination using fluorescence detector. Park et al. analyzed the grape juice and wine for their glutathione, cysteine, methanethiol and ethanethiol contents. Precolumn derivatization of thiols were done using o-phthalaldehyde and 2-aminoethanol, isoindole and derivatized species were separated from each other using reversed-phase HPLC column [296]. In the detection step, fluorescence detector was applied for quantitation measurements. The detection limits for thiols were found as GSH, 3.3 nmol/l (1 µg/l); cysteine, 22 µmol/l (2.7 mg/L); methanethiol, 0.27 µmol/l (12.8 µg/l); ethanethiol, 0.65 µmol/l (11 µg/l) [296]. In another study, reverse phase chromatography coupled on-line with cold vapour generation atomic fluorescence spectrometry was applied for the determination of thiolic groups. Proteins containing thiols were firstly denaturized and derivatized using a phosphate buffer solution including urea and *p*-hydroxymercurybenzoate and then derivatized denatured proteins was online reacted with bromine generated by $KBr/KBrO_3$ in HCl to give inorganic mercury. Hg(II) was reduced using sodium borohydride and reduced mercury was detected by AFS in a Ar/H_2 miniaturized flame [272].

AAS is another method for the determination of thiols. Bramanti and Cavallaro et al. determined cysteine, glutathione, penicillamine, cystein–glycine, homocysteine and *N*-acetyl penicillamine using flow injection CV-AAS [297]. In this method, mercury was reduced to Hg^0 by using $NaBH_4$ and measured using AAS. Detection limit was found to be 2.5 pg, so the risk of interferences caused by complex real matrices can be minimized because of permission for sample dilution. The method was applied to wine samples [297].

Martin et al. used coulometric method for the measurement of plasma total homocysteine. Ion-paired reversed-phase HPLC was used to separate HCys from other possible thiol species; coulometric electrochemical detection, which avoids time-consuming pre or postcolumn derivatization was used. Penicillamine was added to all samples as an internal standard for HCySH determination because of having a similar chemical structure with HCys [289].

GC-MS is one of the combinations especially applied to homocysteine determination in different matrices including human plasma. Valerio et al. described a rapid and precise GC/MS method to determine homocysteine turnover from a relatively low volume of plasma (0.2 mL). In this report, 2-mercaptoethanol was applied to

samples to reduce the disulfide bonds and then bis-tert-butyldimethylsily was added to the solution as a derivatization agent. DL-[3,3,3´,3´,4,4,4´,4´-2H8]-homocystine was used as a internal standard to account for losses associated with each analytical step. A significant ion at m/z 325 ($[M–114)]^+$) was monitored in the GC-MS system [280].

Electrospray MS system has been applied for the thiol speciation. Krupp defined a method for the determination of mercury bound to biothiols. Chromatographic separations of species were carried out within 10 min with formic acid as the mobile phase using a reversed-phase column [298]. HPLC was connected online to ES-MS system. Characterization of mercury and methylmercury complexes derived from their reactions with cysteine and glutathione: $Hg(Cys)_2$, $Hg(GS)_2$, MeHgCys and MeHgGS were done by using electrospray mass spectrometry equipped with an ion trap. In the experimental part, all mercury-thiol compounds for standards were synthetically prepared in solution using stoichiometric amounts. Cys (m/z 122) and GSH (m/z 308) were also monitored to make sure whether the mercury compounds had reacted quantitatively with the organic thiols or not [298]. Tomaiuolo applied liquid chromatography coupled to ion-spray tandem mass spectrometry for the determination of homocystine in human plasma. Quantitative determination of HCys was done using internal standard homocystine-d8. Limit of detection was found to be near to 6.0 fmol/L; a short column was selected to enhance the stability of the signals [232].

Analytes of interest determined in this study are shown in Table 70.

Table 70. Full names, formulas and abbreviations of oxidized and reduced species used in this part of thesis.

Oxidized Species			Reduced Species		
Full Name	Formula	Abbreviation	Full Name	Formula	Abbreviation
Cystine	$C_6H_{12}N_2O_4S_2$	Cys-Cys	Cysteine	$C_3H_7NO_2S$	Cys
Homocystine	$C_8H_{16}N_2O_4S_2$	HCys-HCys	Homocysteine	$C_4H_9NO_2S$	HCys
Selenocystine	$C_6H_{12}N_2O_4Se_2$	$Se(Cys)_2$	Selenocysteine	$C_3H_7NO_2Se$	SeCys
Oxidized Glutathione	$C_{20}H_{32}N_6O_{12}S_2$	GSSG	Glutathione	$C_{10}H_{17}N_3O_6S$	GSH
			Selenomethionine	$C_5H_{11}NO_2Se$	SeMet
			Cysteinylglycine	$C_5H_{10}N_2O_3S$	CysGly

The purpose of this study was to develop new HPLC-ICP-MS and HPLC-ES-MS methods for the determination of total thiols in biological samples in trace amounts.

PART C

CHAPTER 2

C.2. EXPERIMENTAL

C.2.1. Standard and Reagents

Analytical reagent grade chemicals were used throughout this study. DL-Homocsytine (H0501, Sigma), Seleno-L-Cystine (09976, Fluka), L-Glutathione oxidized (49740, Fluka), L-Cystine (C8755, Sigma), Seleno-DL-methionine (S3875, Sigma), DL-homocysteine (H4628, Sigma), L-Cysteine (30089, Fluka), L-Glutathione reduced (G6529, Sigma), CysGly (L0166, Sigma), Tris (2-carboxy-ethyl)phosphine hydrochloride (C 4706, Sigma), 4-(Hydroxymercuri)benzoic acid sodium salt (55540, Fluka) and 1,4-Dithio-DL-threitol (43815, Fluka) were used throughout this study.

In the mobile phase, formic acid (UN 1779, Anachemia, Canada Inc.) and ammonium hydroxide (Anachemia, Canada Inc.) were used. In order to clean the cation exchange column, methyl alcohol (MXC488-1, EMD Chemicals) was utilized.

C.2.2. HPLC-ICP-MS Studies

ELAN 6000 (PE-SCIEX, Thornhill, Ontario, Canada) equipped with Ryton spray chamber was used for ICP-MS measurements. All ICP-MS parameters were daily optimized to find the best sensitivity for mercury using soft-ware program. In the reduction and derivatization of thiols, Branson 3510 sonication instrument and a Wrist Action Shaker, Model 75, Burrell Corporation, Pittsburgh, Philadelphia, U.S.A) were used.

In the HPLC studies, a Dionex BioLC model LCM (Dionex Corp., Sunnyvale, California, USA), fitted with 50.0 µL of loop was applied. Agilent, Zorbax, Eclipse XDB-C8 (150 x 4.6 mm x 5 µm) was used in reverse phase based separations.

Chromatographic signals were processed using in-house software in Excel. In all dilutions, de-ionized water purified to 18 MΩ.cm using a NANOpure water purification system (Barnstead/Thermolyne, Dubuque, Iowa, USA) was used.

In the determination of thiol, mercury was monitored in HPLC-ICP-MS system; ^{201}Hg isotope was used in the calculation, but other isotopes were also monitored throughout this study. At the beginning, thiols were reacted with PHMB to give Thiol-PHMB complex, and then thiol complexes were separated from each other using HPLC. Separated thiol-PHMB complexes were determined in ICP-MS by monitoring Hg in thiol-PHMB complex.

ICP-MS parameters were optimized to find a high sensitivity for Hg. In the optimization, 70.0 ng/mL of Hg prepared in mobile phase, 0.15% (v/v) TFA, was used. All the parameters optimized can be seen in Table 71.

Table 71. Optimization results of ELAN 6000 ICP-MS parameters for Hg.

Optimization Parameter	**Result**
RF Power	1350 W
Sample Flow Rate	1.0 mL/min
Nebulizer Gas Flow	1.0 L/min
Lens Voltage	9.1 V

Sensitivity of the system was periodically checked and in the case of decreasing in sensitivity, all of the ICP-MS parameters were re-optimized.

Separation of thiols from each other is an important step for the speciation analysis of thiols in different matrices. In this study, Reverse Phase-HPLC system was tried to find the best separation conditions. In order to make the analysis of thiols using HPLC-ICP-MS, all thiols in the medium have to be derivatized using the chemical that can be easily analyzed in ICP-MS. For the optimization of HPLC conditions, in initial stages, thiol-PHMB complexes were formed using parameters that were not optimized. For this aim, 9.84 mM of L-glutathione and 10.1 mM of cysteine in 0.05% of formic acid were prepared as single analyte stock solutions. Derivatization of cysteine and glutathione were performed using 9.66 mM of PHMB prepared in 26.0 mM of NaOH. In the derivatization, 1.0 mL of 9.66 mM of PHMB was separately added to 1.0 mL of 10.1

mM of cysteine and 9.84 mM of L-glutathione. Samples were put into sonication bath for 10.0 minutes. Before the HPLC-ICP-MS measurement, proper dilutions were done for samples using de-ionized water. After the optimization of HPLC conditions, derivatization procedures would also be optimized.

C.2.3. HPLC-ES-MS Studies

In the HPLC-ES-MS system, ES-MS measurements were performed using Thermo LTQ-Orbitrap Mass Spectrometry. All ES-MS parameters were optimized to find best sensitivity for analytes using soft-ware program. Electrospray system parameters used in the determination of thiols can be seen in Table 72.

Table 72. Parameters of ES-MS system used in free thiols determination.

ESI Source	
Spray Voltage (kV)	3.04
Spray Current (µA)	5.33
Sheath Gas Flow Rate (L/min)	20.03
Aux Gas Flow Rate (L/min)	5.00
Sweep Gas Flow Rate (L/min)	0.02
Capillary Voltage (V)	36.03
Capillary Temp (°C)	300.01
Tube Lens (V)	90.02

Agilent 1100 Series model HPLC fitted with a 10.0 µL loop was used. Separation by using C8 column was achieved using Agilent, Zorbax, SB-C8 (100 x 2.1 mm x 3.5 µm). HPLC parameters used in the separation of thiols from each other can be seen in Table 73.

Table 73. Experimental parameters for HPLC-ES-MS system.

Parameter	
Column	Agilent, Zorbax, SB-C8 (100 x 2.1 mm x 3.5 µm)
Solvent Program	0-1 min 90% of 0.10% Formic Acid in H_2O 10% of 0.10% Formic Acid in CH_3OH 1-10 min 90-70% of 0.10% Formic Acid in H_2O 10-30% of 0.10% Formic Acid in CH_3OH 10-20 min 70-10% of 0.10% Formic Acid in H_2O 30-90% of 0.10% Formic Acid in CH_3OH 20-27 min 10-90% of 0.10% Formic Acid in H_2O 90-10% of 0.10% Formic Acid in CH_3OH 27-35 min 90% of 0.10% Formic Acid in H_2O 10% of 0.10% Formic Acid in CH_3OH
Flow Rate	0.2 mL/min
Loop Volume	10.0 µL

C.2.3.1. Sample Preparation

All of the system parameters for thiols were optimized to get not only high reduction but also high derivatization efficiencies. All of the optimization experiments are given in “Result and Discussion” part. Under the optimum conditions, thiol species were extracted from yeast samples and then oxidized thiols were reduced using DTT. After the reduction step, thiol species were derivatized using PHMB. Derivatized thiols were separated from each other using RP-HPLC and determined using ES-MS instrument. In sample preparation, thiol content of 25.0 mg sample was extracted by using 10.0 mL of DIW. Samples were sonicated for 5.0 min at room temperature (24-27 ^oC), and then centrifuged for 10.0 min at 6500 rpm to separate supernatant from

residue. Supernatant was decanted into clean 50 mL of centrifugation tube. Supernatant was filtered using Microcon Centrifugal Filters (Ultracel YM-3, Regenerated Cellulose 3.000 MWCO, Millipore) at 13500 RPM for 90 min. After the filtration step, oxidized thiol contents of samples were tried to be reduced using DTT. For this aim, 300 µL of sample was taken and 15.0 µL of 5.0 mM DTT was added into the sample. Reduction was carried out for 60 min. After the reduction step, 15.0 µL of 15.0 mM of PHMB was added to the sample for the derivatization of thiols for 15.0 min. Samples were finally analyzed in HPLC-ES-MS system for the determination of total thiol contents.

PART C

CHAPTER 3

C.3. RESULTS and DISCUSSION

C.3.1. HPLC-ICP-MS Studies

In the optimization of parameters, the elution regimes tested are given in Table 74. In all measurement, all of the Hg isotopes were monitored and ^{201}Hg was generally used in the calculations if there was no problem in signal.

Table 74. Elution regimes tested for RP-HPLC-ICP-MS separation of Cys-PHMB and GSH-PHMB using Agilent, Zorbax, Eclipse XDB-C8 (150 x 4.6 mm x 5 µm), 1.0 mL/min flow rate and 50.0 µL loop volume.

Elution Regime	Given Name	Mobile Phase and/or Solvent Program
Isocratic	RP-MP1	0.10 M Phosphate Buffer, pH 7.0
Isocratic	RP-MP2	0.10 M Phosphate Buffer with 2.0% MeOH, pH 7.0
Isocratic	RP-MP3	10.0 mM ammoniumformate, pH 4.13
Isocratic	RP-MP4	0.05% TFA in 2.0% MeOH, pH Natural
Isocratic	RP-MP5	0.10% TFA, pH Natural
Solvent Programming	RP-SP6	a) 0-4 min, 0.10% TFA, pH Natural, 100% b) 4-6 min, 0.10% TFA, pH Natural, 100-75%, 0.10M Phosphate Buffer with 2.0% MeOH, pH 7.0, 0-25% c) 6-8 min, 0.10% TFA, pH Natural, 75%, 0.10 M Phosphate Buffer with 2.0% MeOH, pH 7.0, 25% d) 8-9 min, 0.10% TFA, pH Natural, 75-100%, 0.10M Phosphate Buffer with 2.0% MeOH, pH 7.0, 25-0%
Isocratic	RP-MP7	0.15% TFA, pH Natural
Solvent Programming	RP-SP8	a) 0-4 min, 0.15% TFA, pH Natural, 100% b) 4-6 min, 0.15% TFA, pH Natural, 100-75%, 0.10 M Phosphate Buffer with 2.0% MeOH, pH 7.0, 0-25% c) 6-9 min, 0.15% TFA, pH Natural, 75%, 0.10 M Phosphate Buffer with 2.0% MeOH, pH 7.0, 25% d) 9-10 min, 0.15% TFA, pH Natural, 75-100%, 0.10 M Phosphate Buffer with 2.0% MeOH, pH 7.0, 25-0%

The Elution regimes named as RP-MP1 and RP-MP2 resulted in chromatograms with no resolution at all. Cys-PHMB and GSH-PHMB were resolved using RP-MP3 and RP-MP4, but the peak shapes were poor with irregularities and broadening. Using RP-MP5 resolution was achieved in 700 s, but there were unknown Hg signals. Using solvent programming RP-SP6, separation was reached in 500 s, but peak shape of Cys-PHMB was not good regarding tailing. RP-MP7 also gave a good resolution, but GSH-PHMB retention time was 1120 s, the chromatogram was obtained in relatively a long period of time. Finally, the most satisfactory result was obtained by using RP-SP8 for Cys-PHMB and GSH-PHMB. Chromatogram is given in Figure 94.

