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SELENOCOMPOUNDS IN SELENIUM-ENRICHED PLANTS DETERMINED BY SPECTROMETRIC METHODS

Doctoral Dissertation

DOLOČANJE SELENOVIH SPOJIN V S SELENOM OBOGATENIH RASTLINAH S SPEKROMETRIČNIMI METODAMI

Doktorska disertacija

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May 2010

MEDNARODNA PODIPLOMSKA ŠOLA JOŽEFA STEFANA
JOŽEF STEFAN INTERNATIONAL POSTGRADUATE SCHOOL
Ljubljana, Slovenia

Try not to become a man of success but
rather try to become a man of value.

Albert Einstein

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Abstract

Selenium (Se) has been demonstrated to be an essential trace element for maintenance of animal and human health. Se is incorporated into proteins to make selenoproteins, which are important antioxidant enzymes. The antioxidant properties of selenoproteins help to prevent cellular damage caused by radicals. Radicals are natural by-products of oxygen metabolism that may contribute to the development of chronic diseases, such as cancer and heart disease. Other selenoproteins help to regulate thyroid function and play a protective role in the immune system.

In majority of countries throughout the world plant foods contain insufficient amount of Se. The content of Se in food depends on the Se content in soil where the plants are grown or animals are raised, and this is mainly poor.

The growth interest in the production of Se-enriched cultural plants and nutritional supplements parallels the need to develop and optimise analytical methodologies for characterization of Se species in these matrices. Several analytical steps were examined in this doctoral dissertation that should make a significant contribution to further research. In this regard, an analytical method for separation and detection of soluble (HPLC-ICP-MS) and volatile Se species (GC-MS) was developed. Before chromatographic separation, the analytes must be extracted from the solid sample in which identification and determination of Se species is required. The most frequently used extraction technique for Se speciation analysis is to transform Se species from protein-bounder to soluble forms with enzymatic hydrolysis. Thus, several commercially available enzymes were evaluated in order to obtain the highest Se extraction efficiency in samples of different origin. Moreover, we checked if commercially available enzymes are pure enough to be used for Se speciation analysis and what contribution could the impurities make to Se determination of a real sample. The stability of Se species in the matrix considered is rarely reported in the literature. The matrix may affect the analyte, therefore we considered the reactions that can take place between the Se species and the matrix components during the extraction. Furthermore, the distribution of Se between insoluble, soluble and volatile fractions (mass balance calculations) is often overlooked, but was considered in this dissertation.

Plants vary considerably in their physiological response to Se; the question of the essentiality of Se in plants still remains unanswered. Although it has not been confirmed to be an essential micronutrient for higher plants, the number of evidence that Se functions as an antioxidant in plants is increasing. Some plant species are Se tolerant and accumulate very high concentrations of Se (Se accumulators), but most plants are Se non-accumulators and are Se-sensitive. However, the specific physiological mechanisms that underlie the positive effects of Se in plants have not been clearly elucidated. This thesis makes contributions to our knowledge of the physiology and biochemistry of Se-enriched non-accumulating plants, often present in Slovenian nutrition (common buckwheat (*Fagopyrum esculentum*) sprouts, cultivar Darja, potato tubers (*Solanum tuberosum* L., cultivar Desiree) and the leafy vegetables: chicory (*Cichorium intybus* L. cv. 'Anivip' and 'Monivip'), dandelion (*Taraxacum officinale Waggner*), garden rocket (*Eruca sativa* Mill.) and wild rocket (*Diplotaxis tenuifolia* DC.)), particularly with regard to Se uptake, incorporation into proteins and volatilisation.

The Se human status can be improved with consumption of food supplements that contain different Se species, and nowadays market is overflowed with them. Since there is no verification of Se and its species content in food supplements, they were studied with regard to quality in meeting declared and recommended intakes.

Povzetek

Selen (Se) je esencialen element za ljudi in živali. Uvrščamo ga v skupino elementov, ki so potrebni v zelo majhnih količinah, saj priporočljiv dnevni vnos znaša 55 μg na dan, medtem ko se znaki toksičnosti pojavijo že pri dnevnem vnosu nad 400 μg. Selenoproteini v organizmu (glutation peroksidaze in selenoprotein P) imajo pomembno antioksidativno vlogo. Antioksidativne lastnosti selenoproteinov pomagajo preprečiti poškodbe celic, ki jih povzročajo radikali. Radikali so stranski produkt kisikovega metabolizma in lahko pospešijo razvoj kroničnih bolezni, kot sta rak in srčna obolenja. Ostali selenoproteini pomagajo uravnavati hormone žleze ščitnice in imajo pomembno vlogo pri vzdrževanju imunskega sistema.

V večini predelov sveta je vsebnost Se v rastlinah nizka, manj kot 0.1 mg/kg, čemur je vzrok nizka vsebnost Se v tleh. Zato bi lahko s Se obogatene gojene rastline uporabili za izboljšanje preskrbljenosti ljudi s Se.

Zagotavljanje kakovostne s Se obogatene hrane, kot tudi prehranskih dopolnil, zahteva razvoj in optimizacijo analitskih tehnik za spremljanje prisotnosti Se spojin, saj kemijska oblika, v kateri je Se prisoten, pogojuje njegovo biorazpoložljivost. Razvili smo analitsko metodo za določanje topnih in hlapnih Se spojin v rastlinskih vzorcih. Separacijo in detekcijo topnih Se zvrsti (Se(IV), Se(VI), SeMet, $SeCys_2$, SeMeSeCys) smo optimizirali na anionski (Hamilton PRP X-100) in kationski (Zorbax 300-SCX) koloni v povezavi z ICP-MS detekcijo, medtem ko smo za separacijo hlapnih Se spojin (DMDSe in DMSe) uporabili kolono z nepolarno stacionarno fazo DB-5 MS v povezavi z masnim spektrometrom (GC-MS). Pred separacijo Se spojin na kromatografski koloni je potrebno imeti analit iz trdnega vzorca v raztopini. V ta namen smo optimizirali postopek encimske hidrolize, za kar smo uporabili različne komercialno dostopne specifične (amilaze, lipaze, celulaze) in nespecifične (proteaza) encime. Velik poudarek smo namenili ugotavljanju Se in njegovih spojin v encimih kot tudi vplivu matrice na stabilnost Se spojin med samo pripravo vzorca (ekstrakcijo) in med njegovim hranjenjem do analize. Pozornost smo namenili tudi masni bilanci, to je porazdelitvi Se med topnimi, netopnimi in hlapnimi spojinami v rastlini.

Fiziološki odziv rastline na dodatek Se je pogojen z njeno vrsto. Kljub temu da so sposobne privzeti večje količine Se in da so se že pojavile prve razlage antioksidativne vloge Se pri višjih rastlinah, zanje esencialnost Se še ni dokazana. Nekatere rastline so sposobne privzeti visoke koncentracije Se (Se akumulatorske rastline), medtem ko večina rastlin spada v skupino Se ne-akumulatorskih rastlin, ki so na Se občutljive, vendar ga privzamejo v manjših količinah, dobro pa uspevajo tudi na področjih z nižjo vsebnostjo Se v tleh. V disertaciji smo ugotavljali vpliv dodajanja Se v različnih oblikah in različnih načinih gojenja na njegove pretvorbe v gojenih rastlinah, katerih pridelava je v Sloveniji široko zastopana (kalice ajde (*Fagopyrum esculentum*), sorte Darja, gomolji krompirja (*Solanum tuberosum* L., sorte Desiree) in solatnice: radič (*Cichorium intybus* L. sorte 'Anivip' in 'Monivip'), regrat (*Taraxacum officinale Waggner*), rukola (*Eruca sativa* Mill.) in divja rukola (*Diplotaxis tenuifolia* DC.)), s poudarkom na privzemu Se v rastlino, njihovo sposobnost vezave v proteine in hlapnostjo.

Za izboljšanje preskrbljenosti s Se se uporabljajo tudi prehranska dopolnila, ki vsebujejo Se v različnih oblikah. Glede na dejstvo da do danes ne obstaja verifikacija Se in njegovih spojin v prehranskih dopolnilih smo razvito tehniko uporabili za analizo nekaj prehranskih dopolnil, da bi preverili ujemanje deklariranih vrednosti z izmerjenimi.

Abbreviations

AAS = Atomic Absorption Spectrometry
AFS = Atomic Fluorescence Spectrometry
Carboxen-PDMS = Carboxen-Polydimethylsiloxane
CE = Capillary Electrophoresis
Cys = Cysteine
DM = Dry Matter
DMDSe = Dimethyldiselenide
DMSe = Dimethylselenide
ES-MS = ElectroSpray - Mass Spectrometry
GC-MS = Gas Chromatography - Mass Spectrometry
GE = Gel Electrophoresis
GPx = Glutathione Peroxidases
HPLC = High Performance Liquid Chromatography
ICP-MS = Inductively Coupled Plasma Mass Spectrometry
LOD = Limit of Detection
LOQ = Limit of Quantification
m. u. = mass unit
MALDI-TOF = Matrix-Assisted Laser Desorption Ionisation - Time of Flight
NAA = Nevtron Activation Analysis
PDMS = Polydimethylsiloxane
PTFE =Teflon – Polyfluoroethylene
RDI = Recommended Daily Intake
Se(IV) = Selenite
Se(VI) = Selenate
SEC = Size Exclusion Chromatography
$SeCys_2$ = Selenocystine
SeMeSeCys = Selenomethylselenocystein
SeMet = Selenomethionine
SIM = Selected Ion Monitoring
SPME = Solid Phase Micro Extraction
t_e = elution time
TIC = Total Ion Chromatogram
t_R = retention time
tr = traces
u. c. = unknown compound
US = Ultrasound
UV-HG-AFS = Ultraviolet unit - Hydride Generation - Atomic Fluorescence Spectrometry
XANES = X-ray Absorption Near-Edge Spectroscopy
XPS = X-ray Photoelectron Spectroscopy
XRF = X-ray Fluorescence

1 Introduction

The history of selenium goes back to 1817, when it was discovered by Swedish chemists J. J. Berzelius and J. G. Gahan, who named it after the Greek moon goddess *Selene*. It is one of the rarest of the elements, since it is about 70th in abundance among the 88 that naturally occur in the earth's crust (Railly, 1996).

Selenium is unevenly distributed in the earth's crust. Its concentration in soil varies markedly between geographic regions. The inorganic Se species most frequently found in soils are selenite and selenate (Rosenfeld and Beath, 1964; Barceloux, 1999; Dhillon and Dhillon, 2003). Selenium has been recognized as an essential trace element for humans and animals based on its presence in antioxidative defence systems (Schwartz and Foltz, 1957; Flohe et al., 1973) and in hormone balance (Arthur et al., 1990). It is a complex trace element. Its function and bioavailability is strongly correlated with its chemical form. Additionally, extremely low and extremely high Se concentrations are detrimental to human and animal health (Combs, 2001). Due to these facts, selenium is one of the most intensively studied elements and it has attracted growing interest in both, human and agricultural field of science.

In plants Se can be found both in inorganic and organic Se forms, including selenoamino acids and methylated compounds. Current interest in Se is focused on the health benefits of high-Se plants as a source of cancerpreventative Se compounds (Finley et al., 2000 and 2001). To do that, the knowledge of Se plant physiology is crucial. Although Se is not classified as a micronutrient for higher plants, numerous studies have shown that at low concentrations, Se exerts a beneficial effect on growth and stress tolerance of plants by enhancing their antioxidative capacity (Hartikainen and Xue, 1999; Xue and Hartikainen, 2000). Similarly, as in human and animal cells, Se increases plant resistance against oxidative stress caused by free oxygen radicals. However, agricultural crops are sensitive to high tissue Se concentrations, but sensitivity varies among plant species.