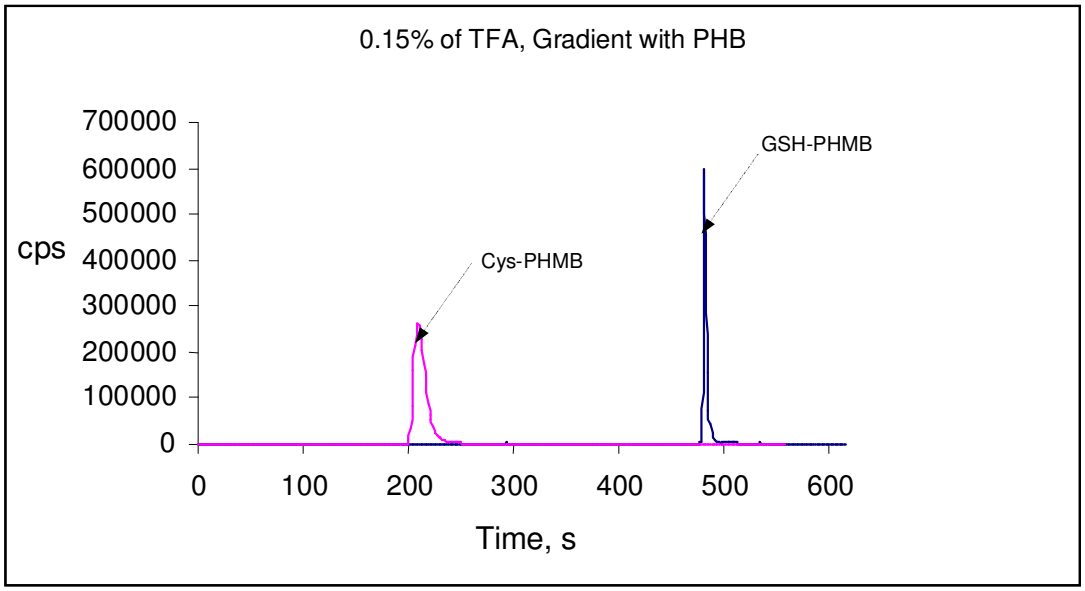

Figure 94. Single analyte chromatograms for Cys-PHMB and GSH-PHMB using the parameters given in Table 74, RP-SP8, by monitoring ^{201}Hg.

As seen in Figure 94, separation of Cys-PHMB and GSH-PHMB species from each other was achieved using the parameters given in Table 74.

In this part of the study, some of the other thiols, namely CysGly and HCys were obtained, so the study was continued using four thiols. For each thiol, 1.0 mM of standard solution was prepared using appropriate amount of solid samples. 0.50 mL of 1.0 mM PHMB as a derivatization agent was added into 0.50 mL of each analyte solution for the derivatization. Samples were sonicated throughout 10.0 minutes for the derivatization. After the derivatization step, solutions were diluted as required and

injected to HPLC-ICP-MS system to obtain single analyte chromatograms. The single analyte chromatograms for Cys-PHMB, CysGly-PHMB, HCys-PHMB and GSH-PHMB are shown in Figure 95 using RP-SP8 given in Table 74.

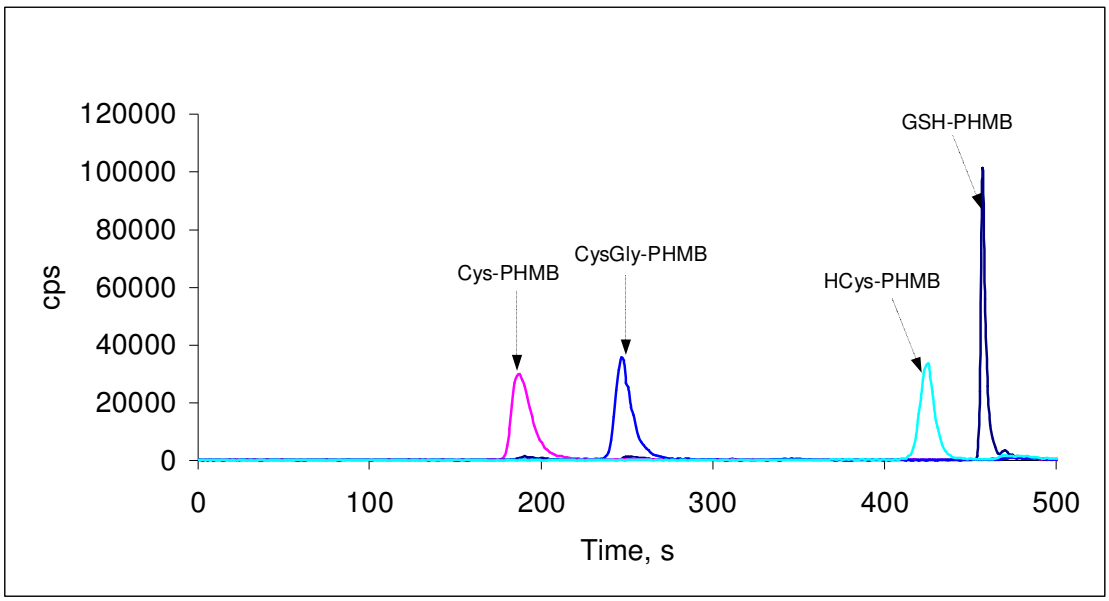

Figure 95. Chromatograms for Cys-PHMB, CysGly-PHMB, HCys-PHMB and GSH-PHMB using the parameters given in Table 74, RP-SP8, by monitoring ^{201}Hg.

As seen in Figure 95, Cys, CysGly, Homo-Cys and GSH species could be separated from each other using the parameters given in Table 74. Retention times for Cys-PHMB, CysGly-PHMB, HCys-PHMB and GSH-PHMB were found to be 195, 250, 415 and 460 seconds, respectively. In addition, a mixed solution containing 1.0 mM of each thiols was prepared and derivatized using 1.0 mM of PHMB. 4.0 mL of 1.0 mM PHMB was added into 1.0 mL of mixed standard solution for the derivatization. Samples were sonicated throughout 10.0 min. After the derivatization step, solutions were diluted as required and injected to HPLC-ICP-MS system. The chromatograms for mixed standard solution are shown in Figure 96.

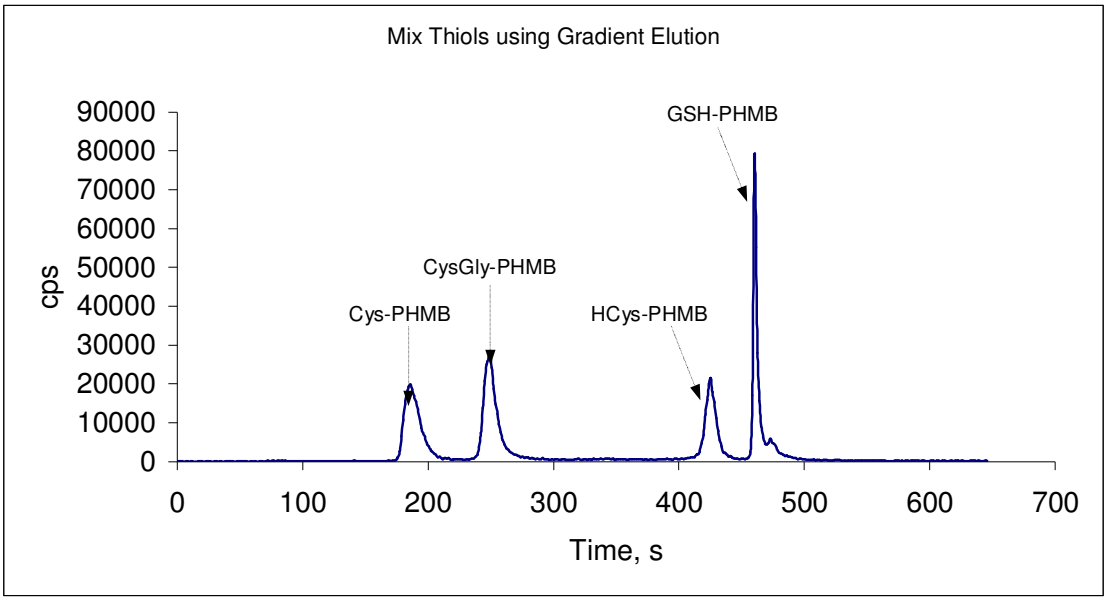

Figure 96. HPLC-ICP-MS Chromatogram of mixed standard solution containing PHMB derivatives of 1.0 mM Cys, CysGly, HCys and GSH using the parameters given in Table 74 as RP-SP8, by monitoring ^{201}Hg.

As seen in Figure 96, resolution of the chromatographic system is good enough to make both qualitative and quantitative measurements of these four thiol species. Peak widths of the signals were not so wide to affect the other analyte signals. Although there is a big difference in the peak height of the signals, peaks areas were found to be close to each other. In addition, there were no other signals in the chromatograms except for the signals obtained from complexes of Cys, CysGly, HCys and GSH with PHMB.

C.3.1.1. Optimization of PHMB Concentration for Derivatization

In order to find the proper concentration of PHMB used in the derivatization step for free thiols, different concentrations of PHMB prepared in 0.10 M NaOH, 1.0, 1.2, 1.5, 2.0, 5.0 and 10.0 mM, were tried. Main stock solutions of thiols were prepared in 0.050% formic acid. In the further dilutions, de-ionized water was used. In the derivatization, 0.80 mL of 1.0, 1.2, 1.5, 2.0, 5.0 and 10.0 mM of PHMB was added to 0.80 mL of analyte solution containing 0.25 mM of each thiol, Cys, CysGly, HCys and

GSH. Samples were sonicated for 30 min for derivatization. After the derivatization step, solutions were diluted as required, and injected to HPLC-ICP-MS system.

1.0 mM, 1.2 mM and 1.5 mM PHMB resulted in clean chromatograms qualitatively similar; separation and quantitative analysis was feasible. Signals of the thiol-PHMB complexes after the derivatization with 1.2 mM of PHMB can be seen in Figure 97.

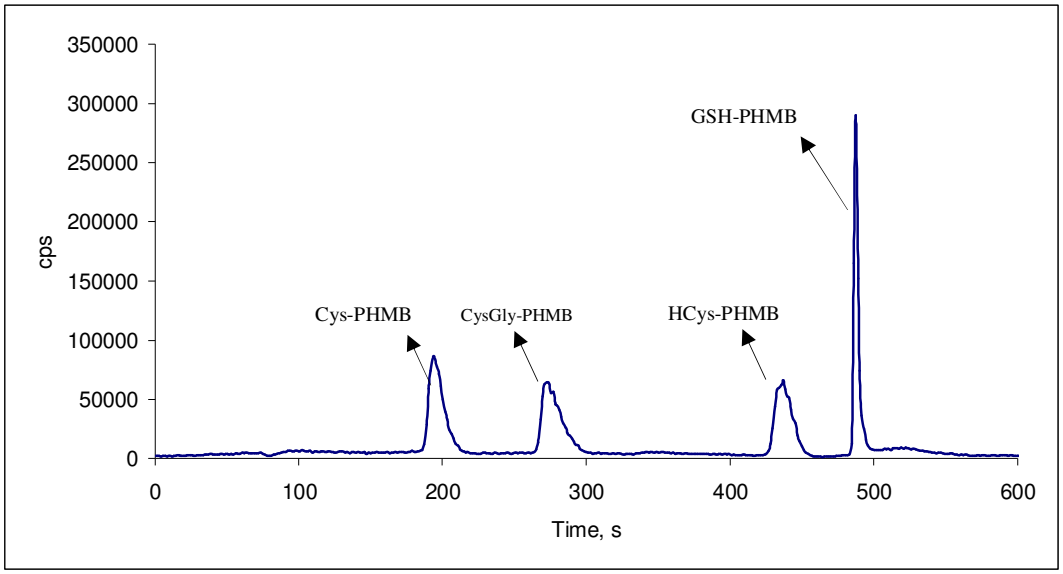

Figure 97. ^{201}Hg-HPLC-ICP-MS chromatogram of mixed standard solution containing 0.25 mM in each of Cys, HCys, GSH and CysGly after the derivatization with 1.2 mM PHMB.

Signals of the thiol-PHMB complexes after the derivatization of 0.80 mL of standard solution containing 0.25 mM each of Cys, Hcys, GSH and CysGly with 0.80 mL of 2.0 mM of PHMB can be seen in Figure 98.

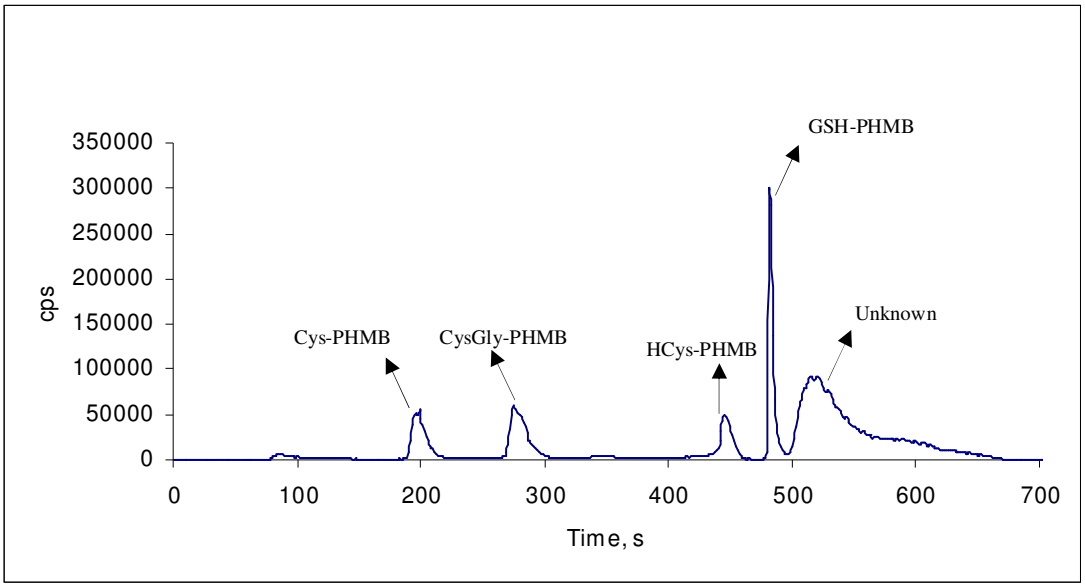

Figure 98. ^{201}Hg-HPLC-ICP-MS chromatogram of mixed standard solution containing 0.25 mM in each of Cys, HCys, GSH and CysGly after the derivatization with 2.0 mM PHMB.

There is a very broad signal appearing at about 520 s in case of 2.0 mM PHMB in derivatization step. Retention time of this broad signal is very close to GSH signal. Hence, GSH signal is affected from the tail of this broad signal. There are no big differences on the other thiol signals. In case of 5.0 mM and 10.0 mM PHMB used in derivatization, the broad unknown peak in Figure 98 become larger and broader; analysis was not feasible.

It can be finally concluded that 1.0, 1.2 and 1.5 mM can be selected as the optimum concentrations for PHMB regarding resolution of the signals, peak shapes and intensity of unknown Hg signals.

C.3.1.2. Determination of Total Thiol

In order to control the reduction of oxidized thiols to reduced thiols, selenocyctine, $Se(Cys)_2$, was used. It is known that we can easily monitor both mercury and selenium simultaneously in ICP-MS. In addition, selenocystine and selenocysteine have different retention times in C8 column used. Hence, decrease in selenocystine signal and increase in selenocysteine signal give us chance to see the reduction efficiency. For the reduction of $Se(Cys)_2$, 0.50 mL of 1.0 mM of TCEP prepared in water

was added to the 0.50 mL of 0.5 mM of $Se(Cys)_2$ standard solution. Reduction was performed throughout 2.0 hours by sonication. After the reduction of $Se(Cys)_2$ to the selenocysteine, a mixed standard solution containing 0.2 mM each of free thiols, Cys, HCys, CysGly, GSH and selenocysteine was prepared. In the derivatization, 1.0 mL of 1.0 mM PHMB prepared in 0.10 M NaOH was added into 1.0 mL of mixed standard solution. Derivatization was performed in sonication instrument for 30 minutes. In addition, reduction of each thiol in single analyte solution was separately realized using the same method. After the derivatization procedure, sample was diluted and injected to C8-HPLC-ICP-MS system. The chromatographic separation condition used before was not stable especially for the retention time of glutathione when reducing agent was used in the medium. Hence, new chromatographic separation parameters were tried to obtain not only a good separation but also stable signals.

In Table 75, the parameters for a new gradient elution with ramping for the separation of thiols are given.

Table 75. Experimental parameters for reverse phase-HPLC-ICP-MS system.

Parameter	
Column	Agilent, Zorbax, Eclipse XDB-C8 (150 x 4.6 mm x 5 µm)
Mobile Phase	a) 0-10.5 min 90% of 0.15% TFA, 10% of 0.10 M Phosphate Buffer with 2.0% MeOH, pH 7.0 b) 10.5-11.5 min, 90-50% of 0.15% TFA, 10-50% of 0.10 M Phosphate Buffer with 2.0% MeOH, pH 7.0 c) 11.5-12.5 min, 50% of 0.15% TFA, 50% of 0.10 M Phosphate Buffer with 2.0% MeOH, pH 7.0 d) 12.5-13.5 50-90% of 0.15% TFA, 50-10% of 0.10 M Phosphate Buffer with 2.0% MeOH, pH 7.0
Flow Rate	1.0 mL/min
Loop Volume	50.0 µL

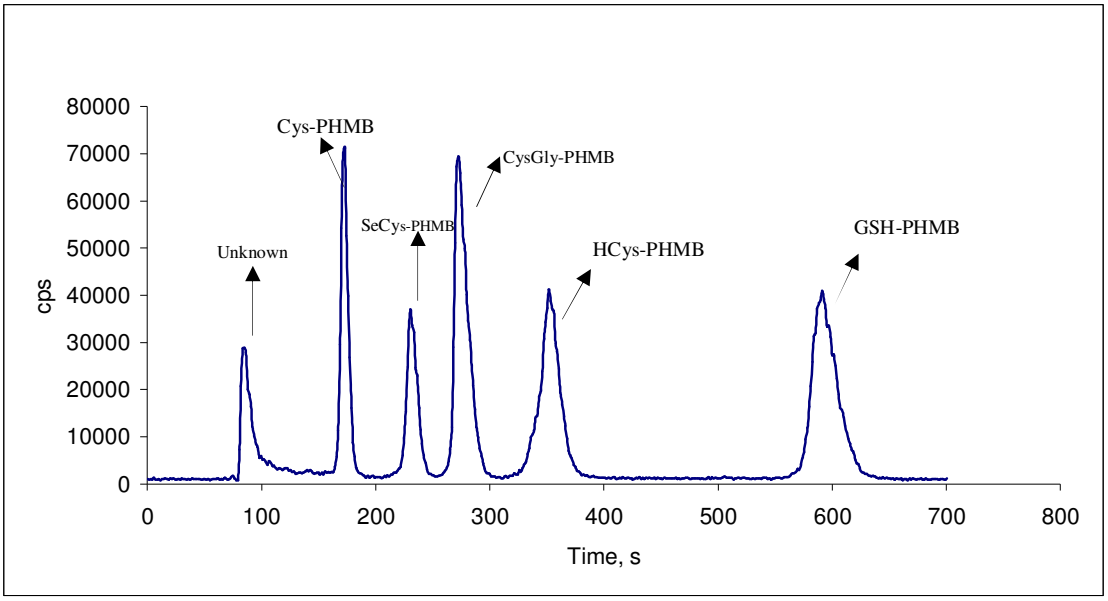

Figure 99. HPLC-ICP-MS chromatogram of mixed standard solution containing 0.2 mM of Cys, HCys, GSH, CysGly and SeCys after the derivatization with 1.0 mM PHMB .

As seen in Figure 99, retention times for Cys-PHMB, SeCys-PHMB, CysGly-PHMB, HCys-PHMB and GSH-PHMB were found to be 180, 225, 285, 350 and 596 seconds, respectively. Separation of all the species from each other was achieved using these chromatographic conditions. Baseline of the chromatographic signals is smooth enough for qualitative and quantitative measurements. Peak shapes of signals are sharp and resolution of the system is well enough. In the new chromatographic separation system, stochiometric ratio of PHMB/Thiol was selected as best one in the derivatization step.

C.3.1.2.1. Optimization of TCEP Concentration in Reduction Step

In order to find the proper concentration of TCEP used to reduce –S-S- bound in the thiols, different concentrations of TCEP, 0.10, 0.20, 0.50, 0.75, 1.0, 2.0, 5.0 and 10.0 mM, were tried. In the reduction, 0.5 mM of $Se(Cys)_2$ was used. 0.5 mL of TCEP at different concentrations was added to the 0.50 mL of $Se(Cys)_2$. Solution was sonicated for 30 minutes. After the reduction step, 0.50 mL of 1.0 mM of PHMB was added to each sample as the derivatization agent. Samples were again sonicated for 10.0 min for

derivatization. After the derivatization step, solutions were diluted as required and injected to HPLC-ICP-MS system. ^{78}Se and ^{82}Se were monitored throughout study to make sure whether all selenocystine is converted to selenocysteine or not. Selenocystine was dissolved in 0.10 M HCl and injected to HPLC-ICP-MS system to find out the retention time of selenocystine. The retention time of $Se(Cys)_2$ is shown in Figure 100.

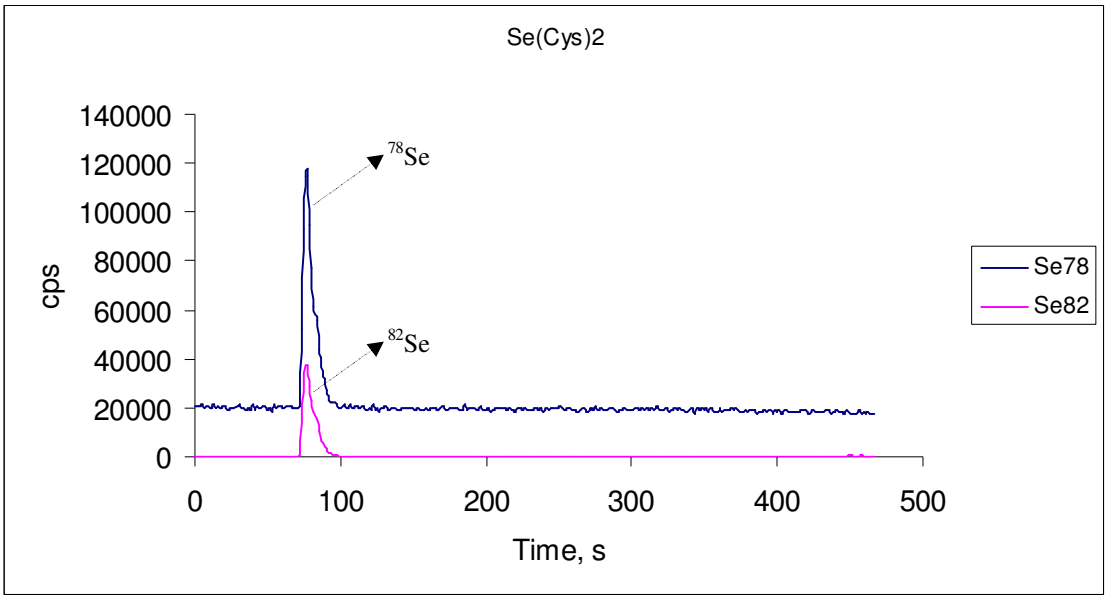

Figure 100. HPLC-ICP-MS chromatogram of 1.24 µM of $Se(Cys)_2$ prepared in 0.10 M HCl .

After reduction of $Se(Cys)_2$ to SeCys, solution was injected to HPLC-ICP-MS system. ^{78}Se, ^{82}Se and ^{201}Hg were monitored to obtain information on the reduction efficiency of $Se(Cys)_2$. Chromatograms obtained using 0.10 mM TCEP are shown in Figure 101.

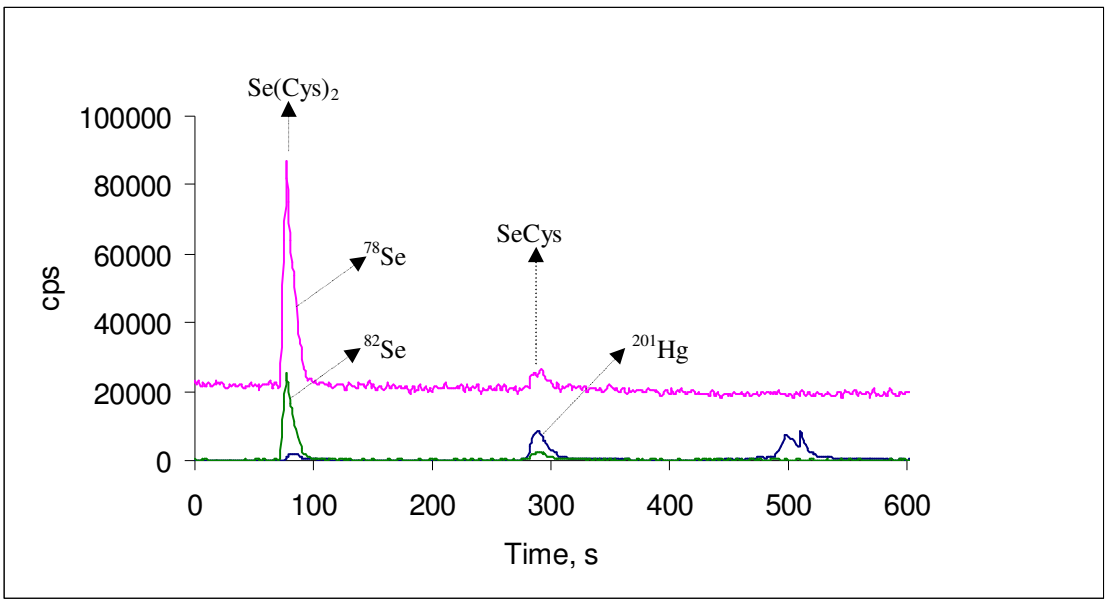

Figure 101. HPLC-ICP-MS chromatogram of $Se(Cys)_2$ using 0.10 mM TCEP in reduction and 1.0 mM PHMB prepared in 0.10 M NaOH in derivatization, 2.0 h reduction period and 30 min derivatization period.

It is clear that there is very small SeCys signal observed by using 0.10 mM of TCEP used as reducing agent in the retention time of 290 s. At the same retention time, ^{201}Hg signal was also observed. This also proved the reduction of $Se(Cys)_2$ to SeCys. Chromatograms obtained using 0.20 mM TCEP had the similar features with SeCys and ^{201}Hg signals increasing with higher TCEP concentrations.

Chromatograms obtained using 0.75 mM TCEP are shown in Figure 102.

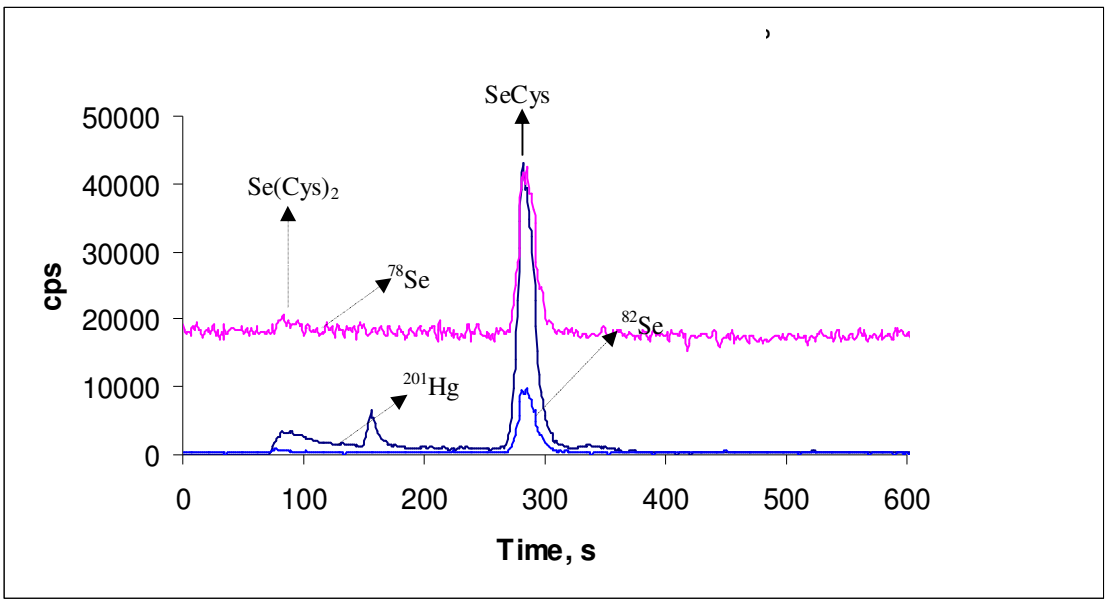

Figure 102. HPLC-ICP-MS chromatogram of $Se(Cys)_2$ using 0.75 mM TCEP in reduction and 1.0 mM PHMB prepared in 0.10 M NaOH in derivatization, 2.0 h reduction period and 30 min derivatization period.

In this chromatogram, it was observed that very small amount of $Se(Cys)_2$ remained that was not reduced to SeCys. The small signal at the retention time of 90 s shows the unreduced $Se(Cys)_2$. TCEP concentration was further increased to 1.0 mM to reduce $Se(Cys)_2$ to get almost 100% reduction efficiency. Chromatograms obtained using 1.0 mM of TCEP are shown in Figure 103.

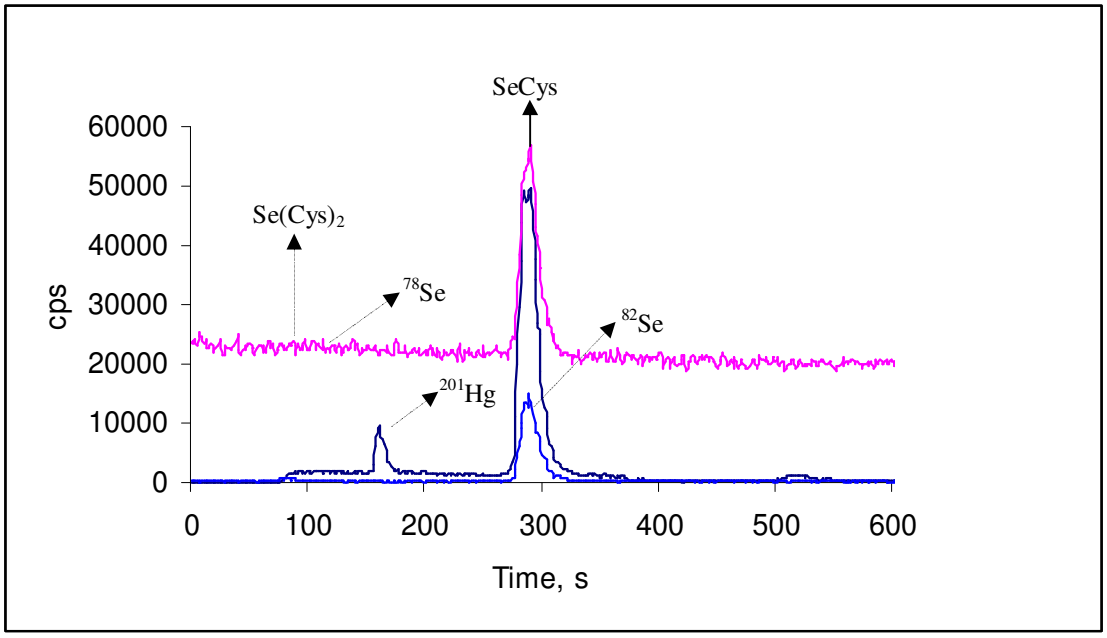

Figure 103. HPLC-ICP-MS chromatogram of $Se(Cys)_2$ using 1.0 mM TCEP in reduction and 1.0 mM PHMB prepared in 0.10 M NaOH in derivatization, 2.0 h reduction period and 30 min derivatization period.