Plants play a unique role in recycling and delivering Se from the soil to the food chain. The concentration of Se in agricultural products and fodder depends on the content of Se in the soil and its bioavailability. Se content in Slovenian soils is relatively low as a result of reduced weathering status (Pirc and Šajn, 1997). In humans this leads to low Se intake, increasing risk of cardiovascular disease, coronary heart disease and cancer (Salonen et al., 1984) and results in nutritional disorders in animals (Reilly, 1996). Se supplements can be beneficial to enhance the Se daily intake. As an alternative, crops and food enriched with selenium during cultivation could be an effective way of producing selenium-rich foodstuffs with benefit to health. Considering these facts, selenium-enriched plants, based on selenate or selenite, can be in future a substitute to selenium supplements.

The growth interest in the production of Se-enriched food and nutritional supplements parallels the necessity to develop and optimise analytical methodologies for Se species characterization in these matrices. Accurate determination of selenium compounds in these samples is not straightforward. There are several reasons for this. Important ones are the low concentration of Se in samples, low extraction efficiencies of Se compounds from the sample matrix and the instability of Se compounds during the whole process from sampling to measurement (Uden et al., 2004a). Neutral conditions should be used due to the instability of Se compounds in acidic and basic conditions. Water extractions were used when water-soluble compounds were investigated (Uden et al., 1998; Roberge et al., 2003), and enzymatic hydrolysis, mostly with the nonspecific enzyme protease, for Se compounds bound to proteins (Uden et al., 1998; Ximenez-Embun et al., 2004; Smrkolj et al., 2006). For further separation of soluble selenium species, ion exchange or ion pairing reverse phase HPLC have been used most often (Caruso et al., 2003). A very sensitive detection system is needed for the detection of Se in the low concentration range (few ng/g) in the eluent, for which ICP-MS is mainly used (Michalke, 2003). Besides, HG-AFS can also be used (Mazej et al., 2006). To avoid Se volatilisation, which can easily occur during plant growth or sample preparation, SPME in combination with GC-MS or GC-ICP-MS is mainly used (Meija et al., 2002).

1.1 Se and its species in biological systems

Selenium has an atomic weight of 78.96 and its atomic number is 34. It is positioned between sulphur and tellurium in Group VI and between arsenic and bromine in Period 4 of the Periodic Table of the elements. This position accounts for many of its biological interrelations with sulphur, and also with arsenic and its neighbour phosphorus. Its chemical properties are intermediate between those of sulphur and tellurium, and its compounds resemble the corresponding sulphur and tellurium compounds in behaviour (Railly, 1996).

Selenium forms many inorganic and organic compounds that are similar to those of sulphur (Table 1). Normally occurring oxidation states of selenium in elemental and combined forms are –2 (e.g. Na_2Se, sodium selenide), 0 (Se, elemental selenium), +4 (e.g. Na_2SeO_3, sodium selenite) and +6 (e.g. Na_2SeO_4, sodium selenate). Of particular interest are selenoaminocarboxylic acids, selenium-containing peptides and selenium derivatives of nucleic acids and other compounds. These compounds, such as selenomethionine and selenocysteine, occur naturally in cells and tissues and are incorporated into proteins, including enzymes (Railly, 1996).

Table 1: Se compounds in biological systems (Pyrzynska et al., 1996; Uden et al., 2004a and 2004b).

Group	Se compound (abbreviation)	Chemical form
inorganic forms	elemental Se	Se
	selenide	Se^{2-}
	selenite	SeO_3^{2-}
	selenate	SeO_4^{2-}
simple organic species	dimethylselenide (DMSe)	$H_3C\text{-}Se\text{-}CH_3$
	dimethyldiselenide (DMDSe)	$H_3C\text{-}Se\text{-}Se\text{-}CH_3$
	trimethylselenonium ion	$(CH_3)_3Se^+$
	methyl selenol	$H_3C\text{-}SeH$
	seleno urea	$Se{=}C(NH_2)_2$
	dimethyl selenoxide	$(H_3C)_2SeO$
	methyl selenonyl ion	$H_3CSe(O)O^-$
	dimethyl selenosulfide	$H_3CSSeCH_3$
	selenocyanate	RSeCN
	selenometionine (SeMet)	$H_3CSe\text{-}CH_2CH_2CH(NH_2)\text{-}COOH$
	selenocysteine (SeCys)	$HSe\text{-}CH_2CH(NH_2)\text{-}COOH$
	selenocystine ($SeCys_2$)	$COOH\text{-}CH(NH_2)CH_2\text{-}Se\text{-}Se\text{-}CH_2CH(NH_2)COOH$
	selenomethyl selenocysteine (SeMeSeCys)	$H_3C\text{-}Se\text{-}CH_2CH(NH_2)\text{-}COOH$
	selenomethyl selenometionine (SeMeSeMet)	$[(H_3C)_2Se^+\text{-}CH_2CH_2CH(NH_2)\text{-}COOH]X^-$
	seleno homocystine	$HOOC\text{-}CH(NH_2)CH_2CH_2\text{-}Se\text{-}Se\text{-}CH_2CH_2CH(NH_2)COOH$
	selenocystationine	$HOOC\text{-}CH(NH_2)CH_2\text{-}Se\text{-}CH_2CH_2CH(NH_2)COOH$
	selenomethionine selenoxide	$H_3C\text{-}SeO\text{-}CH_2CH_2CH(NH_2)\text{-}COOH$
	Selenoglutathione (GSSeH)	$HOOC\text{-}CH(NH_2)\text{-}CH_2CH_2CO\text{-}NH\text{-}CH(CH_2\text{-}S\text{-}Se\text{-}H)\text{-}CO\text{-}NH\text{-}CH_2COOH$
	selenobetaine	$[(H_3C)_2Se^+CH_2COOH]X^-$
	seleno holine	$[(H_3C)_2Se^+ CH_2CH_2OH]X^-$
	γ-glutamyl-Se-methylselenocysteine	$H_3N^+\text{-}CH_2CH_2CO\text{-}NH\text{-}CH(COO^-)CH_2\text{-}Se\text{-}CH_3$
	Se-adenosyl seleno homocysteine	$H_2N\text{-}CH(COOH)CH_2CH_2SeCH_2C_4H_5O_3C_5N_4NH_2$
	selenocysteine acid	
	S-(seleno methyl)cysteine	$H_3CSe\text{-}S\text{-}CH_2CH(NH_2)\text{-}COOH$
	Se-methyl-N-acetyl galactosamine	
	Se-methyl-N-acetylglucosamine	
complex organic species	seleno peptide	
	selenoproteins	
	selenoenzymes	
	selenosugars	
	Se-metal metallotioneine	

1.1.1 Se in human nutrition, health and disease

Selenium distribution within the body, and also its absorption and excretion, depend on several factors, particularly on the chemical forms or speciation and the total quantity of the element in the diet (Figure 1). In addition, intake can be affected by the presence of certain other components of food, including sulphur, heavy metals and vitamins.

Foods normally (mainly) contain organoselenium compounds. Inorganic compounds of the element, such as sodium selenite, only enter the diet as supplements or contaminants. The Se species are transformed to the common intermediate, hydrogen selenide (H_2Se) and then used for the synthesis of selenoproteins (glutathione peroxidase (GPx), iodothyronine 5'-deiodinases (TDI), tioredoxin reductases (TR), selenoprotein P, W and R, etc.) or excreted after successive methylations. The inorganic Se species (selenate and selenite) are reduced to H_2Se by glutathione (GSH). Selenomethionine can be incorporated into proteins non-specifically in place of methionine or alternatively, it can be converted to selenocysteine through the transselenation pathway. Selenocysteine can than be degradaded to H_2Se by a specific lyase enzyme that is used for the synthesis of the t-RNA^{SeCys} complex and incorporated into selenoproteins through the SeCys codon (UGA). SeMeSeCys is degraded by a lyase to methylselenol (CH_3SeH). The methylation of H_2Se detoxifies excess selenium, yielding CH_3SeH, dimethylselenide $(CH_3)_2Se$ and trimethylselenonium ion $(CH_3)_3Se^+$; the latter two metabolites are excreted in breath and urine respectively (Suzuki, 2005; Finley, 2006). Recently a monomethylated selenium compound in urine was identified as a selenosugar (Francesconi and Pannier, 2004). The oxidation of excess H_2Se leads to production of superoxide and other reactive oxygen species (ROS) (Finley, 2006) (Figure 1).

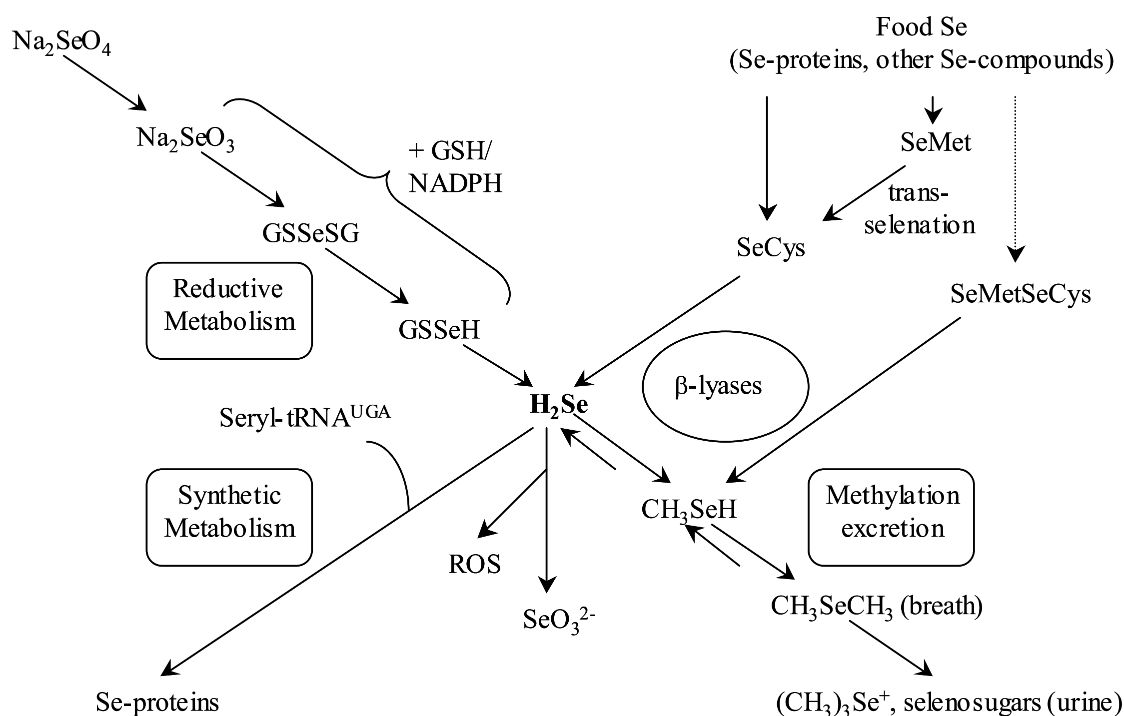


Figure 1: Pathways of Se metabolism (Combs, 2001; Francesconi et al., 2004).