All of the $Se(Cys)_2$ species could be reduced using the 1.0 mM of TCEP as reducing agent. It is clear in the figure that there is no selenium signal at the retention time of $Se(Cys)_2$ while there is a big signal at the retention time of SeCys. In addition, ^{201}Hg signal coming from SeCys-PHMB complex was observed at the same retention time of SeCys. This also proved the reduction of $Se(Cys)_2$ to SeCys. Higher concentrations of TCEP were also used in the reduction. Chromatograms obtained using 2.0 mM of TCEP had features similar to those in Figure 103.

5.0 mM of TCEP was also tried in the reduction and the chromatogram shown in Figure 104 was obtained.

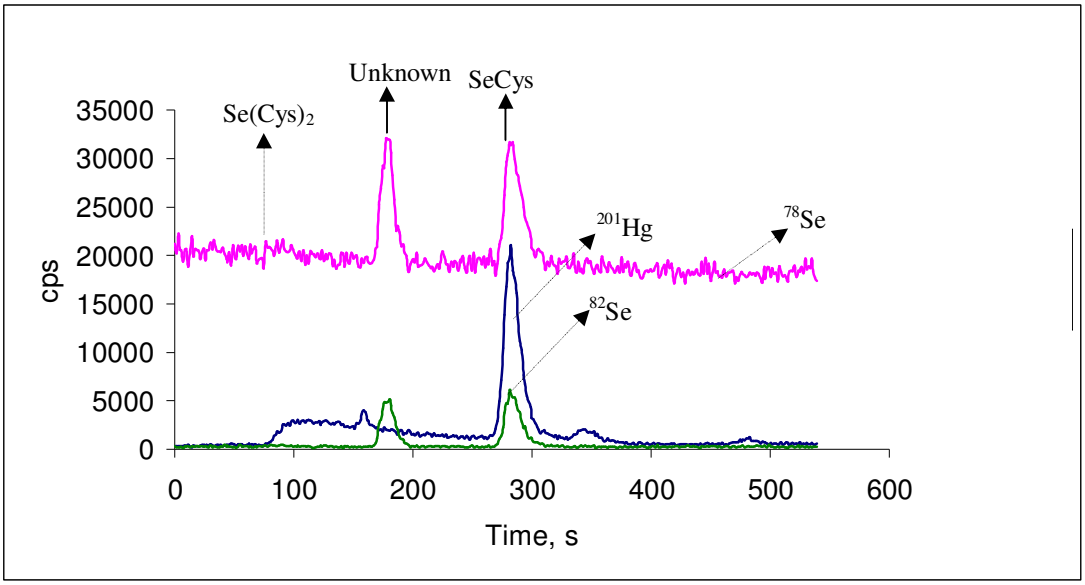

Figure 104. HPLC-ICP-MS chromatogram of $Se(Cys)_2$ using 5.0 mM TCEP in reduction and 1.0 mM PHMB prepared in 0.10 M NaOH in derivatization, 2.0 h reduction period and 30 min derivatization period.

Unknown products were formed and the desired properties of chromatogram could not be obtained. Chromatograms obtained using 10.0 mM of TCEP can be seen Figure 105.

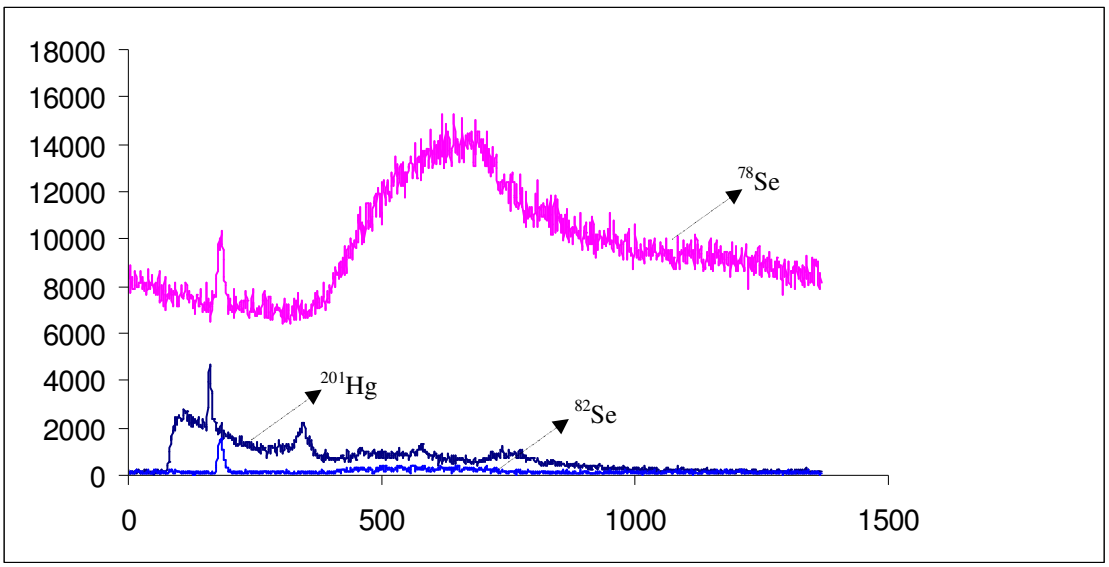

Figure 105. HPLC-ICP-MS chromatogram of $Se(Cys)_2$ using 10.0 mM TCEP in reduction and 1.0 mM PHMB in derivatization, 2.0 h reduction period and 30 min derivatization period.

As seen in Figure 105, there are 2 selenium signals in the chromatogram where 10.0 mM of TCEP was used and one of them having 600 second as retention time had a very wide peak width. In addition, there was no Hg signal at 300 seconds. Hence, it can be concluded that reducing behavior of TCEP in 5.0 mM or higher concentration is not effective for the $Se(Cys)_2$. As an optimum value 1.0 mM TCEP was adopted for the reduction of $Se(Cys)_2$.

In the previous studies, PHMB was prepared in 0.10 M NaOH. In this part of study, PHMB was prepared in water and used for the derivatization of SeCys obtained after reduction with 1.0 mM TCEP to find out whether there is a difference in the reduction efficiency or not.

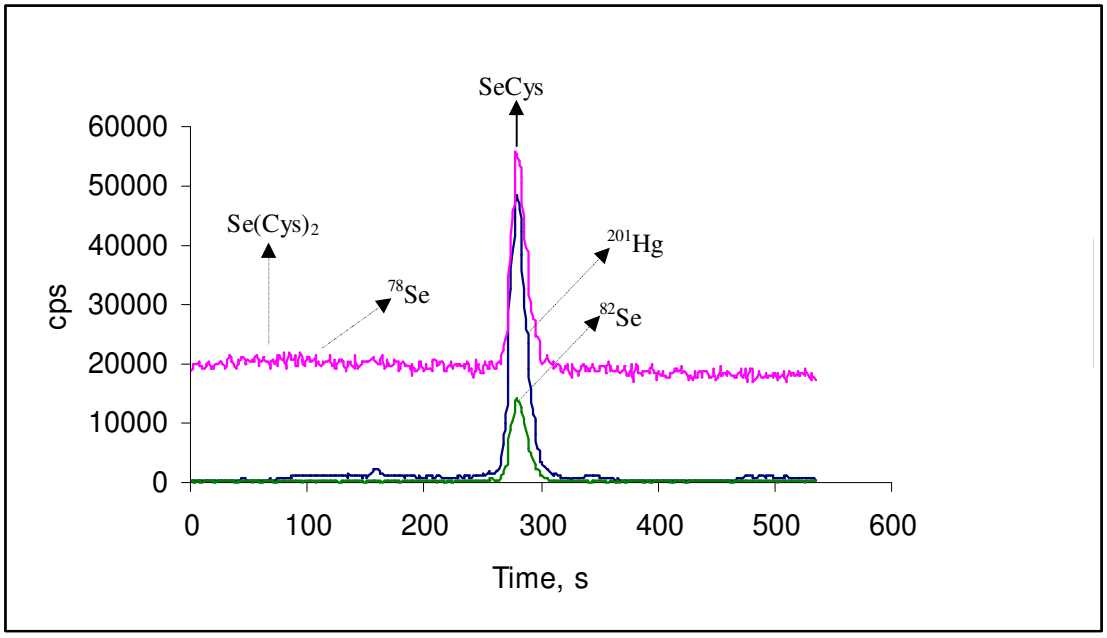

Figure 106. HPLC-ICP-MS chromatogram of $Se(Cys)_2$ using 1.0 mM TCEP in reduction and 1.0 mM PHMB prepared in H_2O in derivatization, 2.0 h reduction period and 30 min derivatization period.

As seen in Figure 106, there is no signal observed at the retention time of 90 seconds where unreduced $Se(Cys)_2$ was eluted from column. It is clear that all of the $Se(Cys)_2$ is reduced to selenocyteine. Hence, 1.0 mM TCEP prepared in H_2O was selected as a feasible concentration in the reduction of selenocystine by using 1.0 mM PHMB.

C.3.1.2.2. Reduction of Thiols using $NaBH_4$

In this part of study, $Se(Cys)_2$ and oxidized glutathione, GSSG, were tried to be reduced using $NaBH_4$. For this aim, 6.0 M $NaBH_4$ was prepared and diluted (1+1) using dimethylsulfoxide. In the reduction, 150 µL of 0.5 mM GSSG and $Se(Cys)_2$ were separately placed into two glass tubes and then 100 µL of 6.0 M $NaBH_4$ (diluted 1+1 using dimethylsulfoxide) and 50 µL of 3.0 M HCl were added into each tube. Each mixture was sonicated for 30 minutes. After the reduction, 60 µL of 3.0 M of HCl were added to each mixture in order to decompose excess $NaBH_4$. For the derivatization, 150 µL of 1.0 mM of PHMB prepared in water were added into each tube and mixture was sonicated for 10.0 min. After the derivatization step, proper dilutions were done and

samples were injected to HPLC-ICP-MS system under the optium parameters given in Table 75.

It was found that $NaBH_4$ is not a proper reagent for the reduction of GSSG and $Se(Cys)_2$. There are no SeCys-PHMB and GSH-PHMB signals observed in respective chromatogram after the reduction using $NaBH_4$.

C.3.1.2.3. Reduction and Derivatization of Oxidized Thiols for Reduction Efficiencies

As given above, 1.0 mM was selected as the optimum concentration of TCEP for the reduction of selenocytine to selenocysteine. The same amount of TCEP was also used for the reduction of other thiols. In order to find out the reduction efficiency, free thiols were also derivatized and determined using optimum parameters. In the reduction, 0.50 mM each of Cys-Cys, HCys-HCys, GS-SG was used. In addition, 1.0 mM each of Cys, HCys, GSH were used to check the reduction efficiencies. Reduction and derivatization periods were used as 120 and 30 minutes, respectively. For the each oxidized thiol, 0.5 mL of sample was placed into a glass tube and then 0.5 mL of 1.0 mM TCEP (pH 7) were added for reduction. The mixture was sonicated for 120 minutes. After the reduction, 1.0 mM of PHMB (pH 7) was added and mixture was sonicated 30 minutes. After the derivatization step, proper dilutions were done and samples were injected to HPLC-ICP-MS system using the optimum parameters given in Table 75. For the free thiols, 0.50 mL of 1.0 mM of each free thiol was derivatized using 0.50 mL of 1.0 mM PHMB for 30 min.

For Cys, peak areas of free Cys and Cys after the reduction of Cys-Cys were found to be very close to each other. The reduction efficiency of Cys-Cys was found to be about 100% using 1.0 mM of TCEP (pH 7) in reduction step (120 minutes) and 1.0 mM of PHMB prepared in H_2O in derivatization step (30 minutes). It was also observed that there were 2 additional Hg signals having the retention times of 155 and 340 s. These signals are most probably due to either unreacted PHMB or other unknown mercury compounds.

Peak heights of reduced GSH and free GSH are found to be very close to each other at the same retention time, 660 seconds. It means that reduction efficiency of

GSH was about 100% like Cys using 1.0 mM TCEP (pH 7) in reduction step (120 minutes) and 1.0 mM of PHMB (pH 7) in derivatization step (30 minutes). It means that ratio of TCEP/oxidized thiol should be 2 to reduce the all GS-SG to GSH. In addition to GSH signal, there were some other unknown signals having the same retention times with the unknown signals in Cys chromatograms.

For HCys, signal was observed at 435 seconds in the chromatograms of reduced and free HCys. In addition, there are 3 additional signals in both reduced and free thiol chromatograms having the same retention times with the signals observed in GSH and Cys chromatograms. It was observed that all of the HCys-HCys are reduced to HCys under these conditions by considering the peak areas of both reduced and free thiol signals.

There is no oxidized CysGly sold in market. Hence, free CysGly was prepared and injected to HPLC-ICP-MS system to get the information about retention time of this species. Retention time of CysGly is about 255 seconds. There are also other 3 mercury signals observed at 90, 160 and 340 seconds. Retention times of these unknown mercury species are not close to CysGly signal. Hence, there is no problem to make qualitative and quantitative measurments of this species.

Selenocystine was also reduced and derivatized using the same method that was applied to other thiols. Selenocysteine is not commercialy produced. Hence, pure SeCys could not be used to find reduction efficiency of $Se(Cys)_2$. In the determination of reduction efficiency of $Se(Cys)_2$, method described before was used. In this method, $Se(Cys)_2$ and SeCys signals were used by monitoring ^{78}Se, ^{82}Se and ^{201}Hg. There was no signal observed at 90 seconds where selenocystine is eluted from system; therefore, all of the selenocystine should be reduced to selenocysteine.

Single analyte chromatograms of reduced Cys, reduced HCys, reduced GSH, reduced $Se(Cys)_2$ and CysGly obtained in HPLC-ICP-MS system using the optimum parameters given in Table 75 can be seen in Figure 107. For this aim, each oxidized thiol was reduced and derivatized separately, and then injected to HPLC-ICP-MS.

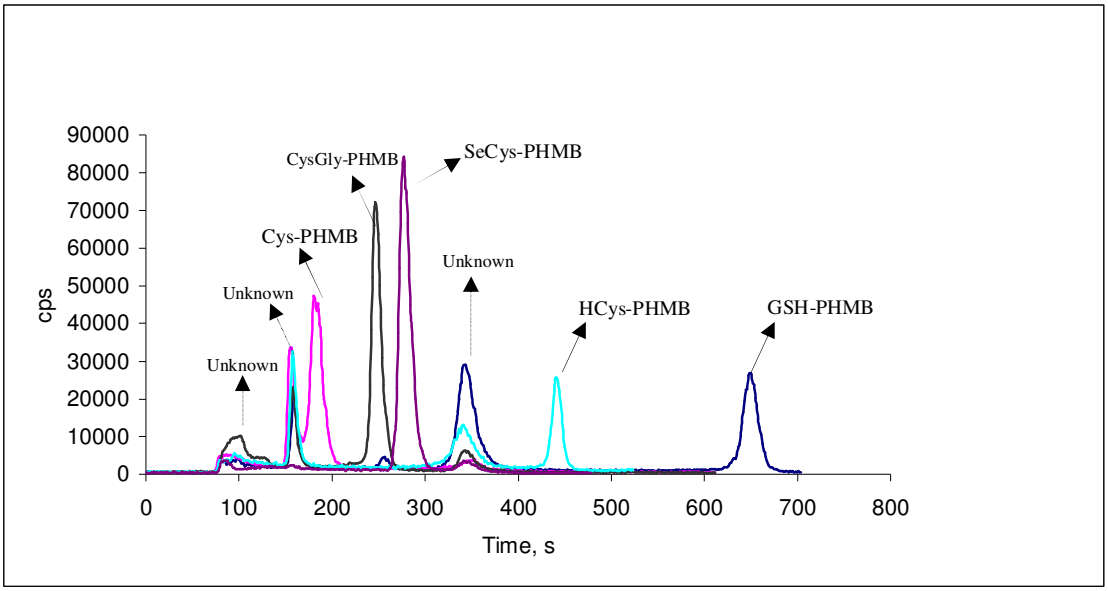

Figure 107. HPLC-ICP-MS chromatograms of 1.0 mM of single analyte solution of Cys, HCys, GSH, CysGly and SeCys using 1.0 mM TCEP in reduction, 1.0 mM PHMB in derivatization, 120 min reduction period and 30 min derivatization period.