The major fate of all selenium absorbed by the body, whatever its original form when ingested, is incorporation into proteins. Several different types of selenium-containing proteins occur in the body, but it appears that only one type, which contains selenocysteine encoded by a UGA codon in mRNA, is specific for the element and is regulated physiologically. These specific selenocysteine-containing proteins are referred as *selenoproteins*, in contrast to other types of selenium-containing proteins which lack specificity for the element and are not encoded by a unique codon in mRNA (*selenium-binding proteins*) (Reilly, 1996).

The first of these functional selenoproteins to be identified were the glutathione peroxidase enzymes, an important antioxidative enzyme that catalyzes the destruction of hydrogen peroxide generated during oxidative metabolism in human and animal cell (Rotruck et al., 1973). Since then, Se was identified as an

essential component in human and animal cells of thioredoxin reductase (Tamura and Stadman, 1996), Type I, II, III, iodothyronine deiodinases (Arthur et al., 1990) and for a number of selenoproteins (Gladyshev et al., 1998; Allan et al., 1999; Behne et al., 2001; Kryukov et al., 2003) (Table 2). Their variety indicates the wide range of biochemical pathways and physiological functions to which Se contributes.

Table 2: Some of known selenoproteins that carry out the metabolic functions of selenium (Behne et al., 2001; Rayman, 2002 and 2005).

Selenoprotein	Function
Glutathione peroxidases GPx (4 forms)	Antioxidant enzymes: remove H_2O_2, lipid and phospholipid hydroperoxides
(Sperm) mitochondrial capsule selenoprotein	Form of GPx (GPx 4): shields developing sperm cells from oxidative damage and later polymerises into a structural protein required for stability/motility of mature sperm
Sperm nuclei GPx	Present in sperm nuclei and is considered necessary for sperm maturation and male fertility
Iodothyronine deiodinases (3 forms)	Production and regulation of level of active thyroid hormone, triiodothyronine, from thyroxine
Thioredoxin reductases (3 forms)	Reduction of nucleotides in DNA synthesis; regeneration of antioxidant systems; maintanance of the intracellular redox state, critical for cell variability and poliferation; regulation of gene expression
Selenophosphate synthetase (SPS2)	Required for the biosynthesis of selenophosphate, the precursor of selenocysteine and therefore for selenoprotein synthesis
Selenoprotein P	Found in plasma and associated with endothelial cells. Antioxidant and transport functions. Scavenger of peroxynitrite, particularly at the endothelium
Selenoprotein W	Belived to be involved in skeletal and cardiac muscle metabolism
15 kDa selenoprotein	Probably involved in the regulation of protein folding. Different expression in normal and malignant tissues. May protect prostate cells against development of carcinoma
DNA-bound spermatide selenoprotein (34 kDa)	GPx like activity. Found in stomach and in nuclei of spermatoaza. May protect developing sperm
18 kDa selenoprotein	Found in kidney and large number of tissues. Preserved in selenium deficiency

More recently, the interest has focused on specific methylated forms of Se, such as semethylselenocysteine, which have been shown to provide chemoprotective effects against certain types of cancer in humans (Finley et al., 2000 and 2001; Whanger, 2002; Ellis and Salt 2003). In the 1930s Se was found to be an environmental toxin responsible for health problems in livestock grazing on soils with a high Se content (Brown and Shrift, 1982). At Se concentrations of 3−15 mg/kg DW in tissues, some plants can cause toxic symptoms in animals. The range between requirement and toxic doses of Se is narrow. The Se requirement for most farm animals is between 0.1 and 0.3 mg/kg of feed. The RDI of Se for human is 55 µg per day for adults. The minimum Se dietary requirement is approximately 20 µg per day for adults (RDI, Dietary Reference Intake 2000). The Food and Nutrition Board has set the Tolerance Upper Intake Levels (UL) for Se at 400 µg per day for adults (Food and Nutrition Board and Institute of Medicine, 2005). However, Se intakes greater than those set by RDI may be beneficial to human. Clark et al. (1996) demonstrated that dietary supplementation of 200 µg of Se per day significantly reduced lung, prostate, and colorectal cancer in humans. Chronic Se toxicity is caused by intakes of 2–4 mg per day or prolonged intakes of 1 mg per day. Chronic symptoms of excessive consumption of Se include morphological changes in fingernails, nail brittleness and loss of hair, as well as nausea, vomiting and skin lesions. Symptoms of

Se deficiency include muscle pain, weakness, and loss of pigment in hair and skin, and whitening of nail beds (Subcommittee of Selenium, Committee on Animal Nutrition, Board of Agriculture National Research Council 1983).

1.1.2 Se in plants

While higher plants are the primary distributors of selenium from soil to foods, which provide for human nutritional needs, it seems that they themselves do not require the element for their own metabolism. Low-selenium soils do not inhibit plant growth and crop yields are not affected by deficiency of the element. Even though not required for growth by higher plants, selenium is still taken up and accumulated in their tissues. From the literature it is seen that plants vary greatly in their ability to absorb selenium from the soil. They appear to use the same transport mechanism for absorption and internal movement for both selenium and sulphur. Thus, plants that normally are rich in sulphur, such as members of the Liliaceae family (onions and garlic) and members of the Cruciferae family (including cabbage and broccoli) can be expected also to be reasonably rich in selenium (Reilly, 1996).

The Se concentration in plants depends on the chemical form of Se, its concentration and bioavailability in soils and the accumulation capacity of the plant. The uptake of selenate into roots and its distribution in plants is much faster than that of selenite (de Souza et al., 1998; Cartes et al., 2005). De Souza et al. (1998) reported that total Se accumulation in a plant was about 10-fold higher from selenate than from selenite. It was proposed that selenate, chemically analogous to the sulphate ion, is actively transported into roots via sulphate transporters and subsequently quickly transported into shoots. The mechanism of selenite uptake is not fully understood, but it is assumed to be passive. In addition, plants can actively take up organic forms of Se, such as selenomethionine (Terry et al., 2000).

The transport of Se from roots to shoots is thought to occur via xylem. Higher plants have differing capacities to accumulate and tolerate Se. They are classified into non-accumulators, indicators and accumulators (Terry et al., 2000; Dhillon and Dhillon, 2003; White et al., 2004). Some particular plant species are termed Se hyperaccumulators. The largest group of hyperaccumulators belongs to the genus *Astragalus* and *Stanleya*. The Se hyperaccumulators are placed into two groups: the primary Se accumulators are able to accumulate thousands of milligrams of Se per kg (> 4000 mg/kg), and the secondary accumulators hundreds of milligrams Se per kg. Brassicaceae species including Indian mustard (*Brassica juncea* L.), broccoli (*Brassica oleracea botrytis* L.) and canola (*Brassica napus* spp. *oleifera* L.) have been classified as primary accumulators. Plant species with a high Se capacity to accumulate and tolerate Se could be used in the phytoremediation of Se-contaminated sites (Terry et al., 2000; Berken et al., 2002). However, most cultivated crop plants have a low tolerance of high Se levels. Generally, they contain less than 25 µg Se/g DM and are considered to be non-accumulators. Although non-accumulators are sensitive to high Se concentration, they can tolerate as well as accumulate even high concentrations of Se without growth reduction when grown in Se-enriched soils (Rani et al., 2005).

The proposed pathway of Se in higher plants is shown in Figure 2. Se is primarily taken up from the soil by plants as selenate or selenite. Selenate directly competes with sulphate for uptake by plants. After uptake selenate is primarily transported into the chloroplasts, where it is processed by sulphur assimilation pathway. Selenate is thought to be activated by ATP sulfurylase, forming adenosine 5'-phosphoselenate (APSe). In the presence of glutathione, bound APSe is non-enzymatically reduced to selenite. Once selenate is reduced to selenite, it is non-enzymatically reduced to selenide by glutathione. The synthesis of selenocysteine from O-acetyl serine (OAS) and selenite is only in the presence of purified cysteine synthase, glutathione reductase, glutathione and NADPH. Selenocysteine was synthesized from OAS and selenide by cysteine synthase. Ng and Anderson concluded that the only source of selenide in this assay was the non-enzymatic reduction of selenite by glutathione (Ng and Anderson, 1978). The existence of this non-enzymatic pathway for the reduction of selenite to selenide explains why selenite is more readilly assimilated by plants than selenate.

Onward, differences occur in metabolism of Se accumulator and non-accumulator plants. In non-accumulator plants, SeCys can be non-specifically incorporated into proteins or transformed to the selenoamino acid, selenomethionine. Once synthesized by the methionine biosynthetic pathway, selenomethionine can be methylated and converted to DMSe and then volatilised. The first step in the biosynthesis of DMSe is the methylation of selenomethionine to form methylselenomethionine. The conversion to DMSe is probably carried out by S-methyl methionine hydrolase, which normally converts S-methylmethionine to dimethylsulfide (DMS). The non-specific incorporation of the selenoamino acids (selenomethionine and selenocysteine) into proteins is thought to be the major cause of Se toxicity in non-

accumulator plants supplied with a high Se dose (Brown and Shrift, 1982). Lyons et al. (2005) suggested that one explanation for higher toxicity of selenite compared to selenate is that after uptake selenite is incorporated faster than selenate into selenoamino acids in roots. In Se accumulator plants, SeCys can be transformed to several non-protein aminoacid, such as selenocystationine, SeMeSeCys and γ-Se-glutamyl-SeMeSeCys, where SeMeSeCys is the predominated organic Se constituent. It is produced from the methylation of selenocysteine. SeMeSeCys is transformed to SeMeSeCys Se-oxide, which is converted to volatile dimethyldiselenide (Terry et al., 1992; de Souza et al., 1998).

Figure 2: Simplified scheme summarizing a proposed Se assimilation and volatilisation pathway in higher plants (modified from Terry et al., 2000; Berken et al., 2002).

Recently, Se volatilisation by plants has received attention as a possible method for the phytoremediation of Se-contaminated soils. Phytovolatilization would have the advantage over phytoaccumulation of not producing highly Se-enriched plant material that would require special disposal (de Souza et al., 1998).

Whether Se is essential in higher plants is still a controversial issue. However, there are indications that Se might be an essential micronutrient for accumulator plants species such as *Astragalus* (Trelease and Trelease, 1938). In addition, it was shown that Se is required for a growth of a green alga (*Chlamydomonas reinhardtii*) from which Se-dependent GPx was identified (Fu et al., 2002). In contrast, Se non-accumulator

plants, including most crop species, do not appear to require Se for their growth (Terry et al., 2000). However, lately Malorgio et al. (2009) showed that the addition of 0.5 and/or 1.0 mg Se per L to the nutrient solution resulted in a positive effect on the plant yield.

The ability to accumulate and tolerate high Se levels is related to differences in Se metabolisms between accumulator and non-accumulator plant species. Se accumulators and some secondary accumulators limit the integration of selenoamino acids into proteins by converting Se into soluble non-protein selenoamino acids like Se-methylselenocysteine, γ-glutamyl-Semethylselenocysteine, and selenocystathionine (Terry et al., 2000). The Se-methylselenocysteine is the most predominant selenoamino acid in the Se-accumulators such as garlic (*Allium sativum* L.), onion (*Allium cepa* L.), broccoli (*Brassica oleracea* L.) and wild leek (*Allium tricoccum* L.) (Neuhriel et al., 1999). In non-accumulator plants, selenomethione has been found to be the main Se species in seeds of cereals (Stadlober et al., 2001) and in seed and leaves of pea (*Pisum sativum* L.) plants (Smrkolj et al., 2006). Several authors (Mazej et al., 2006; Smrkolj et al., 2006) have reported that various cultural vegetables grown in selenium-enriched culture media did not apparently exhibit the symptoms of toxicity. High Se concentrations were shown to provoke oxidative stress responses such as increased lipid peroxidation in plants (Hartikainen et al., 2000).

The ability of non-accumulator plants to accumulate higher amounts of Se is one possibility to enhance the Se status in food and consequently in human. There are different techniques available to enhance the Se content in plants, such as Se addition to the soil, foliar treatment of plants with Se solution, soaking seeds in Se solution and hydroponic or aeroponic cultivation in a nutrient solution containing Se (Teiz and Zeiger, 2002). Since fall 1984 all agricultural multinutrient fertilizers in Finland have been supplemented with sodium selenate in an attempt to improve the nutritional quality of local foodstuffs known to be exceptionally low in Se (Varo et al., 1988). However, selection of an appropriate method must be done with care due to possible harmful effects on the environment. The use of Se fertilizers caused run-off of the element in the USA resulting in its accumulation in aquatic biota. The chemical form of added selenium affects the distribution of selenium species and also the translocation of essential elements from roots to aerial parts. These Se-enriched plants could be in future a substitute to food supplements. However, not the total amount but its species content determines Se bioavailability.

1.2 Determination of Se species in plants

Trace metals in the environment may have associated risks or benefits. Although the need for trace and ultratrace analyses is clear, the required information about mobility, bioavailability, and finally the impact of elements on ecological systems or biological organisms is not necessarily given by total element concentrations alone. The knowledge of the elemental species provides a better understanding of chemical and biochemical processes and bioavailability and provides more complete information about toxicity or essentiality. Elemental species may involve their oxidation states, organometallic forms, isotopic composition, or complexation states. A meaningful risk assessment should then require speciation analysis (Caruso et al., 2003).

A *chemical species* is defined as a specific form of a chemical element, such as molecular or complex structure or oxidation state. Consequently, the *speciation of an element* is the distribution of defined chemical species of an element in a system. And finally, for elucidating speciation, a *speciation analysis*, defined as the analytical activity of identifying and measuring species is necessary. This term includes appropriate sampling, quantification, and quality-controlled analytical methods (IUPAC, 1999).

Complete speciation schemes consist of sampling, sample storage, sample preparation, species analysis, and evaluation. Most critical step is sampling, where concerns arise about contamination, representativeness of the sample, species preservation or adequate tracking, and precipitation or wall effects (from sample containers). Without proper sampling and sample preparation procedures, there is little chance that any speciation analysis will provide reliable data upon which human health or environmental decisions can be based. Alterations and errors produced at this step are typically irreversible. Storage should be for as short duration as possible and preferably at low temperature. Clean room conditions should be used if possible and as necessary. Sample preparation needs to be as simple as possible to reduce steps in the process that may lead to contamination or species instability (Caruso et al., 2003).

Even today, with all the advantages of modern analytical equipment available to us, the determination of selenium in food materials is a particular challenge because of its often very low concentrations and the ease with which it can be lost during sample preparation.

Therefore, a key aspect of Se determination in biological materials is the need for mass balance calculations. It demands the decomposition of the sample either to make selenium available for the

analytical measurement or to destroy the organic matrix of sample to avoid interference. This destructive step usually consist of an oxidation which can be in fusion or in wet procedure.

One of the oldest used techniques is wet decomposition in an open system (under reflux), which may give rise to systematic errors due to contamination caused by reagents and container material, losses of elements caused by adsorption on the surface of the vessel or by reaction with vessel material, and losses of elements by volatilisation. These systematic errors of wet digestion in open vessel led to development of pressure decomposition techniques in closed system. The main advantages of closed systems are the absence of volatilisation losses of elements, shorter reaction times and improvement of decomposition because of the high temperature, and the lower blank values due to the reduced reagent quantities and reduced contamination from external sources. Microwave procedure has been frequently adopted as a rapid method of sample digestion. This method often reduces the reaction timescales from several hours to few minutes and is considered to improve the level of reaction and process control (Richter et al., 2001).

There are many different detection techniques for selenium determination. Analytical methods used are limited due to the normally low concentrations of elements (requiring analytical methods with low limits of detection). Determination of the metal content is usually performed by neutron activation analysis, fluorescence spectrometry, atomic absorption spectroscopy (flame (FAAS), electrothermal (ETAAS), hydride generation (HG-AAS)), atomic fluorescence spectrometry, hydride generation atomic fluorescence spectrometry, inductively coupled plasma (optical emission spectrometry (OES), atomic emission spectrometry (AES), mass spectrometry (MS)), gas chromatography (electron capture detection (ECD), MS), colorimetric determination, X-ray emission spectrometer, X-ray fluorescence (XRF), gel electrophoresis and others. The detection system chosen will depend on several factors such as the nature of samples to be analysed, the level of sensitivity needed, the number of samples and what other elements are also to be determined (Table 3).

Table 3: Some detectors type vs attributes or performance (Caruso et al., 2003).

	AAS	**AFS**	**ICP-OES simultaneous**	**ICP-MS quad**	**ICP-MS TOF**
Investment cost	1	1	3	3	3
Operating cost	1	1	3	3	4
Required operator expertise	2	2	3	4	5
Portability	1 - 3	1 - 2	4 - 5	4 - 5	5
Sensitivity	2 - 4	2	2 - 3	1 - 2	2 - 3
Linear range	5	3 - 4	2 - 3	1	1
Robustness	4	3	1	2	2
Interferences	3	2 - 3	1 - 2	2 - 3	1 - 2
Multielement/ transient signal	NA	NA	1	2	1
Isotopic composition	NA	NA	NA	3	3

Note: 1, excellent; 2, very good; 3, good; 4, fair; 5, poor; NA, not applicable.

1.2.1 Sample pre-treatment for Se speciation analysis

The selection of sample preparation procedure depends on the matrix, chemical form of selenium expected in the sample and the instrumentation selected for further separation and identification of the species. Sample treatment is the first stage of the process, selenium species should be quantitatively extracted avoiding species interconversion.

Selenium in biological samples are mainly presented as selenoamino acids, either or nor incorporated to proteins. Several approaches have been used for selenoamino acids extraction from samples: aqueous leaching, acid, basic and enzymatic approaches, the latter being the method of choice for majority of researchers.

Some selenoamino acids are water soluble, and they can be extracted with hot water with good recovery values if selenium species are not incorporated to proteins (selenomethylselenocysteine, γ-glutamylselenomethylselenocysteine). This extraction procedure has been successfully applied in different

types of samples with recoveries ranged from 10–25% (selenized yeast) (McSheehy et al., 2002) to higher recoveries in plants (Zhang and Frankerberge, 2001). To increase the low yield of aqueous leaching procedures, more aggressive leaching media has been applied from several authors. The addition of SDS, increases the yield of selenium by realizing selenoproteins from yeast to the aqueous phase (Casiot et al., 1999). The use of acidic (with HCl) or alkaline hydrolysis (tetramethylammonium hydroxide) has been applied for selenium extraction with good recovery values but degradation or transformation of species can easily occur (Casiot et al., 1999).

As previously mentioned, enzymatic hydrolysis is most often the method of choice for selenium species extraction in biological samples. Among the advantages are that it allows selenium species extraction under mild operation conditions of temperature (37 °C) and pH (7.0), what consequently prevents degradation of the original species. The first attempt to use enzymes in Se speciation was reported by Gilon et al. (1995). They found that the recovery of selenoamino acids can increase to 95% by using a mixture of proteolytic enzymes. Proteolytic enzymes (proteinase K, (Kannakumarath et al., 2002), subtilisine (Quijano et al., 2000), pepsine (Dernovics et al., 2002) and protease (Larsen et al., 2003; Uden et al., 2004; Mazej et al., 2006), or mixture of proteolytic enzymes protease and lipase) have been widely applied in a wide variety of samples for extraction of selenoamino acids incorporated to proteins by cleaving peptide bonds. Non-proteolytic enzyme as driselase has been applied for selenium species extraction entrapped physical or chemically within the cell wall. The employ of sequential extraction procedure by sequentially adding leaching reagents is a useful tool for better understanding of how and where selenium is present in the samples. Casiot et al. performed a sequential extraction for selenium in yeast consisting of three steps: (1) hot water, (2) driselase and (3) SDS for proteins solubilization in the solid residue (Casiot et al., 1999).

A particular case of sequential extraction application is the establishment of selenium bioaccesibility by using in vitro gastrointestinal digestion method. There are several approached established, among which the one developed by Luten et al. (1987) is being the most often applied. The procedure implies two steps: (1) gastric digestion: sample is submitted during 4 h at 37 °C at the action of gastric juice, which consist of 6% of pepsine and (2) gastrointestinal digestion where solid residue from step one is treated with intestinal juice containing 1.5% pancreatine and 0.5% amylase. Selenium bioaccesibility is calculated as the percentage of selenium present in the fraction with respect to the total content in the sample. Information of species integrity during digestion is evaluated by performing speciation in the resulting gastric and gastrointestinal fraction.

What should be emphasised is, that there are no data until now on the selenium content present in form of impurities in enzymes, used for hydrolysis. This enzyme characterization could be relevant especially when dealing with low-level Se speciation analysis. Montes-Bayon et al. (2006) studied different extraction methodologies (0.1 M HCl, 25 mM ammonium acetate buffer (pH 5.6) and protease in aqueous solution) for the extraction of Se-amino acids such as Se-methyl selenocysteine and Se-methionine from selenium-enriched plant tissues. In the case of protease, Se recoveries ranged from 103 to 127%, indicating that recoveries higher than 100% could be due to contamination of the protease used for enzymatic extraction.

Due to the instability of Se compounds during the analytical procedure, accurate determination of selenium compounds is not straightforward. Owing to this, evaluation of species stability under several conditions before their determination is a necessary preliminary step. Several authors reported that the sample preparation procedure may affect the accuracy of the results for various reasons, such as species inter-conversion, volatilisation, adsorption, microbial activity, temperature, pH, light action, etc. (Munoz Olivas et al., 1998; Gomez Ariza et al., 2000; Lindemann et al., 2000; Moreno et al., 2002; Pyrzynska, 2002; Roberge et al., 2003; Palacios and Lobinski, 2007). The studies reported have been mainly performed on the stability of selenium in aqueous solutions (Munoz Olivas et al., 1998; Lindemann et al., 2000) and samples of animal origin (Moreno et al., 2002), but information about Se species stability in plant material is scarce. Since plant samples represent a complex matrix, it is necessary to evaluate the stability of the species in the sample of interest. Furthermore, in cases in which sample treatment is time-consuming (at least 24–48 h of treatment at 37 °C), an evaluation of how to maintain species integrity in the extracts is of paramount importance (Mazej et al., 2006; Smrkolj et al., 2007).

In order to decrease time during sample treatment several approaches have been performed. One of them is the use of ultrasonic energy combined with enzymatic hydrolysis. Ultrasound energy has been used in the analytical field for different purposes: dispersion of agglomerates, homogenization, cell disruption and emulsification. These effects are based on the cavitation process where high temperature and pressure are generated. The enzymatic incubation using the ultrasonic probe allows extraction of selenium (75–120%) from the green part of two types of selenium-enriched plants, garlic (*Allium sativum*) and Indian mustard (*Brassica juncea*) in 3 min time (Montes-Bayon et al., 2006). The method is noncomplex and rapidly performed compared with the conventional enzymatic hydrolysis which takes at least 24 h to obtain the same results. The noticeable decrease in time may be attributed to the cell disruption capability of the US probe favouring the contact between the enzymes and cellular components. Although enzymatic probe

sonication has become a conventional tool for selenium species extraction, species transformation could occur in some cases due to the nature of samples.