As seen in the Figure 107, separation of 5 species from each other was achieved using parameters mentioned above. Retention times for Cys, CysGly, SeCys, HCys and GSH were found to be 185, 235, 285, 435 and 660 seconds, respectively. The signals belonging to unreacted PHMB were eluted at 90, 170 and 350 seconds and they do not affect the signals of analytes with the exception of Cys. Tail of the signal at the retention time of 170 seconds overlaps partly with the cysteine signal, but it does not cause a serious problem in qualitative and quantitative measurement of cysteine since there is no effect of this tail on the peak height of cysteine. In addition, baseline of the chromatographic signals is smooth enough for qualitative and quantitative measurements for each thiol species. Peak shapes of signals are sharp and resolution power of the system is well enough.

C.3.1.3. Analytical Figures of Merit for HPLC-ICP-MS System

Analytical performance of the system was determined by using the peak height measurement for ICP-MS signals for ^{201}Hg for each species. In the calculations, standard solutions of thiol species were used. It was found that limit of detection values

of HPLC-ICP-MS for the species were changed between 440 and 1110 fmol. All the LOD and LOQ values for the HPLC-ICP-MS system can be seen in Table 76.

Table 76. Analytical figures of merit for HPLC-ICP-MS system for selected thiols.

	Cys	**HCys**	**SeCys**	**GSH**	**CysGly**
LOD, fmol	730	1110	440	1110	580
LOQ, fmol	2430	3690	1480	3710	1940

Although the analytical figures of merits for HPLC-ICP-MS system was sufficient to determine the thiol species given above in trace levels, unknown mercury signals might cause problem for real sample measurements. In addition, it was impossible to use excess amount of TCEP in reduction as well as excess amount of PHMB in derivatization step that prevents chromatographic separation and detection of thiol species in HPLC-ICP-MS system. In the real sample measurements, excess amounts of TCEP and PHMB should be used to make sure that all of the oxidized thiol species are reduced and derivatized.

C.3.2. HPLC-ES-MS Studies

Determination of the thiol species were also done by using HPLC-ES-MS system. In literature, there are not many studies where this system was used for the determination of different thiols. Although identification of thiol species were done using ^{201}Hg signals as a marker at different retention times in HPLC-ICP-MS system, it is very easy to determine thiol species by monitoring molecular masses of thiols using HPLC-ES-MS system. In HPLC-ICP-MS system, unreacted PHMB can cause some interference problems for the analyte species. All of the mercury compounds are monitored in ICP-MS, so all of the retention times of the mercury compounds should be different from each other to obtain resolved signals and to make qualitative and especially quantitative measurements. This is not a problem for HPLC-ES-MS system because all of the masses can be monitored separately and the resolution of the ES-MS instrument is high enough to separate the two very close signals having the very similar masses.

C.3.2.1. Determination of Total Thiols using HPLC-ES-MS System

The method involves reduction of oxidized thiol using DTT and derivatization of reduced thiols with PHMB. Cystine, homocystine, selenocystine and oxidized glutathione are the analytes used in the method developments for the reduction. Identification of analytes is based on retention times in the positive-ion ES–MS chromatogram. In addition to cystine, homocystine, selenocystine and oxidized glutathione, we also determined some free thiols including CysGly and selenocysteine. We know that yeast samples contain also selenomethionine. Hence, we add selenomethionine to list of analytes. In order to find out the retention time of each thiol, a mixed standard solution including all the analytes were prepared and all of the system parameters were optimized.

C.3.2.1.1. Free Thiol

C.3.2.1.1.1. Optimization Studies

C.3.2.1.1.1.1. Optimization of Derivatization Conditions

In order to do the determination of thiols using HPLC-ES-MS, all of the thiols in the medium should be derivatized not only to get lower detection limits but also to increase stability of thiols. 20.0 mM of main stock solution for each of Cys, HCys, GSH and CysGly were prepared in 0.05% formic acid (v/v) to minimize possible oxidation of thiols. 1.0 mM single analyte solution were prepared in de-ionized water from main stock solutions. Seleno-DL-methionine was also included in analytes in addition to the thiols. For this aim, 1.0 mM of seleno-DL-methionine was prepared in de-ionized water. For the reduction of $Se(Cys)_2$ to the selenocysteine, procedure given in “HPLC-ICP-MS” part was used. After reduction of $Se(Cys)_2$ to SeCys, 10.0 µM mixed standard solution containing Cys, HCys, GSH, CysGly, SeCys and SeMet was prepared in water. In order to find out the best derivatization procedure, three different methods, namely standing at room medium, sonication and shaking, were applied to the samples. In all the methods, a mixed standard containing 10.0 µM each of thiol was used. The molar ratio of PHMB/thiols was applied as 20. Derivatization time was kept constant, 30 minutes, in

all methods. In the first method, “Standing at room medium”, 50 µL of 20.0 mM PHMB was added to 1.0 mL of mixed thiol solution containing 10.0 µM each of Cys, HCys, GSH, CysGly, SeCys and SeMet. Derivatization was performed in ambient conditions for 30 min. In the second method, “Sonication”, derivatization was performed in sonication for 30 min while shaker was used for 30 min in the last method, “Shaking”.

Table 77. Peak area of signals in the optimization study of derivatization type.

	Cys	**HCys**	**SeCys**	**GSH**	**SeMet**	**CysGly**
Standing at room medium	1722355	2777616	1992998	4416668	888384	2367876
Sonication	1636172	2647822	1891580	4261233	871373	2331841
Shaking	1643949	2621315	1888049	4219414	887525	2318152

As seen in Table 77, there are no large differences between the results, but “Standing at room medium” method looks more proper than others. Therefore, standing samples at room medium without using any instruments was selected.

C.3.2.1.1.1.2. Optimization of Derivatization Period

Different derivatization periods were applied to thiols to find the best derivatization period. For this aim, the molar ratio of PHMB/free thiols was kept 20. 50 µL of 20.0 mM PHMB was added to 1.0 mL of mixed thiol solution containing 10.0 µM each of Cys, HCys, GSH, CysGly, SeCys and SeMet. “Standing at room medium” method was used in derivatization. Samples were derivatized for 5.0, 10, 15, 30, 60 and 120 minutes.

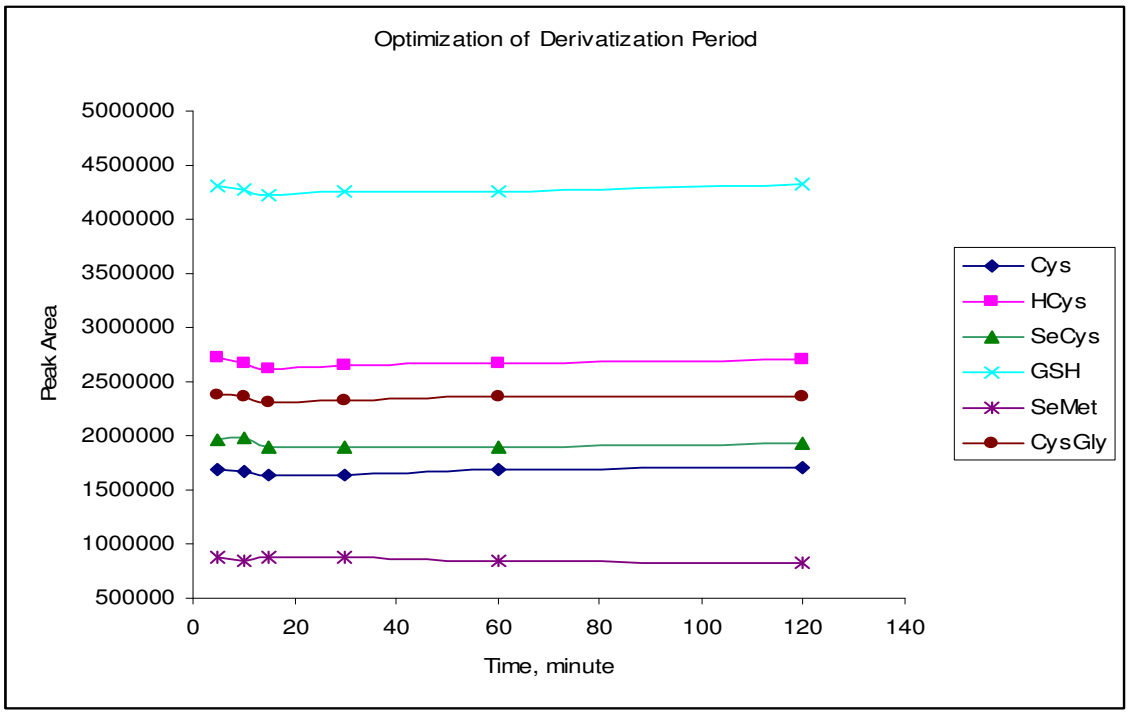

Figure 108. Optimization of derivatization period using "Standing at room medium" method, 1.0 mL of mixed thiol solution containing 10.0 µM each of Cys, HCys, SeCys, GSH, SeMet, CysGly and 50 µL of 20.0 mM PHMB.

As see in Figure 108, no big differences in peak areas of thiols were observed using different derivatization period. Hence, 5.0 minutes were selected as optimum derivatization period.

C.3.2.1.1.1.3 Optimization of PHMB/Free Thiol Ratio

In the optimization of PHMB concentration, molar ratios of PHMB/free thiols were kept at 0.20, 0.40, 1.0, 2.0, 4.0, 10, 15, 20, 30, 40. 100 mM of NH_3 was used to prepare 0.20, 0.40, 1.0, 2.0, 4.0, 10, 15, 20, 30, 40 mM of PHMB to improve the dissolution of PHMB. In the derivatization, 50 µL of PHMB in different concentrations given above were added to 1.0 mL of mixed thiol solution containing 10.0 µM each of Cys, HCys, GSH, CysGly, SeCys and SeMet. Derivatization was performed in ambient conditions for 5.0 minutes. After derivatization procedures sample were injected to HPLC-ES-MS system for measurement.

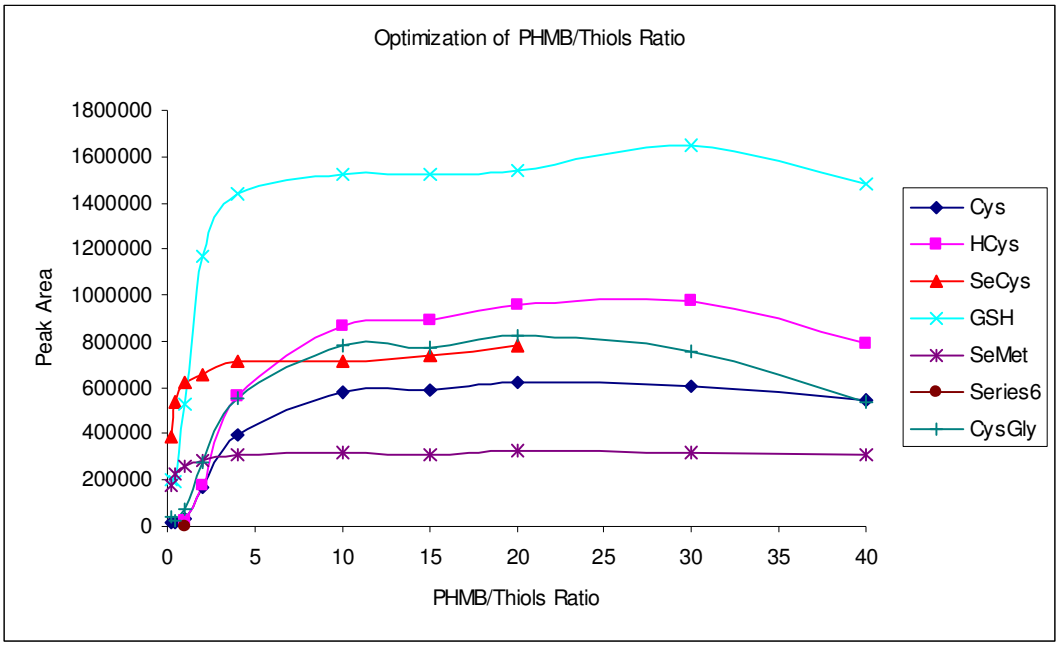

Figure 109. Optimization graph of PHMB/thiol.

In the case of 20 fold excess of PHMB, peak shapes of selenocysteine and CysGly were not good. Hence, 15 were selected as best PHMB/Thiol ratio in derivatization step.

All of the optimization results for the free thiol determination can be seen in Table 78.

Table 78. Optimization results of free thiol derivatization.

Parameter	**Result**
Derivatization Type	Standing at room medium
Derivatization Period	5.0 min
PHMB/Thiol Ratio	15.0

Under the optimum conditions, the chromatograms shown in Figure 110 were obtained for mixed free thiol solution containing 10.0 µM of Cys, HCys, SeCys, GSH, SeMet and CysGly. PHMB/Thiol Ratio was used as 15, and analytes were derivatized for 5.0 min by standing at room medium.

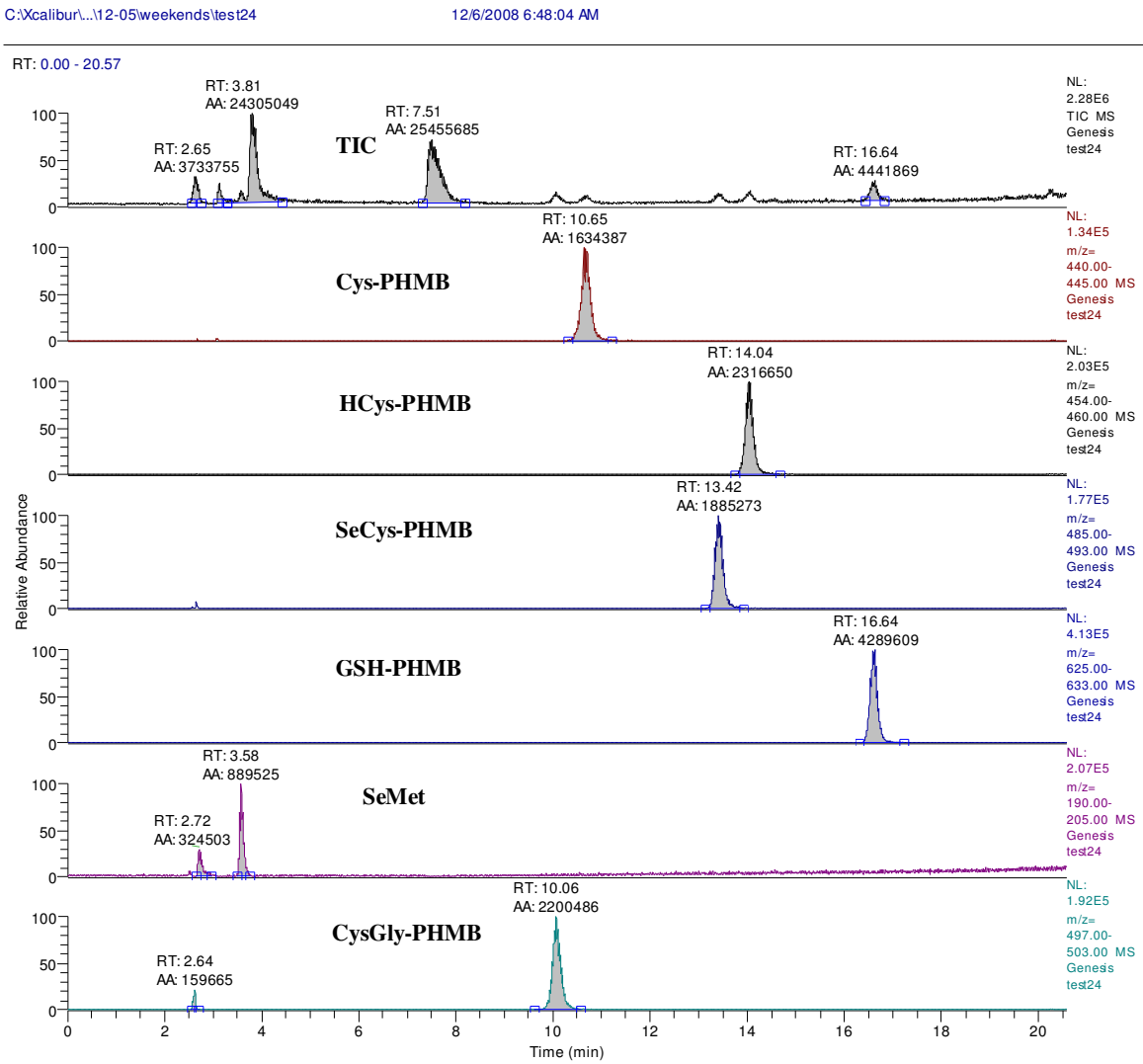

Figure 110. HPLC-ES-MS chromatograms of a mixed standard containing 10.0 µM Cys, HCys, SeCys, GSH, SeMet and CysGly using 15.0 as PHMB/Thiol Ratio, 5.0 min derivatization by standing at room medium.