1.2.2 Separation and detection of Se species

Once the species have been isolated from the matrix, they have to be separated, identified and quantified. The successful approach for speciation depends on two factors: selectivity (to be sure of determining the proper species) and sensitivity (to match the analytes level in the samples). Without doubt, the breakthrough of the evolution of speciation analysis took place with the use of hyphenated techniques, which allow identification and quantification of the individual species in real time. The hyphenated techniques imply the combination of a powerful separation technique with an extremely sensitive detector. For selenium, the most frequently used hyphenated techniques have been separation techniques (gas chromatography, liquid chromatography and electrophoretic techniques) coupled to a great variety of atomic detector (ICP-AES, HG-AFS, ICP-MS, MIP-AES) (Table 4).

Gas chromatography with use of different detectors (MS, ICP-MS, MIP-AES and AFS) has been mostly applied for selenium volatile compounds among which are methylselenol (CH_3SeH), dymethylselenide $(CH_3)_2Se$ and dimethyldimethyldiselenide ($CH_3SeSeCH_3$). One elegant approach is the use of SPME for preconcentration. Carusos's group (Meija et al., 2002) performed determination of several volatile alkylselenides and their sulphur analogues by SPME-GC-ICP-MS. Application was focussed on selenium metabolism in *Brassica juncea* seedlings, finding that the primary volatiles measured in headspace were dymethylselenide and dimethyldiselenide. Dietz et al. (2004) used the same approach for the determination of volatile selenium compounds in lupine, yeast, Indian mustard and garlic forming during growing process in selenium-enriched media. GC has also been applied to non-volatile selenocompounds after derivatization. Selenoamino acids have been derivatized with methyl chloroformiate (Yang et al., 2004a) and with cyanogen bromide (CNBr) (Yang et al., 2004b). However, the use of GC-based methods for non-volatile selenium species is time-consuming and may fail for oligopeptides. Therefore, for selenoamino acids and selenopeptides HPLC is the separation method of choice (Pedrero and Madrid, 2009).

HPLC is a powerful separation technique that offers several advantages over other separation techniques as for instance gas chromatography and electrophoretic methods. It can be directly applied to non-volatile compounds of high and low molecular weight providing a great versatility derived from different separation modes (reverse phase, ion exchange and size exclusion) and can be easily "on line" interfaced to the ICP-MS and UV-HG-AFS. Selenium in biological samples mainly occurs in form of non-volatile compounds such as: inorganic species, small metabolites, selenoamino acids or even polypeptides. From all mentioned, HPLC-ICP-MS used to be the method of choice for selenium speciation in biological and food samples. The type of liquid chromatography used for a specific application is chosen according to relative polarity, solubility, and molecular weight of the species of interest (Caruso et al., 2003).

Separation by SEC is based on the differences in the molecular weight of the analytes (fractionation). SEC is often applied for biomolecules with a molecular weight between 10 and 1000 kDa. This technique is not appropriate for separation of compounds with low molecular weight because adsorption or ionic exchange process can take place as secondary separation mechanism. SEC-ICP-MS has gain on popularity for speciation since it allows the detection of metal bound to macromolecular ligands. However, its application to selenoproteins has been limited because its lack of sensitivity and resolution. Mounicou et al. (2004) presented the use of SEC for screening and resolution of several selenium-containing proteins present in *Brassica juncea*.

Size exclusion has been widely applied as purification step followed by complementary liquid chromatography in multidimensional schemes (speciation). By means of these procedures the selenocompounds are firstly fractionated by SEC and then the collected fractions are processed by other type of LC chromatography such as reverse phase or ion exchange. This multidimensional separation schemes has been applied for increasing resolution and for characterization of isolated compounds by tandem mass spectrometry. For instance, SEC-ICP-MS was first applied for fractionation of aqueous yeast extract into three to six containing fractions. Each of them was further fractionated by anion exchange. Cation or uncharged compounds coelute in the void volume. The fractions obtained after 2D HPLC for anionic compounds and after 3D HPLC for other compounds are usually analysed by ESI-MS (Polatajko et al., 2005). The multidimensional schemes are necessary not only for improving resolution in the separation of selenocompounds but also to remove the matrix components, which would affect electrospray ionization. The described multidimensional scheme has been applied to the identification of selenocompounds in yeast (Polatajko et al., 2005), garlic and other vegetables (Ogra et al., 2004; Montes-Bayon et al., 2006).

Reverse phase is probably the most popular HPLC modality and involves the use of non-polar stationary phase (octadecyl C18 or octyl C8 chains) and a polar liquid mobile phase. The elution is carried out by reducing the polarity by adding organic modifier (methanol, acetonitrile, isopropanol). The separation takes place according to the compounds polarity. Reverse phase ion-pairing chromatography is a special mode of reverse phase used for separation of ionic compounds in which an ion-pair is formed between the solute and the counter ion present in the polar phase. Because of its ability to separate a very broad range of compounds with different polarity (anionic, cationic and neutral molecules) this particular separation mode has been adopted for selenium speciation. Various ion-pairing agents have been applied for selenium speciation being trifluoracetic acid (TFA) and heptafluorobutanoic acid (HFBA) the most applied (Kotrebai et al., 2000).

Selenium speciation has also been performed by using ion exchange chromatography. Ionic exchange has been not only used for the separation of inorganic ions but also for easily ionizable compounds, and it can be used in two modes: anion exchange and cation exchange. At injection, the mobile-phase ions are joined with functional group counter-ions, and charge neutrality is maintained. Upon injection of analyte(s), these ions compete with mobile-phase ions for the functional group sites. The separation of analyte ions (different species) takes place when analyte species displace mobile-phase ions. Since the different analyte ions will often have greater or lesser retention on the column than others, a separation takes place (Mayer, 1988; Caruso et al., 2003).

Another separation technique is electrophoresis (capillary and gel electrophoresis). Capillary electrophoresis offers attractive features for species separation: high resolution, low amount of sample (below 1 ng), which provides good absolute detection limits (pg levels) and the possibility of analysing relatively labile species. CE-ICP-MS has been applied for chiral separation of L- and D-selenomethionine previously derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide in enzymatically digested yeast samples, being only L-selenomethionine detected in the samples (Day et al., 2002). Monicou et al. (2002) applied the technique for selenoamino acids separation in water-soluble fraction of selenized yeast. However, about 30% of the selenium compound did not elute from the column within 30 min. Bendal et al. (2001) applied a capillary coated with polyvinilsulphonate for separation of selenium species in nutritional supplements tablets based on selenized yeast. More than 20 compounds were separated in aqueous extracts yeast within 13 min and detection limits in the low µg/L range.

Gel electrophoresis (native and SDS PAGE) in one or two-dimensional format is a powerful tool for selenoprotein and Se-containing proteins separation providing better resolution than HPLC. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is the most common separation method. In this case, secondary protein structure is denaturized, and consequently its application is restricted to analytes covalently bound into proteins. Therefore, it can be used for selenium speciation without risk of analyte losses. The location of Se-containing bands in 1D GE or spots in 2D GE has been done by INAA, autoradiography of radioactive (e.g. ^{75}Se-labelled) species and laser ablation ICP-MS (LA-ICP-MS) (Kyriapolus et al., 2002).

One of the main problems related with selenium speciation is the lack of available selenocompound standards that makes identification of each individual species difficult. Inductively coupled plasma mass spectrometry has become an essential tool in selenium speciation but it does not provide structural information for the identification of known, unknown or unpredicted compounds and, as a consequence, it is impossible to identify species for which standards are unavailable. Chromatographic separation coupled to ICP-MS usually produces a number of peaks which identification should be considered with extreme care in order to avoid data misinterpretation. The assignment of analytes on the basis on retention times can often lead to wrong identification. Some selenocompounds are hardly retained and it can easily coelute from chromatographic column leading to erroneous identification. For instance, it is well documented in the literature that by using Hamilton PRP-X 100 column (one of the most popular separation column for selenium speciation) the eluted Se peaks appearing around 3–5 min can be attributed to $SeCys_2$ and SeMetO. In fact, these species are hardly retained in anion exchange and coelute close to the chromatographic void volume. A proper identification of the species implies application of at least two chromatographic columns with different separation mechanisms (Pedrero and Madrid, 2009).

However, the number of Se-species detected by chromatographic separations coupled to ICP-MS is growing, while the number of available Se-compound standards is quite limited, resulting the use of alternative chromatographic techniques being inadequate for solving most of identification problems in biological and food matrices. These problems can be alleviated by measuring, not only the elements but also the molecules of which they form part. This can be done by electrospray mass spectrometry (ES-MS, ES-MS-MS) or matrix-assisted laser desorption ionization time-of flight (MALDI-TOF) measurements. Both techniques have an important advantage because of the possibility of following the isotope pattern and therefore, allow species identification (Pedrero and Madrid, 2009).

Selenocompounds cannot be directly analysed by ES-MS because of the presence of the matrix

composed of high molecular weight compounds and salts suppressing the signal. The identification of selenocompounds in biological matrices usually is based on the purification of the compounds of interest by multidimensional chromatography followed by the characterization of isolated compounds by mass spectrometry. MALDI is less vulnerable to matrix suppression than electrospray and produced mostly single charged ions. These characteristics make an attractive identification of target ions for structural study that would otherwise remained undetected with ES tandem MS. The major part of molecular MS application for structural elucidation has been performed in selenized yeast and plants. McSheehy et al. (2001) used sequential extraction and the fractionation of the yeast water extract by size-exclusion chromatography to isolate low molecular weight. Other authors suggest further purification of low molecular weight compounds by HPLC (e.g. anion exchange or reverse phase) Se-adenosylhomocysteine was identified by ES-MS previous separation by multidimensional chromatography SEC-RP (Casiot et al., 1999). Later a higher resolution SEC separation of aqueous yeast extract provided a low molecular weight fraction containing only adenosyl-containing species. Anion-exchange of this fraction gave two peaks: the first containing dehydrated Se adenosylhomocysteine and the second containing Se-adenosylhomocysteine (Chassaigne et al., 2002). Also, it allowed the identification of Se-methylselenomethionine in *Brassica juncea* roots (Grant et al., 2004), γ-glutamyl-Se-methylselenocysteine in green onions (Shah et al., 2004) and garlic (Polatajko et al., 2005). Although great advances have been achieved in this area most of the work done is limited to high-Se food supplements: high-Se yeast, garlic, onions and Brazil nuts where total selenium concentration is high enough to form the analytical features of ES-MS. Further strategies for a proper identification of species would require the development of efficient sample clean-up and preconcentration methods.

Many of the materials that are the subject of Se speciation studies are solids, whereas many of the techniques that are brought to bear the measurement challenge are only capable of accepting liquid samples. Additionally, chemical and physicochemical data, in addition to the MS data are often required to propose a reliable structure of detected Se species. The use of complementary techniques (e.g. NMR) and direct speciation methods using either X-ray absorption or diffraction techniques (extended X-ray absorption fine structure (EXAFS), X-ray absorption near-edge spectroscopy (XANES), X-ray diffraction (XRD), microprobe analyses, X-ray photoelectron spectroscopy (XPS)) that give access to structural information of solid sample are important. The techniques have become increasingly popular due to the minimal sample preparation needed and the resulting decreased risk of species transformation (Caruso et al., 2003). XANES and X-ray fluorescence analysis were used for the *in vivo* investigation of the distribution and the local speciation of Se in roots and leaves of onion *Allium cepa L* (Bulska et al., 2006). Moreover, X-ray spectroscopy, and in particular XANES, have been used to monitor elemental speciation in biological tissues of *Astragalus* plants (Sors et al., 2005) and selenium-rich lentils *Lens culinaris L.* (Thavarajah et al., 2007). However, while some of the techniques allow high spatial resolution for solid samples, the direct quantification of certain species can be difficult and is typically not very sensitive (Caruso et al., 2003).

Table 4: Se species in different plant samples, with plant growing conditions, extraction of Se species from plants, separation and detection system used.

Plant	Growing conditions	Extraction/ preconcentration procedure	Separation technique	Detection system	Se species: LOD	Reference
Chicory (*Cichorium intybus* L.)	aeroponic treatment (SeO_4^{2-})	water extraction, protease XIV; incubation	HPLC: ion-exchange (Hamilton PRP-X 100; Hamilton PRP-X 200)	UV-HG-AFS	Se(IV): 2.3 ng/g, Se(VI): 5.7 ng/g, SeMet: 9 ng/g, SeMeSeCys: 6 ng/g	Mazej et al., 2006
Wheat (*Triticum aestivum*)	hydroponic treatment (SeO_3^{2-}; SeO_4^{2-})	water extraction; sonication 30 min	HPLC: anion-exchange column (Dionex AS14)	ICP-MS	SeO_3^{2-}: SeMet, SeMeSeCys, SeMet Se-oxide, Se(IV)	Li et al., 2008
Indian mustard (*Brassica juncea)* Garlic leaves (*Allium sativa*)	hydroponic treatment (SeO_3^{2-})	0.1 M HCl, 25 mM ammonium acetate buffer, protease; ultasonic probe or incubation	HPLC: ion-pairing reversed phase (C8 Alltima) and size exclusion/ ion exchange (Shodex Asahipak)	ICP-MS	SeMet, SeMeSeCys	Montes-Bayon et al., 2006
Onion leaves (*Allium fistulosum*)	foliar spraying (SeO_3^{2-})	0.1 M NaOH, proteinase K, protease XIV; incubation	HPLC: ion-pairing reversed phase (C8 Alltima) and size exclusion (Superdex Peptide HR 10/30)	ICP-MS ES-ITMS	inorganic Se, SeCys, SeMeSeCys, SeMet γ-glutamyl-SeMeSeCys	Shah et al., 2004
Onion leaves (Allium cepa L.)	hydroponic treatment (SeO_3^{2-})	0.1 M NaOH, proteinase K, protease XIV; incubation	HPLC: size exclusion (Superdex Peptide HR 10/30); ion pairing (C8 Alltima); ion-exchange (Dowex 1X8)	ICP-MS	SeMet, SeMeSeCys, SeCys/$SeCys_2$	Wrobel et al., 2004
Brazil nut (*Bertholletia excelsa*)		protease XIV; simulated gastric fluid and simulated intestinal fluid; incubation	HPLC	ICP-MS ES-MS-MS	SeMet, $SeCys_2$	Dumont et al., 2005

Carrot (*Daucus carota*)	foliar spraying (SeO_3^{2-}; SeO_4^{2-})	protease XIV; incubation	HPLC: ion-exchange (Chrompack IonoSpher 5C; ION-120, Transgenomic)	ICP-MS	roots: SeO_3^{2-} or SeO_4^{2-}: Se(VI), Se(IV), SeMet: 72 ng/g, γ-glutamyl-SeMeSeCys leaves: SeO_3^{2-} or SeO_4^{2-}: Se(VI), Se(IV), SeMet	Kapolna et al., 2009
				ES-MS-MS	SeMet	
Indian mustard (*Brassica juncea)*	hydroponic treatment (SeO_3^{2-}; SeO_4^{2-}; SeMet; KSeCN)	HS-SPME (Carboxen-PDMS)	GC; capillary column (DB-5)	ICP-MS	DMeSe: 7ppb, DMeDSe: 1ppb, DEtDSe: 1ppb	Meija et al., 2002
			GC; capillary column (RTX-5 SILMS)	MS	DMeSe, DMeDSe, DEtDSe	
High-Se yeast		derivatization (ethyl chloroformate, ECF) extraction with $CHCl_3$ or 0.05M HCl solution, ultrasonic bath	GC; capillary column (HP-5MS)	MS	SeMet: 2.26 mg/mL, $SeCys_2$: 2.89 mg/mL	Iscioglu et al., 2004
Onion leaves (Allium cepa L.)	hydroponic treatment (SeO_3^{2-}; SeO_4^{2-})			μ-XANES	SeO_3^{2-}: SeMeSeCys	Bulska et al., 2006
				μ-XRF	SeO_4^{2-}: Se(VI)	
Lentil (Lens culinaris L.)				XANES	SeMet, Se(VI)	Thavarajah et al., 2007

2 Aims and Hypothesis

The goals of the thesis were:

- to check if commercially available enzymes are pure enough to be used for Se speciation analysis
- to optimise the extraction of Se species from plant samples (using optimal enzyme, incubation, ultrasound-assisted extraction, solid-phase micro extraction)
- to develop analytical methods for the separation and determination of soluble Se species by HPLC-ICP-MS
- to compare the performance of HPLC-UV-HG-AFS with the analytical method developed (HPLC-ICP-MS)
- to develop analytical method for the separation and determination of volatile Se species by GC-MS
- to check the stability of extracted Se species in water and enzymatic plant extracts
- to determine Se content in control and Se-enriched plants
- to determine distribution of selenium between insoluble, soluble and volatile part in the selected plants
- to identify the Se species present in selected Se-enriched plants
- to check what is the influence of Se addition on content of sulphur, essential elements like Cu, Zn, Mo, Fe, Mn and toxic element Cd in selected Se-enriched plants

The hypothesis considering selenium species present in selected plants were as follows:

- The main selenium species in Se-enriched plants (potato, buckwheat and leafy vegetables) will be in organic form, as Se-methionine. Lower part will be present as inorganic selenium and Se-methyl-Se-cystein. Share of each selenium species will be plant dependent.
- The stability of Se species in extracts will be plant/matrix dependent.
- Additional Se in plants will not have essential effluence on content of sulphur, essential elements like Cu, Zn, Mo, Fe, Mn and toxic element Cd when plants will be treated with low Se content. In contrast, we expect differences when plants will be exposed to high Se content.
- Methods developed will be appropriate not only for determination of Se and its species in plant samples but also for analysis of other matrixes, like food supplements.
- By analysing selected food supplements, confirmation of declared and measured selenium and its species content will be achieved.

3 Materials and Methods

3.1 Samples

Selected plants were grown in collaboration with the Biotechnical Faculty (University of Ljubljana, Slovenia) on their experimental field. Additionally, most spread commercially available food supplements, containing antioxidants, were collected on Slovenian market.

Plants were grown as follows:

Potato tubers (*Solanum tuberosum* L.), cultivar Desiree
Potato tubers were planted on 20th of April, in plastic pots, with an inner volume of 18 × 18 × 18 cm, in a mixture of soil (95%) and crushed peat (5%), one plant per pot, five pots per treatment on the experimental field of the Biotechnical Faculty in Ljubljana. Soil, peat, and irrigation water contained no detectable Se (i.e., soil and peat less than 0.1 mg of Se per kg and water less than 0.5 μg/L). Experiments were performed outdoors under a removable plastic roof, which was automatically positioned in rainy weather. Four combinations of treatments were performed: well-watered plants (W+) with and without Se foliar spraying and drought-exposed plants (W-) with and without Se foliar spraying. Plants emerged on 2th May. All plants were well-watered until 20th June. On 20 June, they were foliarly sprayed with a solution of detergent (Triton X-100, Sigma, 0.2 mg/L) without or with Se (10 mg of Se/L in the form of sodium selenate). Plants were watered daily with an amount of water corresponding to 4 L/m^2 rainfall (well-watered plants) or 1.5 L/m^2 (drought exposed plants) until harvest on 15 August. The growing conditions are described in details elsewhere (Germ et al., 2007; Cuderman et al., 2008).

Common buckwheat (*Fagopyrum esculentum*) sprouts, cultivar Darja
Common buckwheat seeds were bought in a seed company Semenarna Ljubljana, Slovenia. About 500 mL (346 ± 30 g) of seeds (average seed mass was 0.026 ± 0.001 g) were soaked either in 500 mL of MilliQ water, in a solution of sodium selenate (5, 10, 20 mg Se/L), in a solution of sodium selenite (5, 10, 20 mg Se/L), or in solution of selenometionine (10 mg Se/L). Seeds were soaked for four hours, then they were separated from the solution and distributed equally in plastic bowls, which were covered with filter paper.

During germination seeds were treated with tap water (Se content below detection limit), as needed. Buckwheat sprouts from seeds soaked in i) Se(VI) solution, were grown for 22 days in April, when the average day temperature was 13 °C (13 h of daylight) and the average night temperature was 8 °C. Buckwheat sprouts, from seeds soaked in ii) Se(IV) solution were treated for 8 days in May. The average day temperature was 25 °C (15 h of daylight) and the average night temperature was 18 °C. Seeds soaked in iii) solutions of either SeMet, Se(VI) or Se(IV), at a concentration of 10 mg Se/L, were treated for 11 days in July. Sprouts were treated at 22 °C in a bright air-conditioned room (16 h of daylight). For every treatment a control group (seeds soaked in water) was included. Sprout sampling (harvest) was done when the buckwheat sprouts developed two extended cotyledon leafs. The whole sprouts were colected, including the cotyledons and roots. Six replicates of a hundred randomly chosen sprouts were weighed to obtain the average sprout mass. Buckwheat sprouts from seeds soaked in Se(VI), Se(IV) and SeMet solution are named Se(VI), Se(IV) and SeMet sprouts in the following text. The growing conditions are described in details elsewhere (Ožbolt, 2006).

Common buckwheat (*Fagopyrum esculentum*), cultivar Darja
Buckwheat was sown in July on the experimental field of the Biotechnical Faculty in Kleče. At the beginning of flowering (6 weeks after sowing) one group of plants was sprayed with sodium selenate solution containing 10 mg Se per L and detergent (Triton X-100, Sigma; 0.2 mL/L). The control group of plants was sprayed with only a detergent (Triton X-100, Sigma, 0.2 mL/L). The control and treated groups of plants were grown under the same weather conditions. Three random samples (I, II, III) of both Se treated and the control group of plants were colected 10 weeks after sowing, when plants reached their ripe

phase. Plants were air-dried and separated into individual parts: stems, leaves, inflorescences (after removal of ripe and unripe seeds), dehusked ripe seeds, dehusked unripe seeds and their respective husks. The growing conditions are described in details by Vogrinčič et al. (2009).