As seen in Figure 110, peak shapes of all thiols were found to be sharp without tailing. Resolution and S/N ratio of the signals are good enough to make qualitative and quantitative measurements. Retention times of Cys, HCys, SeCys, GSH, SeMet and CysGly were found to be 10.65, 14.04, 13.42, 16.64, 3.58 and 10.06 minutes, respectively. Retention times of all analytes are different from each other.

C.3.2.1.2. Total Thiol

In order to determine the total thiol concentration, all of the procedures including reduction step were optimized. In all optimization, oxidized thiols were used.

C.3.2.1.2.1. Optimization Studies for Total Thiol Determination

All the parameters used in the total thiol determination have been optimized to find not only proper reduction and derivatization parameters but best S/N for each species as well.

C.3.2.1.2.1.1. Optimization of Reduction Conditions

In this optimization, reduction conditions were tried to be optimized. In the reduction of cystine, homocystine, selenocystine and oxidized glutathione to their free thiols, 50.0 µL of 5.0 mM DTT prepared in water was added to the 1.0 mL of standard solution containing 5.0 µM each of cystine, homocystine, selenocystine and oxidized glutathione. Three different methods, namely standing at room medium, sonication and shaking, in the reduction step were tested to find the optimum condition. In the first method, reduction was carried out by standing samples at room medium. Sample was reduced for 2.0 hours. Sonication instrument was applied in the second reduction for 2.0 h like in the first method. In the last method, reduction was performed using shaker for 2.0 h. After the reduction step using any of three methods, 50.0 µL of 15.0 mM PHMB as derivatization agent were added to the samples. Derivatization time was kept constant, 30 minutes, in all three methods. The results obtained are given in Table 79.

Table 79. Peak area of signals obtained in the optimization of reduction conditions.

	Cys	**HCys**	**SeCys**	**GSH**
Standing at room medium	686803	436411	1099595	2281426
Sonication	407006	316906	717343	1397005
Shaking	489235	525103	887987	1892046

As seen in Table 79, “Standing at room medium” method is the best among the methods tested. Although homocysteine signal was found to be lower than that

obtained using shaking method, the highest signals were obtained for Cys, SeCys and GSH using standing at room medium. Hence, standing at room medium was selected as optimum method for the reduction of thiols. The chromatogram obtained using room medium method can be seen in Figure 111.

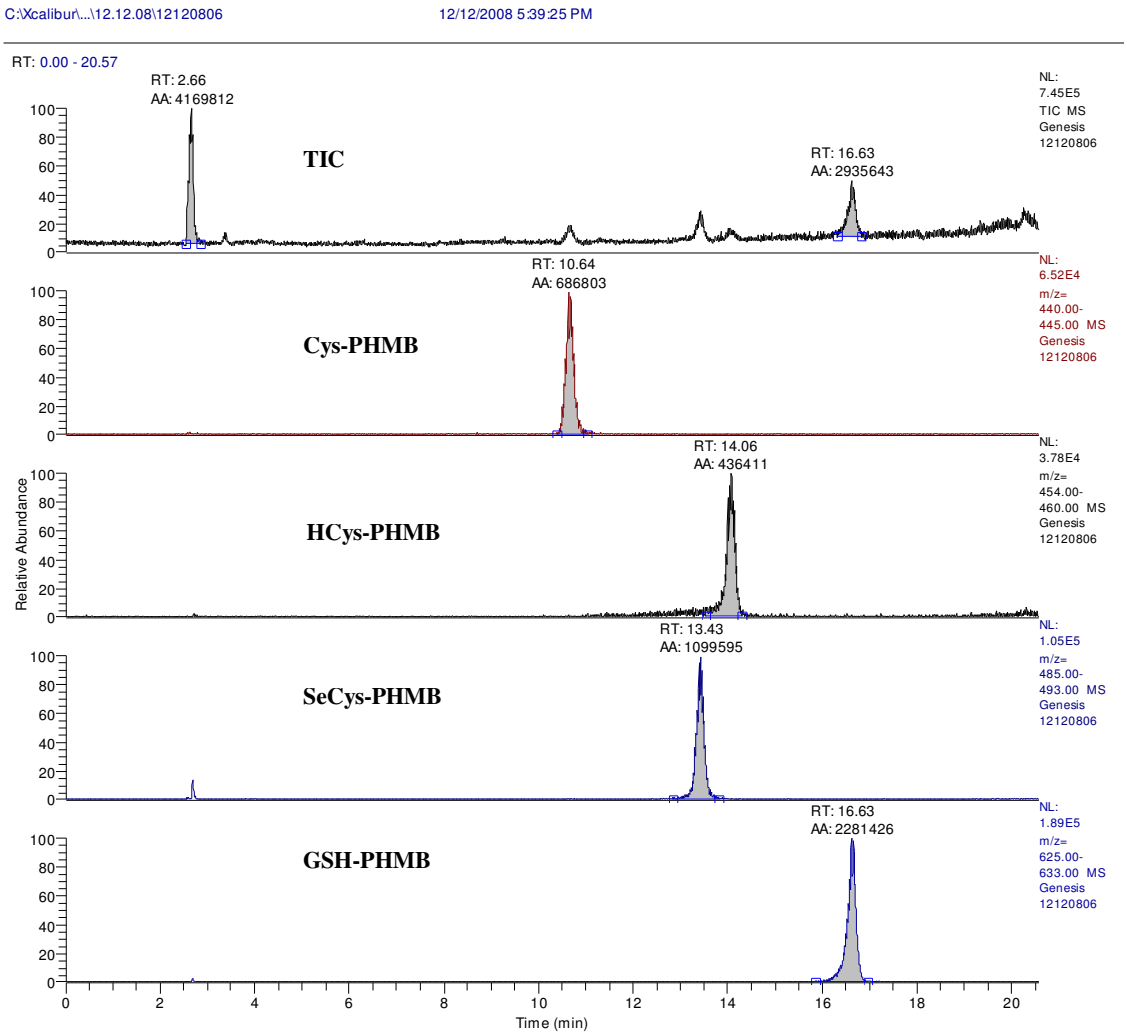

Figure 111. HPLC-ES-MS chromatograms of a mixed oxidized thiol standard containing 5.0 µM Cys-Cys, HCys-HCys, $Se(Cys)_2$ and GSSG using 50.0 µL of 5.0 mM DTT as reducing agent, 2.0 h reduction period, 50.0 µL of 15.0 mM PHMB as derivatization agent, 30.0 min derivatization by standing at room medium.

C.3.2.1.2.1.2. Reduction Medium Temperature

This experiment was carried out to find the effect of the temperature of the reduction medium on reduction efficiency. For this aim, reduction of thiols with DTT was done in 0 ℃ and room temperature, separately. 50.0 µL of 5.0 mM DTT prepared in water was added to the 1.0 mL of solution containing 5.0 µM each of cystine, homocystine, selenocystine and oxidized glutathione. In the study where temperature was kept at ℃, sample was placed in ice bath and reduced for 2.0 h. In the room temperature method, sample was put in room medium (25-27 ^{0}C) and reduced for 2.0 h by standing at room temperature. After the reduction step, 50.0 µL of 15.0 mM PHMB as derivatization agent was added to the samples. Derivatization was performed by standing samples at room medium for 30 minutes. After the derivatization step, samples were analyzed HPLC-ES-MS system. The results can be seen in Table 80.

Table 80. Peak area of signal in the optimization of temperature in reduction step.

	Cys	**HCys**	**SeCys**	**GSH**
Standing sample at room temperature	747067	829723	1285341	2781929
Standing sample at 0 ℃	635003	549221	1125874	2636235

Standing sample at temperature was selected as more sufficient method to obtain high reduction efficiency for oxidized thiols.

C.3.2.1.2.1.3. Optimization of Reduction Period

Different reduction periods were applied to thiols to find the optimum reduction period. 50.0 µL of 5.0 mM DTT prepared in water was added to the 1.0 mL of standard solution containing 5.0 µM each of cystine, homocystine, selenocystine and oxidized glutathione. Oxidized thiols were reduced by standing at room medium for 5.0, 15, 30, 60, 120 minutes. After the reduction step, 50.0 µL of 15.0 mM PHMB as derivatization agent were added to the samples, and thiols were derivatized for 30 minutes. Samples were analyzed after the derivatization procedure.

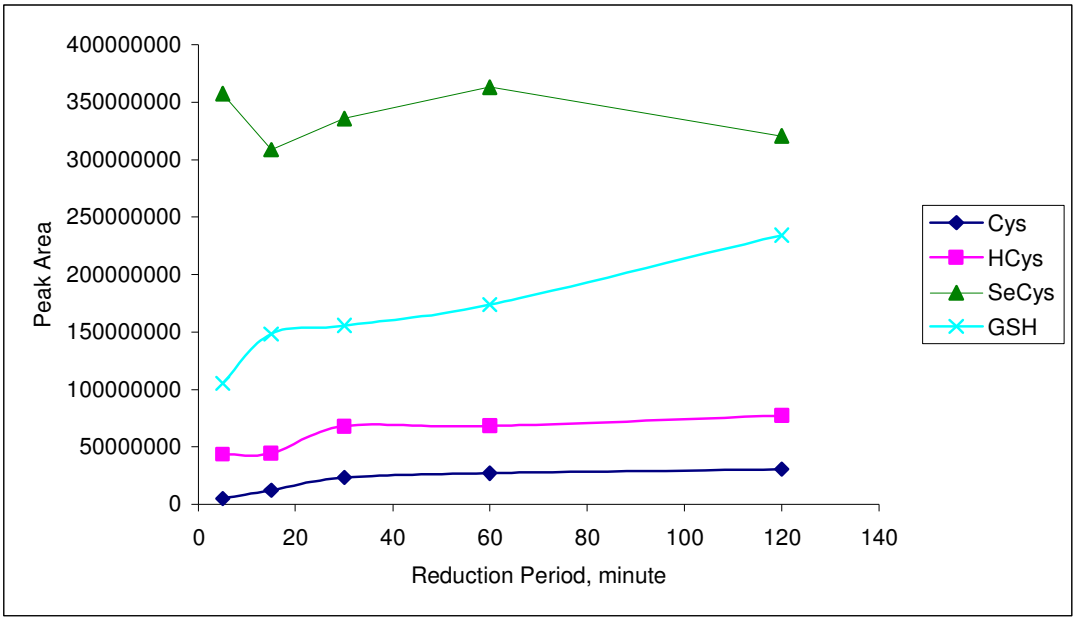

Figure 112. Optimization of reduction period using mixed oxidized thiol standard containing 5.0 µM Cys-Cys, HCys-HCys, $Se(Cys)_2$ and GSSG, 50.0 µL of 5.0 mM DTT as reducing agent, different reduction periods, 50.0 µL of 15.0 mM PHMB as derivatization agent, 30.0 min derivatization by standing at room medium.

As seen in Figure 112, 60 min are the optimum reduction period for all species. Although reduction efficiency of GSH was found to be higher using 120 min reduction period, peak area of the selenocysteine decreased in case of 120 min reduction period. In addition, there was no big change in the peak areas of HCys and Cys after 60 and 120 min reduction periods. Hence, 60 min were selected as the optimum reduction period for oxidized thiols.

C.3.2.1.2.1.4. Optimization of DTT/Thiol Molar Ratio

In this optimization, DTT/Thiol molar ratio in the reduction step was optimized. Molar ratio was kept at 1.0, 2.5, 6.25, 12.5, 17.5, 25, 37.5, 50 and 100. In the reduction step, 50 µL of DTT in different concentrations was added to 1.0 mL of mixed thiol stock solution containing 5.0 µM of cystine, homocystine, selenocystine and oxidized glutathione. Thiols were reduced by standing at room medium for 60 min. In the derivatization, 50 µL of 15.0 mM PHMB was added to mixed thiol solution.

Derivatization was performed by standing samples at room medium for 30 min. After derivatization procedures sample were injected to HPLC-ES-MS system.

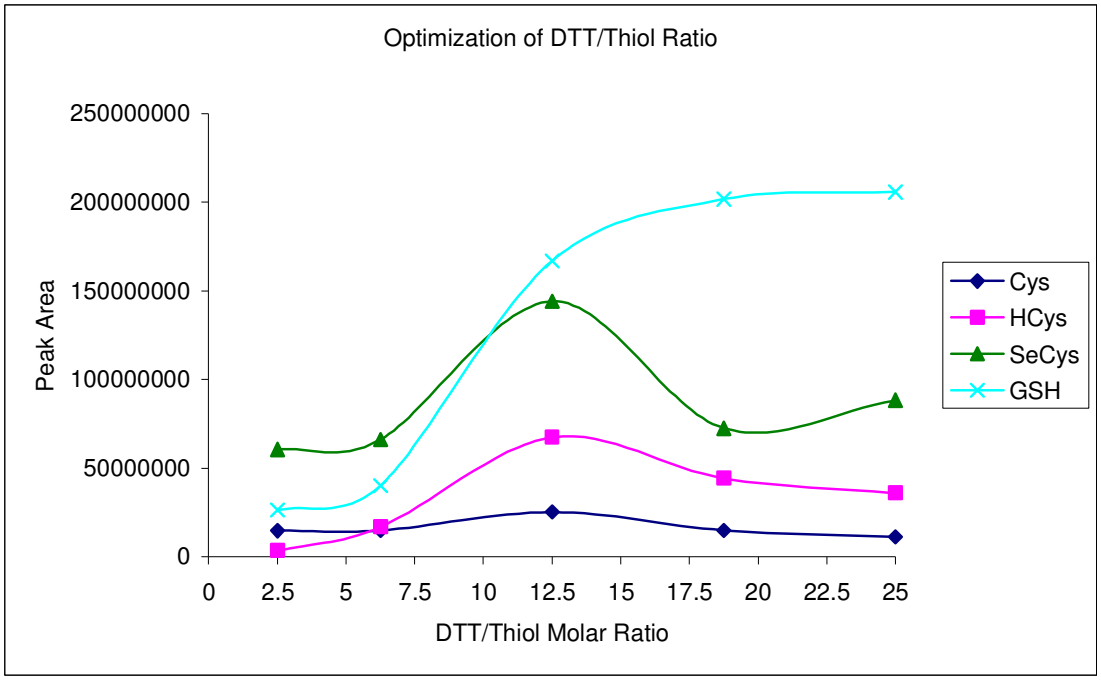

Figure 113. Optimization of molar DTT/Thiol ratio using mixed oxidized thiol standard containing 5.0 µM Cys-Cys, HCys-HCys, $Se(Cys)_2$ and GSSG, 50.0 µL of DTT in different concentrations as reducing agent, 60 min reduction period, 50.0 µL of 15.0 mM PHMB as derivatization agent, 30.0 min derivatization by standing at room medium.

As seen in Figure 113, the molar ratio of 12.5 was selected as the optimum one. In case of using 37.5, 50 and 100 fold excess amount of DTT, there were no peaks for all thiols.