Leafy vegetables: chicory (*Cichorium intybus* L. cv. 'Anivip' and 'Monivip'), dandelion (*Taraxacum officinale Waggner*), garden rocket (*Eruca sativa* Mill.) and wild rocket (*Diplotaxis tenuifolia* DC.)
Seeds of all leafy vegetables were obtained from a commercial source (Slovene market). The greenhouse experiment was conducted in the experimental field of the Biotechnical Faculty in Ljubljana. The experiment was designed as a factorial completely randomised block. In each of four blocks a combination of vegetables and treatment was replicated five times. Each replication consisted of a single tray. Styrofoam trays with 40 cells per tray (each circle cavity measuring 5.5 cm deep and 5.5 cm wide) and 90 ml volume of each cell, were hand-filled with the peat-based growing medium Klasmann Tray substrate (pH 6-6.5; N 180 mg/L; P_2O_5 210 mg/L; K_2O 250 mg/L; MgO 85 mg/L + microelements). Two to three seeds were sown in each plug cell in March (low concentration of Se) or April (high concentration of Se). Thinning was done at the second true leaf stage leaving one plant per cell. The trays were covered with a 10% shade cloth until seed germination was completed. About 2 weeks after sowing, the shade cloth was removed and plants were exposed to natural light conditions. Greenhouse and plant cultivation conditions were kept as near-ideal as possible. The ventilation temperature was set at 2 ^{o}C above the heating temperature set points. The lowest temperature was about 10 °C and the highest between 20-30 °C. Relative humidity was maintained at 75±10% using ventilation throughout the growing season. The greenhouse climate was monitored and controlled by a DGT-Volmatic System. Watering was done as needed (generally four times per week), with tap water containing no detectable amount of selenium (i.e., less than 0.5 µg/L). Once weekly all the plants were supplied with a water-soluble fertilizer Peters Professional, Scotts Company (0.75 g N, 0.55 g P_2O_5 and 1.45 g K_2O/L).

Plants without and with Se foliar spraying were treated as follows: Se-enriched plants were sprayed twice with Na_2SeO_4 solution of various concentrations (1+1; 2+2; 5+5; 0+10; 10+10 and 10+50 mg Se/L). At the time of first spraying the plants had four leaves. The time interval between first and second spraying was 5 days. During each treatment about 60 mL of Se solution was sprayed onto each plant group, carefully observing that the solutions were evenly distributed on all leaves. In addition to the Se treated plants, control plants were grown under the same conditions, but were physically separated to avoid cross-contamination during spraying. Plants were colected from trays in May (spraying with low Se concentration) or June (spraying with high Se concentration). More details are described by Žnidaršič (2009).

After plants were grown, they were lyophilised at -50 °C and 0.050 mbar (CHRIST ALPHA 1-4, LOC-1, freeze-dryer), milled and homogenized in a planetary micro mill (FRITSCH, Pulverisette 7, Idar-Oberstein, Germany) and were afterward ready for determination of Se and its species content.

In contrast, three antioxidant food supplements were selected: Fidi koencim 10 (Fidimed, four lots with serial numbers 1341850001, 1338980001, 0399220100 and 0376380100, tablet mass 0.98 g), Super CoQ10 PLUS (4Life, serial number 07224, tablet mass 0.81 g), Koencim Q10 (Akti Vita, serial number 0908, tablet mass 0.45 g). Beside coenzyme Q10, as the main ingredient, the selected supplements contained selenium, vitamin E, vitamin C, vitamin K and beta-carotene. In the text, these three samples are denoted with the letters (A, B, C).

3.2 Chemicals and standards

For preparation of solutions and sample treatment, ultra-pure water (Milli Q, Millipore Corporation, Bedford, MA, USA) was used. The following chemicals were used: 96% H_2SO_4 (Merck, Suprapur), 65% HNO_3 (Merck, Suprapur), 30% HCl (Merck, Suprapur), 36% HCl (Merck, p.a.), 30% H_2O_2 (Merck, p.a. and Suprapur), V_2O_5 (Merck, p.a.), 40% HF (Merck, Suprapur), NaOH (Merck, puriss p.a.), $NaBH_4$ (Fluka, Purum p.a.), $(NH_4)_2HPO_4$ (Fluka Chemie, puriss p.a.), pyridine (Fluka Chemie, puriss p.a.), diammoniumhydrogen citrate (Fluka Chemie, puriss p.a.), citric acid (Fluka Chemie, puriss p.a.), MeOH (Primar, Fisher Scientific UK, trace analysis grade), rutin (Carl Roth GmbH + Co., >98.5%), tannin (Tannic acid, Merck), coenzyme Q10 (Sigma-Aldrich), coenzyme Q10 (B.M.P. Bulk, Medicines & Pharmaceuticals), beta-carotene (Carl Roth), vitamin E (Fluka).

Enzymes of different origin and classification were used: protease from *Streptomyces griseus* (type

XIV: bacterial, 4.4 units/mg solid; Sigma P-5147) and from subtilisin Carlsberg (11.7 units/mg solid; Sigma P-5380); lipase from wheat germ (type I, 8.2 units/mg solid; Sigma L-3001) and from porcine pancreas (type II, 30-90 units/mg protein, Sigma L-3126); cellulase from *Aspergillus niger* (0.57 units/mg solid; Sigma C-1184); β-amylase from barley (type II-B: crude, 17.6 units/mg solid; Sigma A-7130) and from sweet potato (type I-B, 827 units/mg solid; Sigma A-7005); α-amylase from *Aspergillus oryzae* (53.7 units/mg solid; Sigma A-6211), from *Bacillus species* (type II-A, 839 units/mg solid; Sigma A-6380), from barley malt (type VIII-A, 1.7 units/mg solid; Sigma A-2771) and from porcine pancreas (type VI-B, 19.6 units/mg solid; Sigma A-3176); amyloglucosidase from *Aspergillus niger* (59.9 units/mg solid; Fluka 10115).

For preparation of Se solutions, Na_2SeO_3 (Se(IV), Sigma-Aldrich, >98%), Na_2SeO_4 (Se(VI), Sigma-Aldrich, SigmaUltra), selenomethionine (SeMet, Fluka Chemie, >99%), selenocystine ($SeCys_2$, Fluka Chemie, >98%), selenomethylselenocysteine (SeMeSeCys, Fluka Chemie, >98%), $(CH_3)_2Se$ (DMSe, Fluka, >99%) and $(CH_3)_2Se_2$ (DMDSe, Sigma-Aldrich, 98%) were used.

Stock solutions of Se(VI), Se(IV), SeMet, $SeCys_2$ and SeMeSeCys containing about 1 mg Se/g in water were prepared and kept at 4 °C. For Se speciation analysis standards were prepared at concentrations of approximately 100 ng Se per g for each species in supernatants of the control group of the sample analysed, to check for the possible different retention times of Se species caused by matrix interactions in the measurement system.

Stock solutions of DMSe and DMDSe containig about 1 mg Se/g in MeOH were prepared and kept at 4 °C. For preparation of working standard solutions and sample treatment, ultra-pure water (J. T. Baker, Baker analyzed LC-MS Reagent) was used.

For Se, Mn, Fe, Cu, Zn, Mo, Cd solution, 10 mg/L ICP multi-element standard solution XXI (Merck, Germany) or 100 mg/L ICP multi-element standard solution XVI (Merck, Germany) was used. Adittionally, 10 000 mg/L sulphur ICP standard (Merck, Germany) was used.

Stock solutions of multi-element standard containing about 1 mg Se, Mn, Fe, Cu, Zn, Mo, Cd per g and 1000 mg S per g in 1% HNO_3 were prepared and kept at 4 °C. For preparation of solutions and sample treatment, ultra-pure water (Milli Q, Millipore Corporation, Bedford, MA, USA) was used. Further, the standards were diluted as necessary.

3.3 Procedures

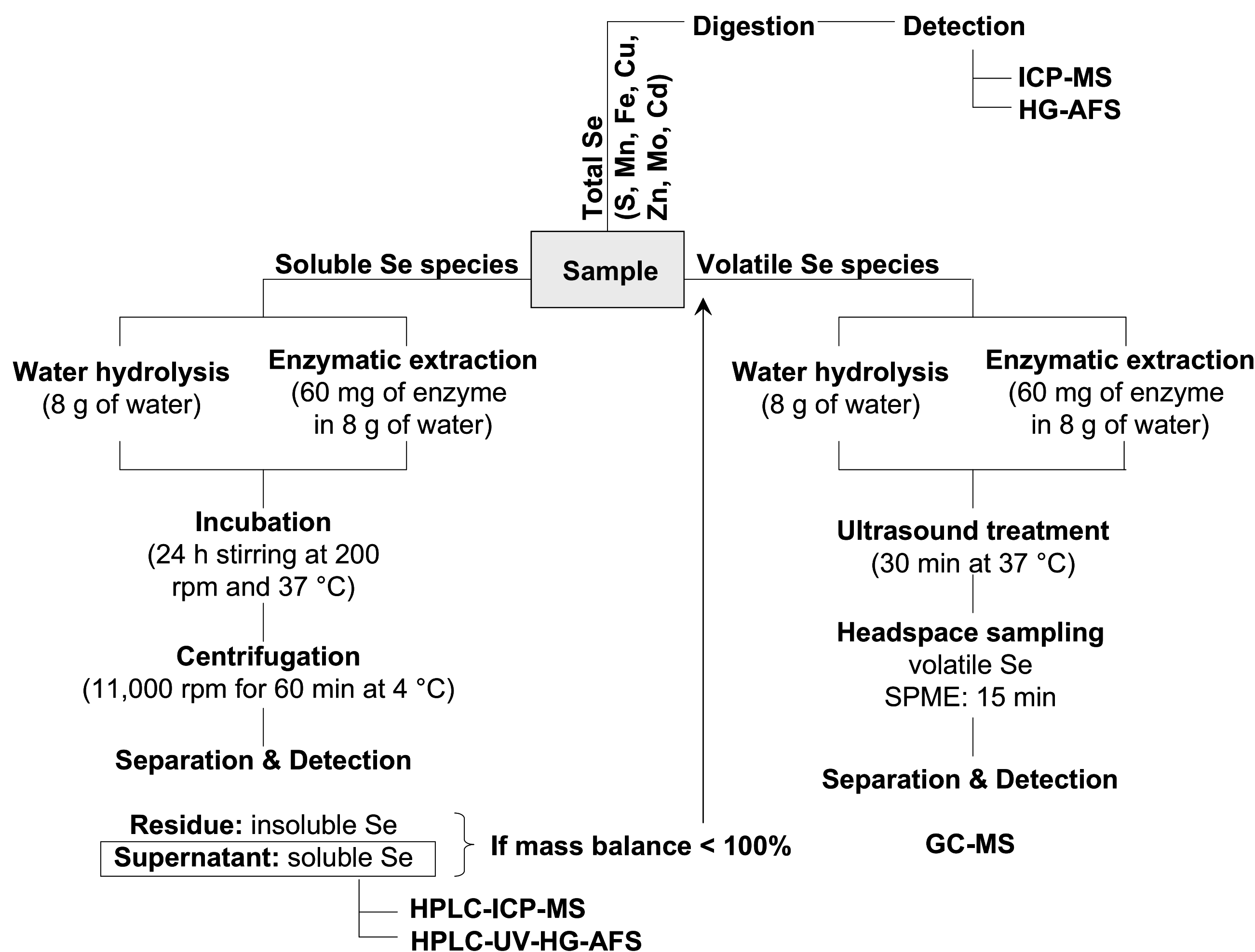


Figure 3: General scheme of Se and its species determination in selected samples.