C.3.2.1.2.1.5. Optimization of PHMB/Thiol Molar Ratio

In the optimization of PHMB/Thiol molar ratio in the derivatization step, 1.0 mL of mixed thiol stock solution containing 5.0 µM of cystine, homocystine, selenocystine and oxidized glutathione was first reduced at optimum DTT/Thiol molar ratio, 12.5. Thiols were reduced by standing at room medium for 60 min. After the reduction step, 50 µL of PHMB in different concentrations were added to mixed thiol solution to obtain

1.0, 2.0, 5.0, 10, 15, 20, and 30 as molar ratios of PHMB/thiols. Derivatization was done by standing samples at room medium for 30 min. After the derivatization procedure, samples were injected to HPLC-ES-MS system.

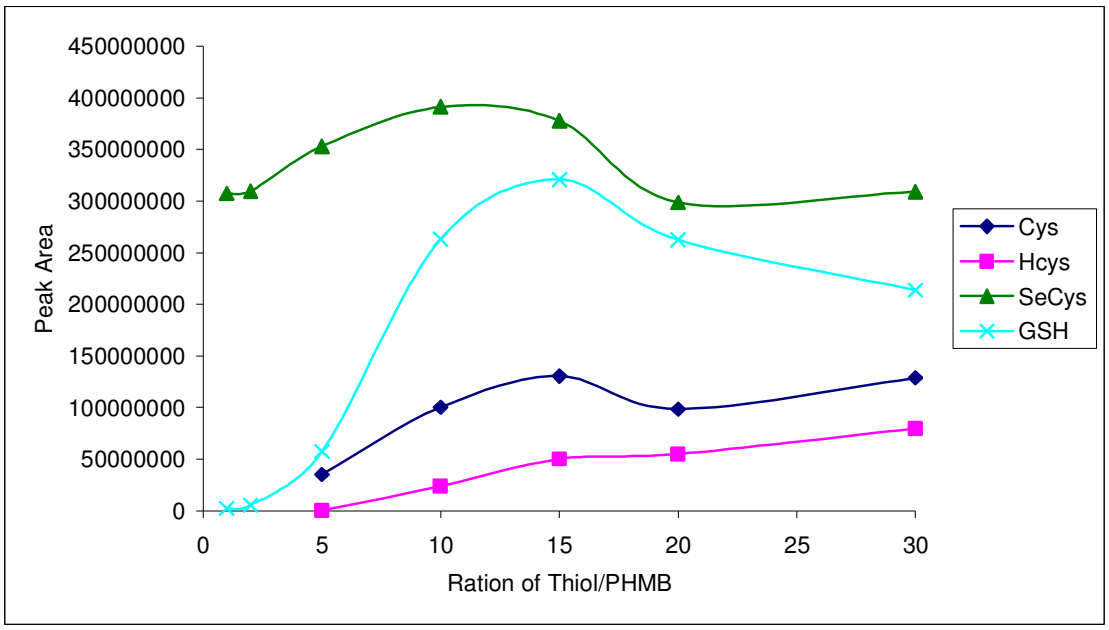

Figure 114. Optimization of molar PHMB/Thiol ratio using mixed oxidized thiol standard containing 5.0 µM Cys-Cys, HCys-HCys, $Se(Cys)_2$ and GSSG, 12.5 as optimum DTT/Thiols molar ratio, 60 min reduction period, 50.0 µL of PHMB in different concentrations as derivatization agent to obtain 1.0, 2.0, 5.0, 10, 15, 20, and 30 as molar ratios of PHMB/thiols, 30.0 min derivatization by standing at room medium.

As seen in the Figure 114, optimum PHMB/Thiol ratio was found to be 15. Although the peak area of HCys was found to be the highest in the ratio of 30, peak shape of HCys at the ratio of 15 was much better than 30.

C.3.2.1.2.1.6. Optimization of Derivatization Conditions

Derivatization condition was tried to be optimized in this part of study. Mixed standard solution containing 5.0 µM each of cystine, homocystine, selenocystine and oxidized glutathione was used in this optimization. DTT/Thiol molar ratio was used as 12.5. Thiols were reduced by standing at room medium for 60 min. After the reduction step, three different methods, namely standing at room medium, sonication and

shaking, in the derivatization step were tested to find the optimum derivatization conditions. Proper amount of PHMB as derivatization agent was added to the samples to obtain 15 of PHMB/Thiol molar ratio. 30 minutes derivatization time was applied to samples for three methods. The results can be seen in Table 81.

Table 81. Peak area of signal in the optimization of derivatization conditions.

	Cys	**HCys**	**SeCys**	**GSH**
Standing at room medium	58169372	166508611	214714302	268578264
Sonication	41052413	161223868	204414874	290234826
Shaking	29687261	170578152	160403329	288411933

It is seen in Table 81 that room medium can be selected as optimum derivatization medium.

C.3.2.1.2.1.7. Optimization of Derivatization Period

Different derivatization periods were applied to thiols to find the optimum derivatization period. In this study, mixed thiol standard containing 5.0 µM each of cystine, homocystine, selenocystine and oxidized glutathione was used. Molar ratio of DTT/Thiol was kept at 12.5 in the reduction step. Reduction of analytes was done by standing at room medium for 60 min. After the reduction step, proper amount of PHMB as derivatization agent was added to the samples to obtain 15 of PHMB/Thiol molar ratio, and thiols were derivatized for 5, 15, 30 and 60 min by standing at room medium. Samples were analyzed after each derivatization period.

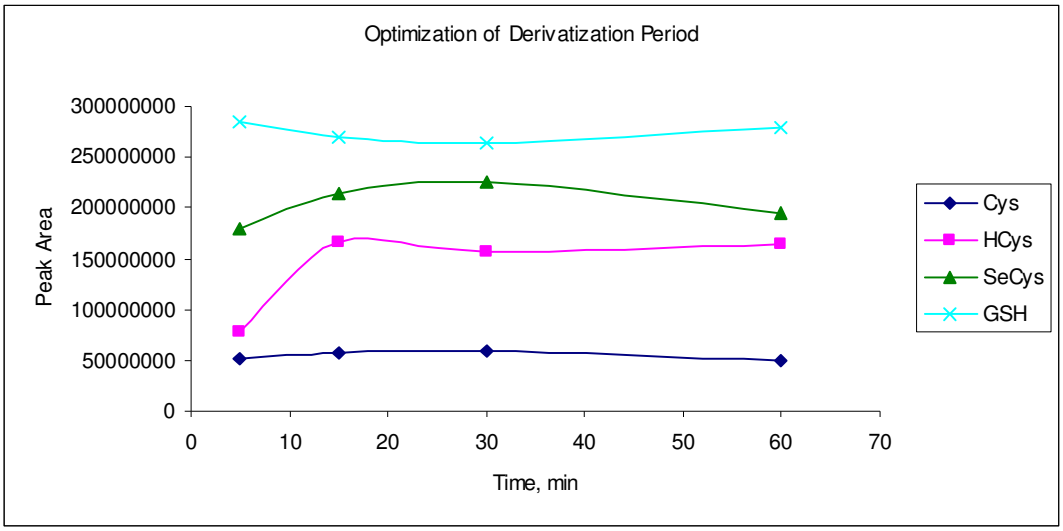

Figure 115. Optimization of derivatization period using mixed oxidized thiol standard containing 5.0 µM Cys-Cys, HCys-HCys, $Se(Cys)_2$ and GSSG, 12.5 as optimum DTT/Thiols molar ratio, 60 min reduction period, 15 as the molar ratio of PHMB/Thiols, different derivatization periods by standing samples at room medium.

As seen in Figure 115, 15.0 min is the optimum derivatization period for total thiol determination. All of the optimization results for the determination of total thiols can be seen in Table 82.

Table 82. Optimization results of total thiol determination.

Parameter	**Result**
Reduction Condition	Standing at room medium
Reduction Temperature	Room Temperature
Reduction Period	60 min
DTT/Thiol Ratio	12.5
Derivatization Condition	Standing at room medium
Derivatization Period	15 min
PHMB/Thiol Ratio	15.0

C.3.2.1.2.2. Determination of Reduction efficiency

Reduction efficiency for each disulfide was calculated in this step. For this aim, 5.0 µM of mixed oxidized thiol standard was prepared and species were reduced with DTT and derivatized with PHMB under the optimum conditions given in Table 82. Free thiol was also used to find the reduction efficiency. For free thiols, 10.0 µM of mixed free thiol standard solution was prepared and species were derivatized with PHMB under the optimum conditions given in Table 78.

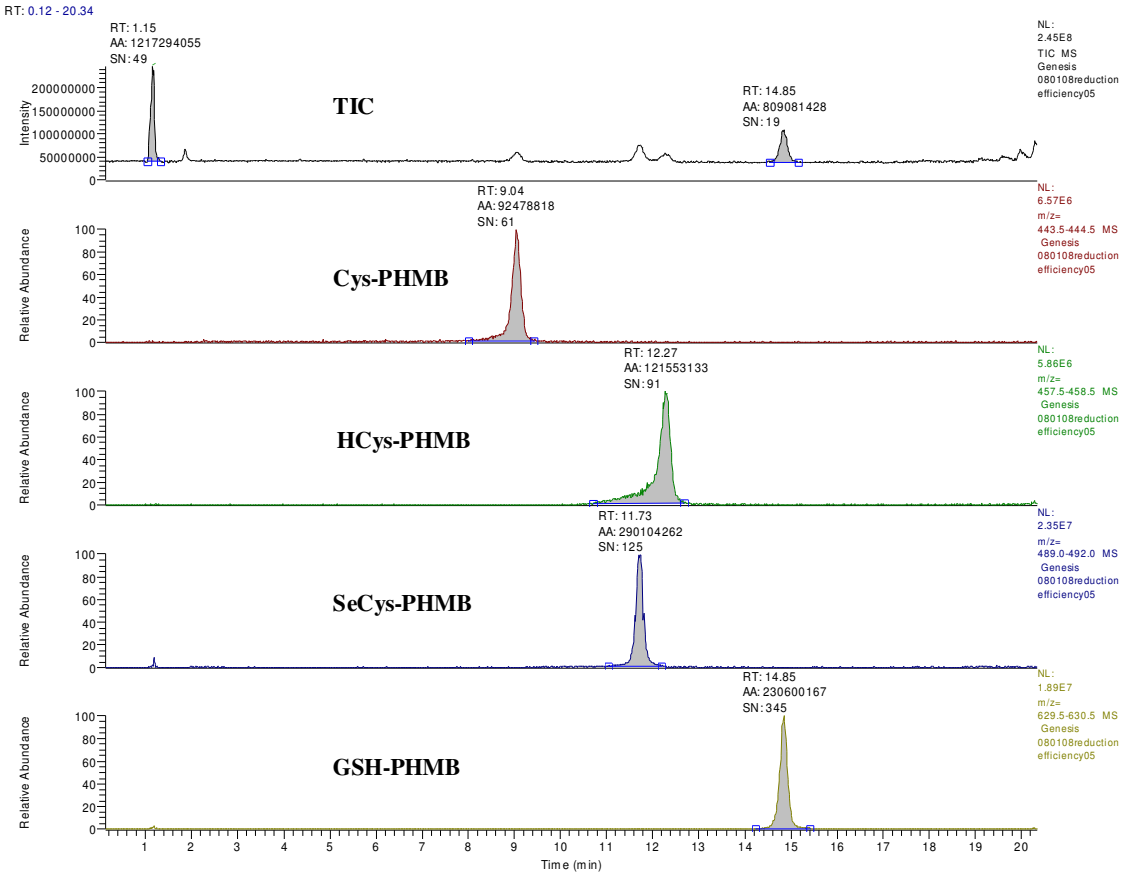

Figure 116. HPLC-ES-MS chromatograms for total thiol measurements using the parameters given in Table 82.

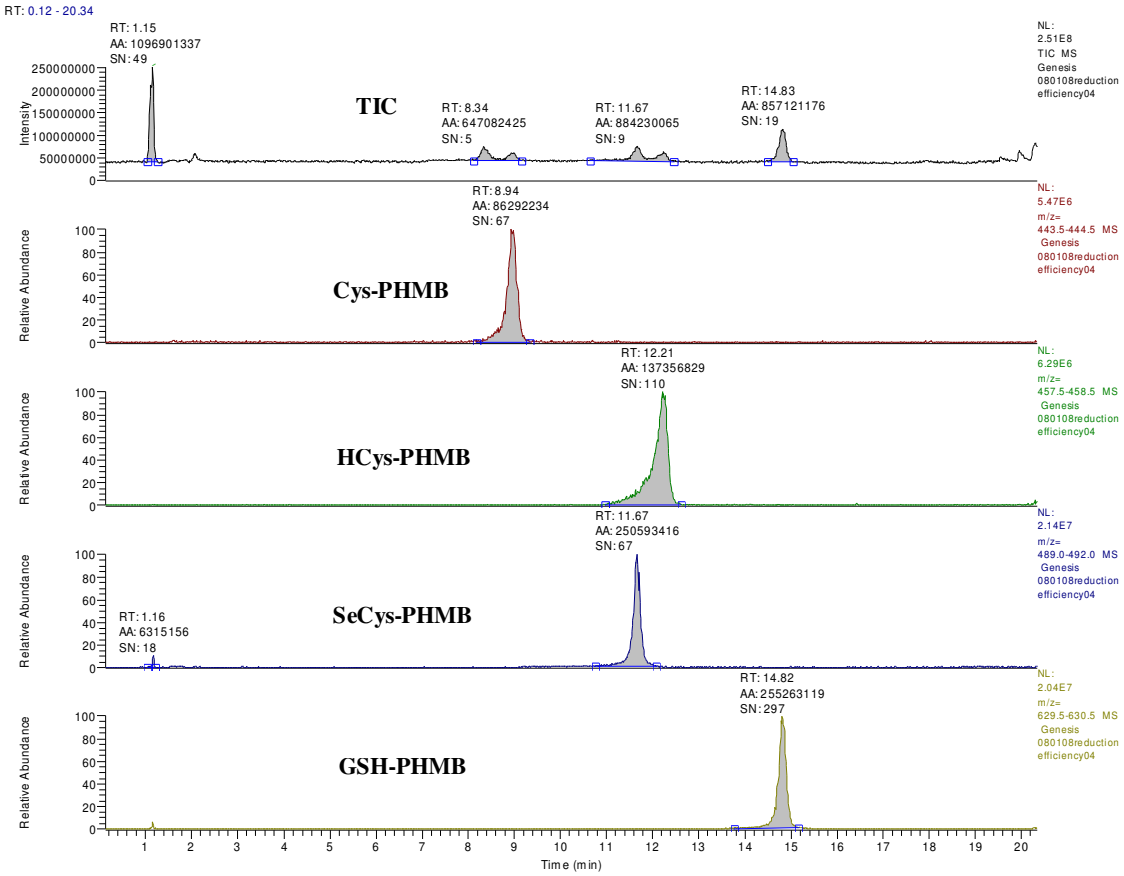

Figure 117. HPLC-ES-MS chromatograms for free thiol measurements using the parameters given in Table 78.

Peak areas of the signals were used to calculate reduction efficiency. Reduction efficiencies for each species can be seen in Table 83.

Table 83. Reduction efficiency for oxidized thiols.

	% Reduction Efficiency, Mean ± S.D., N=3
Cys	107 ± 5
HCys	89 ± 5
SeCys	116 ± 8
GSH	90 ± 5

As seen in Table 83, reduction efficiencies were found to be 89 ± 5 or better as percentage for all oxidized thiol species.

C.3.2.1.2.3. Analytical Figures of Merit

Different concentration of thiol solutions were prepared and injected to the HPLC-ES-MS system. Oxidized species, Cys-Cys, HCys-HCys, $Se(Cys)_2$ and GSSG were reduced and derivatized using the parameters given in Table 82. For CysGly, there is no reduction. This species was deirvatized using the parameters given in Table 78. There is no reduction and derivatization for SeMet. It was added to analyte list to see whether yeast samples contain water soluble SeMet or not. In the calculation of LOD and LOQ, blank was injected to HPLC-ES-MS system 9 times, and peak heights at specific retention times of analytes were used. The analytical figures of merit of HPLC-ES-MS system for total thiol measurement are shown in Table 84. The following formulas were used to calculate LOD and LOQ values.

LOD= 3xStandard Deviation/Slope

LOQ= 10xStandard Deviation/Slope

Table 84. Analytical figures of merit of HPLC-ESMS system for total thiol determination.

	Cys	**HCys**	**SeCys**	**GSH**	**SeMet**	**CysGly**
Equ.	y=241063x -8526.2	y=327596x -2931.1	y=299899x -2618.4	y=754628x +16794	y=41519x +295.39	y=348579x -23074
R^2	0.9995	0.9997	0.9997	0.9996	0.9988	0.9999
Linear range, µM	0.01-2.5	0.02-5.0	0.02-5.0	0.005-10.0	0.05-5.0	0.05-10.0
LOD, nM	1.79	3.42	3.87	1.15	12.8	10.3
LOD, fmol	17.9	34.2	38.7	11.5	128	103
LOQ, nM	5.96	11.4	12.9	3.83	42.7	34.4
LOQ, fmol	59.6	114	129	38.3	427	344

C.3.2.1.2.4. Sample Preparation Studies

SELM-1 as a CRM was used to find the proper sample preparation method. Five different calibration methods were applied to SELM-1. External calibration (Method 1), standard addition using free thiols before extraction step (Method 2), standard addition using oxidized thiols before extraction step (Method 3), standard addition using free thiols after extraction step (Method 4) and standard addition using oxidized thiols after extraction step (Method 5) were tried. External calibration method (Method 1) was selected as the optimum method for the real sample analysis by considering peak areas and reproducibility of the results.

C.3.2.1.2.5. Real Sample Analysis

For the real sample analysis, yeast samples were selected. Three different commercial yeast samples were bought from a local store at Ottawa, Canada for the determination of total thiol contents. Thiol determination in SELM-1 was also carried out; the thiol content in this CRM has not been certified yet.

In the sample preparation and measurement steps for the total thiol determination, five replicates for CRM sample and two replicates for each commercial yeast sample were prepared for the total thiol measurements. Procedure given in “Experimental” part was applied to samples. 25.0 mg sample was extracted by using 10.0 mL of DIW. After the filtration step, oxidized thiol contents of samples were reduced using DTT. For this aim, 300 µL of sample was taken and 15.0 µL of 5.0 mM DTT was added into the sample. Reduction was carried out for 60 min. After the reduction step, 15.0 µL of 15.0 mM of PHMB was added to the sample for the derivatization of thiols for 15.0 min. Thiol contents were finally determined by using HPLC-ES-MS system. HPLC-ES-MS chromatograms of SELM-1 and Brand A are shown in Figure 118 and Figure 119.

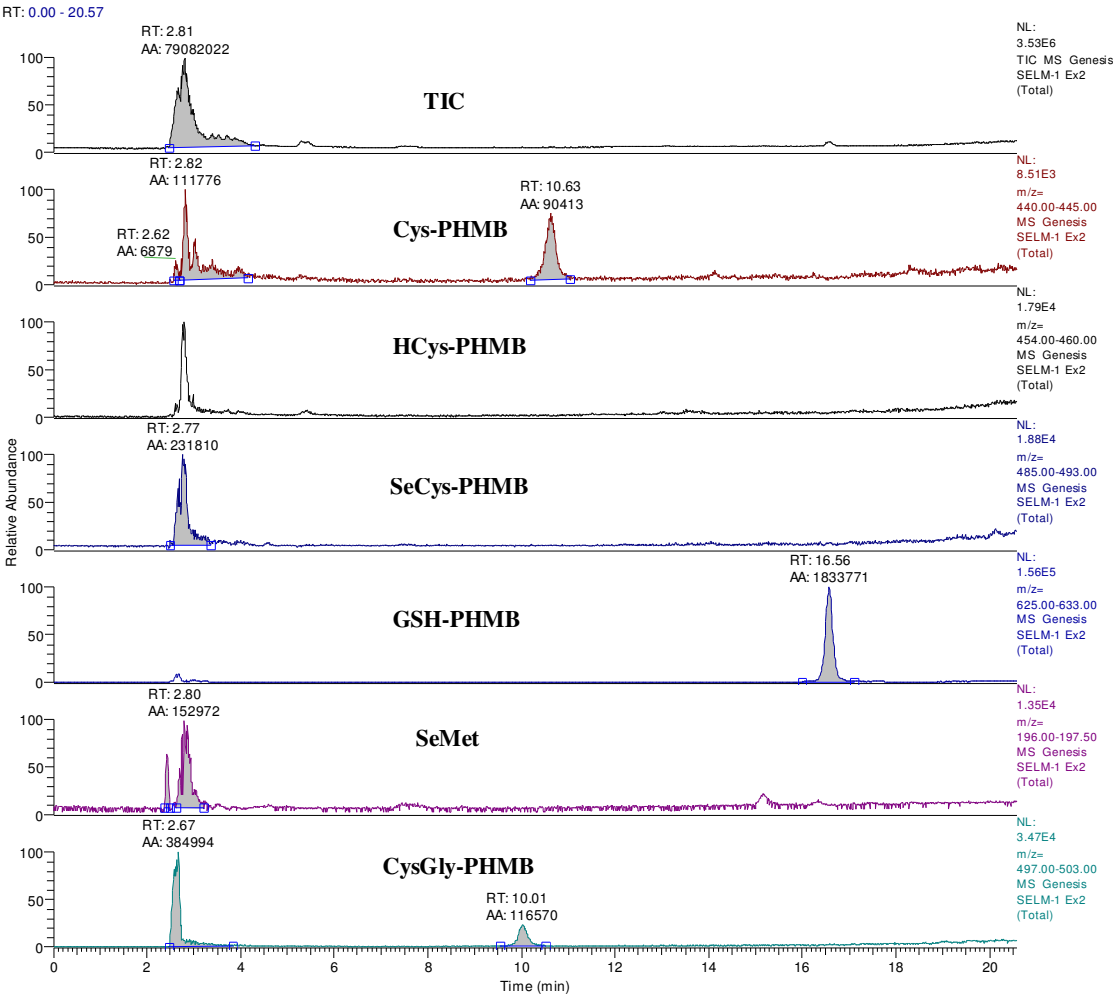

Figure 118. HPLC-ES-MS chromatograms of SELM-1 for the measurement of total thiol contents.

As seen in Figure 118, signals obtained from Cys, GSH and CysGly are fairly sharp and there are no tails. Total amount of GSH in SELM-1 was found the lowest among all samples while some CysGly signal was observed in the extract of SELM-1 unlike the other three yeast samples. HCys, SeCys and SeMet were not observed in the chromatogram of SELM-1. Total thiol content of SELM-1 can be seen in Table 85.

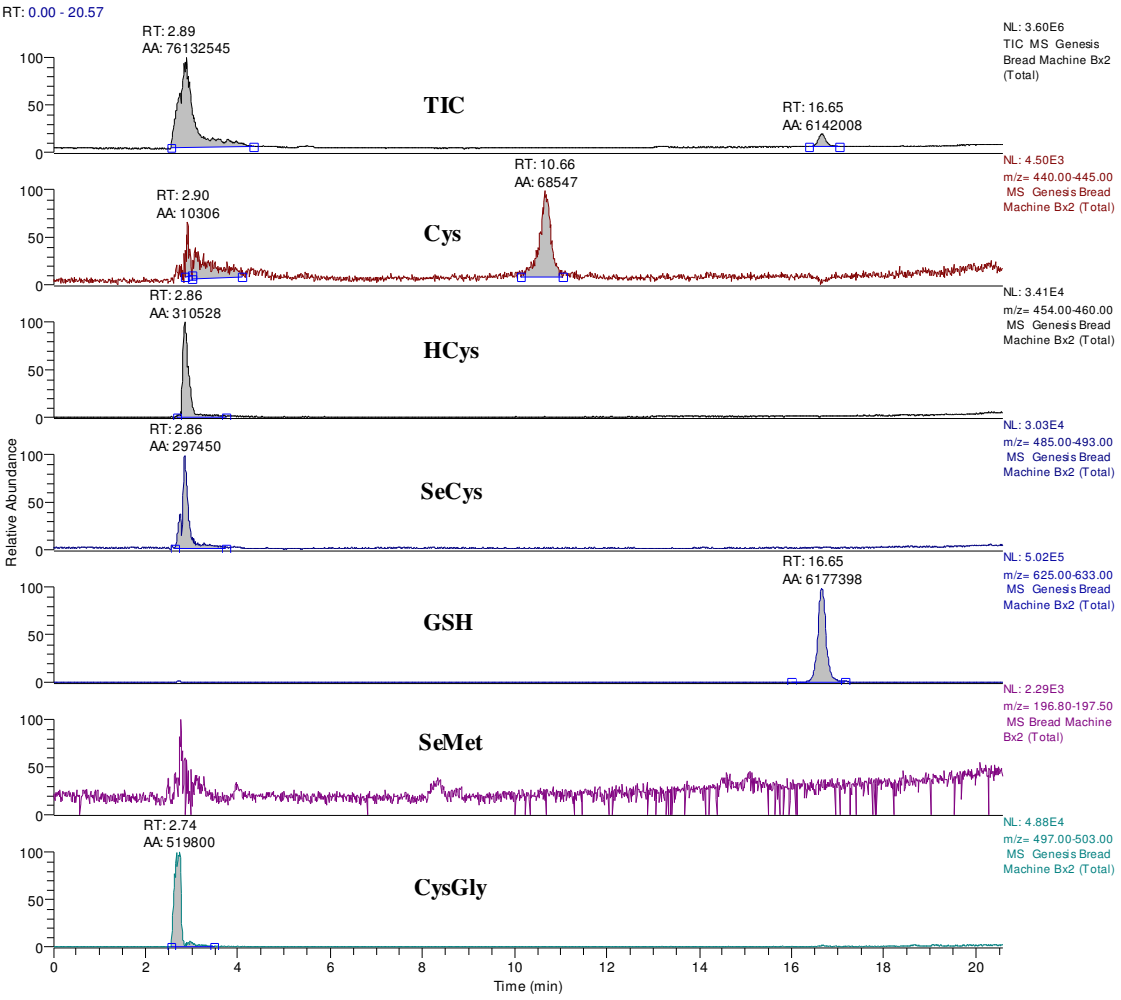

Figure 119. HPLC-ES-MS chromatograms of Brand A for the measurement of total thiol contents.

SeMet, HCys, SeCys and CysGly were not detected in the extract of Brand A. While SELM-1 contains some CysGly, Brand A dose not contain any CysGly. GSH concentartion was found to be one of the highest among the 4 yeast samples. Concentration of GSH was found as 17.87 ± 0.72 µmol/g while this value for Cys was 0.88 ± 0.04 µmol/g. Total thiol content of Brand-A can be seen in Table 85.

For Brand B, cysteine signal was found to be biggest among all yeast samples. 1.32 ± 0.12 µmol/g is the concentration of cysteine in this yeast sample. In addition, Brand B is the other yeast having one of the highest concentrations of GSH. Like the

other commercial yeasts, HCys, SeCys, SeMet and CysGly were not detected. Total thiol content of Brand-B can be seen in Table 85.

For Brand C, only cysteine and gluthathione can be detected. Cysteine concentration in Brand C is the lowest in all analyzed yeast brands. In addition, this yeast sample has the second lowest GSH content. Total thiol content of Brand-C can be seen in Table 85.

Table 85. Total thiol contents of 4 yeast samples obtained by using HPLC-ES-MS system.

Concentration Unit		Cys	GSH	CysGly	SeCys	HCys	SeMet
Mean ± S.D., µmol/g	SELM-1	1.16 ± 0.02	4.45 ± 0.37	0.60 ± 0.02	N.D.	N.D.	N.D.
	Brand A	0.88 ± 0.04	17.87 ± 0.72	N.D.	N.D.	N.D.	N.D.
	Brand B	1.32 ± 0.12	16.73 ± 0.14	N.D.	N.D.	N.D.	N.D.
	Brand C	0.61 ± 0.05	10.36 ± 0.51	N.D.	N.D.	N.D.	N.D.
Mean ± S.D., mg/kg	SELM-1	140 ± 3	1368 ± 114	106 ± 4	N.D.	N.D.	N.D.
	Brand A	107 ± 4	5491 ± 223	N.D.	N.D.	N.D.	N.D.
	Brand B	160 ± 14	5141 ± 44	N.D.	N.D.	N.D.	N.D.
	Brand C	74 ± 6	3184 ± 157	N.D.	N.D.	N.D.	N.D.

N.D.: Not Detected

PART C

CHAPTER 4

C.4. Conclusion

Analytical methods were developed for the determination of total thiols in biological samples. Reversed phase chromatograph coupled to ICP-MS and ES-MS systems were used for the separation and detection of thiols. For the total thiol determination, oxidized thiols were reduced using DTT in HPLC-ES-MS system and TCEP in HPLC-ICP-MS system. Reduction efficiencies for species of interest were found to be 89 ± 5 or better as percentage. Reduced and free thiols were derivatized before introduction on the column by *PHMB* prior to separation on a C8 column. Optimization of extraction, separation and detection steps of HPLC-ICP-MS and HPLC-ES-MS methods were carried out. Length of analysis, LODs and RSDs are higher in HPLC-ICP-MS system. In addition, unknown peaks in chromatograms cause problems in quantitation. Hence, HPLC-ES-MS system was used in the determination of the total thiols in real samples. Three different commercial yeast samples and a selenized yeast CRM, SELM-1, were analyzed. In HPLC-ES-MS as analytical method for real samples, PHMB was used for the derivatization of thiols after reduction of oxidized thiol to free thiol by DTT. Species were separated from each other using reversed phase column and then detected by ES-MS system. Concentration of HCys, SeCys, SeMet and CysGly were found to be lower than detection limits in three commercial yeast samples. Cysteine concentration of Brand B was the highest among the others. CysGly was found only in SELM-1 in low concentration if compared with Cys and GSH. GSH amount in Brand A was found to be one of the highest values among the four yeast samples. Detection limits of cysteine, homocysteine, selenocysteine, glutathione, selenomethionine and cysteinyl-glycine were found to be 17.9, 34.2, 38.7, 11.5, 128

and 103 fmol, respectively, in the thiol determination using HPLC-ES-MS system. This study may be one of the pioneering attempts for the certification of thiol species.

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BS	Fırat University, Elazığ Department of Chemistry (*First-ranking student*)	2001
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WORK EXPERIENCE

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AWARDS

1- **Excellent Poster Award**, Bor Elementinin Erkeklerde Prostat Büyüklüğü ve Alt Üriner Sistem Semptonlarına Etkisi, XI. Ulusal Spektroskopi Kongresi, 23-26 Haziran 2009, Ankara, Türkiye.
2- **Perkin Elmer Excellent Poster Award,** Interference Studies for Bismuth Determination by Tungsten Trap Atomic Absorption Spectrometry, Colloquium Spectroscopicum Internationale XXXV, 23-27 September 2007, Xiamen, China.
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Projects:

1. Şehir Atık Suyu Arıtma Tesisi Lağım Çamurundaki Kurşun'un Kimyasal Yapısının Belirlenmesi, TÜBİTAK Üniversite Öğrencileri Araştırma Projeleri, 2000.

2. İnsanların Bir Günde Aldıkları Bor Miktarının Saptanması ve Bunun İnsan Sağlığına Etkisinin Belirlenmesi, Eti Maden İşletmeleri Genel Müdürlüğü, Proje No: 204.C.11.0010, June 2006.

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Refereeing in Journals:

1. Polish Journal of Environmental Studies
2. Fresenius Environmental Bulletin
3. Environmental Geochemistry and Health
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