3.3.1 Se determination

Determination of Se, S, Mn, Fe, Cu, Zn, Mo, Cd with ICP-MS

For the determination of Se, S, Mn, Fe, Cu, Zn, Mo and Cd with ICP-MS the microwave digestion (ETHOS, Milestone, N. America) was employed. Approximately 0.25 g of dried sample was weighed in a Teflon tube and digested using 4 mL HNO_3 and 1 mL H_2O_2. The mineralization program used for microwave digestion involved two steps. Firstly, the temperature and power gradiently increased to 200 °C and 1500 W in 20 min time, respectively, and were further held constant for another 20 min. The end-solutions were diluted with MilliQ water up to 30 mL, with additional dilution of 1.5 mL to 10 mL. The reagent blanks were digested in the same way.

The total concentrations were directly determined by ICP-MS (Agilent 7500ce, Tokyo, Japan). The ICP-MS operating conditions were as follows: RF power 1500 W, plasma argon flow 15 L/min, nickel sampler and skimmer cones, dwell time 0.1 s. The following isotopes were used for the measurement: ^{111}Cd, ^{95}Mo, ^{78}Se, ^{66}Zn, ^{63}Cu, ^{57}Fe, ^{55}Mn, $^{34}S^{16}O^+$, using octopole reaction cell. Hydrogen was used as a collision/reaction gas at 4.5 mL/min for ^{78}Se determination, while for ^{111}Cd, ^{95}Mo, ^{66}Zn, ^{63}Cu, ^{57}Fe, ^{55}Mn helium gas with the same flow rate (4.5 mL/min) was used. Sulphur was determined as its oxide ($^{34}S^{16}O^+$), therefore 15% of oxide was added as a gas before sample reached the plasma. The rest of the parameters were optimised on a daily basis using ICP-MS tune solution, containing 1 ppb Li, Mn, Y, Ce, Tl, Co in 2% HNO_3.

Working standards solutions of Se, S, Mn, Fe, Cu, Zn, Mo, Cd were prepared daily by dilution of a stock standard solution with a solution containing appropriate amounts of HNO_3 to obtain the same acidic media as in the samples. To check the accuracy and precision of the method a standard reference material representing a similar matrix (NIST SRM 1570a, Trace Elements in Spinach Leaves; NIST RM 8436, Durum Wheat Flour; NIST RM 8414, Gluten) was analysed simultaneously (optimisation data for determination of S, Mn, Fe, Cu, Zn, Mo and Cd in plant samples are not shown).

Determination of Se with HG-AFS

Total Se was determined in homogenized freeze-dried samples using HG-AFS as described in more detail by Smrkolj et al. (2004). Mineralization was carried out on 0.2 g of sample. In the case of Se determination in enzymes, mineralization was carried out on 60 mg of enzyme. This was weighed in a Teflon tube, and mineralization was performed using concentrated HNO_3 (1.5 mL) and concentrated H_2SO_4 (0.5 mL) by heating the closed tube in an aluminium block, kept at 80 °C overnight and then for 1 h at 130 °C. After cooling, 2 mL of hydrogen peroxide was added and the tubes were heated for 15 min at 115 °C. This step was repeated. After the solution had cooled to room temperature, 0.1 mL of V_2O_5 in H_2SO_4 was added and the tube was reheated at 115 °C until the solution turned blue in colour. To reduce selenate to selenite, 2.5 mL of concentrated HCl was added to the solution and heated at 90 °C for 10 min. Samples were afterwards diluted with MilliQ water. Sensitive detection was achieved by HG-AFS with the chemical and instrumental operating conditions according to Smrkolj et al. (2004).

Working standard solutions of Se(IV) were prepared daily by dilution of a stock standard solution with a solution containing appropriate amounts of H_2SO_4 and HCl to obtain the same acidic media as in the samples. To check the accuracy and precision of the method the standard reference material representing a similar matrix (NIST SRM 1570a, Trace Elements in Spinach Leaves; NIST RM 8436, Durum Wheat Flour; NIST RM 8414, Gluten; NRC CRM SELM-1, Selenium Enriched Yeast) was analysed simultaneously.

Determination of Se in Residue and Supernatants with HG-AFS

Selenium in the residue (after extraction) was determined by the same procedure as that for total selenium described above.

Se content in the supernatants was determined by digestion with HNO_3. To 0.5 g of supernatant 1 mL of concentrated HNO_3 was added and heated for 30 min at 80 °C and then for 15 min at 160 °C. A total of 1.5 mL of H_2O_2 was added 3 times, and the solution was kept at 120 °C until it evaporated to 0.5-0.6 g of sample. Concentrated HCl (0.5 mL) was added for reduction of Se(VI) to Se(IV), which was then determined by HG-AFS. Working standard solutions of Se(IV) were prepared daily by dilution of a stock standard with 0.5 M HCl (Smrkolj et al., 2004).

3.3.2 Determination of soluble Se species

3.3.2.1 Extraction of soluble Se species

Enzymes

For extraction of Se species from enzymes the same enzyme/water ratio was used as for Se speciation analysis in real samples. For selenium speciation 60 mg of each enzyme or a combination of two enzymes were dissolved in 8 mL of water. Each enzyme was stirred at 200 rpm at 37 °C for 24 h (SW 22, Julabo) and centrifuged at 11,000 rpm for 60 min at 4 °C (5804R, Eppendorf). The supernatant was filtered through 0.45 and 0.22 μm Millex GV filters (Millipore Corporation) and used for selenium speciation analysis by HPLC-ICP-MS. Supernatants and residues were stored at –20 °C until further analysis of total Se by HG-AFS.

Plant samples

For selenium speciation analysis, plant samples were prepared as follows: 8 mL of water without or with 60 mg of enzyme (protese from *Streptomyces griseus*) was mixed with 0.6 g of lyophilised sample. All of the samples were stirred at 200 rpm at 37 °C for 24 h. After extraction, each sample was centrifuged at 11,000 rpm for 60 min at 4 °C. The supernatant was filtered through 0.45 and 0.22 μm Millex GV filters and used for selenium speciation analysis by HPLC-ICP-MS or HPLC-UV-HG-AFS. Supernatants and residues were stored at -20 °C until analysis of total Se by HG-AFS was carried out.

Food supplements
The supplements, sold as tablets or pills, were homogenized. In the case of oil tablets extraction with diethyl-ether (Merck, p.a.) was performed to remove oils and fats. 1.5 g of tablet was shaken with 12 mL of diethyl-ether. The mixture was centrifuged for 5 min at 5,000 rpm. The soluble part was separated from the insoluble and the procedure was repeated for three more times. Diethyl-ether was evaporated from the soluble part to dryness at room temperature. Both phases were analysed for Se content by HG-AFS (see above). The insoluble part was used for Se speciation analysis.

For Se speciation analysis, 8 g of water without or with 60 mg of protease from *Streptomyces griseus* was added to 0.6 g of defatted or non-defatted sample. The mixture was shaken for 24 h at 37 °C. After this extraction procedure, the extract was centrifuged at 11,000 rpm for 60 min at 4 °C. The supernatant was filtered through 0.45 and 0.22 μm Millex GV filters and subjected to selenium speciation analysis by HPLC-ICP-MS or HPLC-UV-HG-AFS. Supernatants and residue were stored at minus 20 °C until analysis of total Se by HG-AFS.

3.3.2.2 Separation and detection of soluble Se species

HPLC-ICP-MS
For soluble Se species determination, a Hamilton PRP-X 100 anion-exchange column (4.1 mm × 250 mm × 10 μm) and a Zorbax 300-SCX cation-exchange column (4.6 mm × 250 mm × 5 μm) were used. Anion-exchange chromatography was used for Se(IV), Se(VI), SeMeSeCys, and SeMet determination, and cation-exchange chromatography was used for SeMet, SeMeSeCys, and $SeCys_2$ determination. Citrate buffer was selected as the mobile phase for anion-exchange chromatography (gradient elution: 3 mM and 10 mM citric buffer, pH 4.8), and 3 mM pyridine (pH 2.1) solution was used as an eluent for cation-exchange chromatography. To increase the sensitivity of the selenium signal, methanol (2% solution) was added to the mobile phase. The flow rate was 0.5 mL/min, and the volume of the sample injected was 50 μL.

The chromatographic system consisted of a high-performance liquid chromatography pump, Series 1100, from Agilent (Waldbronn, Germany), equipped with a Rheodyne (Cotati, CA) Model 7725i injector using a 50 μL loop. The outlet of the column was directly connected to the concentric nebulizer and the Scott-type spray chamber of the ICP-MS (Agilent 7500ce, Tokyo, Japan). Treatment of data was performed with Agilent ChemStation software. Data processing was based on the peak area.

The ICP-MS operating conditions were as follows: RF power 1500 W, plasma argon flow 15 L/min, octopole reaction cell using hydrogen gas 4.0 to 4.5 mL/min, nickel sampler and skimmer cones, dwell time 0.3 s, isotope monitored ^{78}Se. In some cases sulphur was analysed simultaneously, using the same operation conditions. Isotope monitored ^{34}S.

The separation detection conditions described enabled separation of the Se species (Se(IV), Se(VI), SeMet, SeMeSeCys, and $SeCys_2$) using both columns. Standards were prepared at concentrations of approximately 100 ng of Se/g for each species in supernatants of the control group of the sample analysed, to check the different retention times of Se species caused by matrix interactions in the measurement system. Moreover, the Se species in the supernatant were confirmed by the standard addition method.

HPLC-UV-HG-AFS
Se species in supernatants were also determined by HPLC-UV-HG-AFS. HPLC-UV-HG-AFS was used because it was already introduced to our laboratory. It is a cheaper technique and easier to deal with. Therefore, it was used, in the case of high Se content in plants.

The separation system consisted of a high-pressure pump (Varian ProStar 210), a Rheodyne 7725i injector, and ion (anion, Hamilton PRP X 100; cation, Zorbax SCX-300) exchange columns. The chromatographic system was connected to a UV-HG-AFS system used for online detection, for which the operating conditions are described in detail elsewhere (Mazej et al., 2006) and are summarized in Table 5. Optimal conditions for the anion exchange column (Hamilton PRP-X 100) were achieved with 40 mM phosphate buffer (pH=6) at a flow rate of 0.5 ml/min (Mazej et al., 2006). The same authors used the Hamilton PRP-X 200 as a cation exchange column with 3 mM pyridine (pH=2.4) and 0.5 ml/min as mobile phase. In this work the Agilent Zorbax 300-SCX was used with the same operating conditions (Table 5). The standards were prepared in the same way as for ICP-MS detection (described above).

Table 5: Optimal conditions for Se species determination with HPLC-UV-HG-AFS (Mazej et al., 2006).

Parameter	Value
HPLC	
instrument: Varian ProStar 210	
Anion exchange chromatography:	
Hamilton PRP-X 100 column	4.1 mm x 250 mm x 10 µm
mobile phase	40 mM phosphate buffer (pH 6.0)
flow rate (mL/min)	0.5
injected volume (µL)	100
Cation exchange chromatography:	
Zorbax 300-SCX column	4.6 mm x 250 mm x 5 µm
mobile phase	3 mM pyridine solution (pH 2.4)
flow rate (mL/min)	0.5
injected volume (µL)	100
UV-HG-AFS	
instrument: PS Analytical	
carrier gas flow rate (mL/min)	1
argon gas flow rate (L/min)	0.26
nitrogen gas flow rate (L/min)	4
reduction	conc. HCl (flow: 3 mL/min) and UV
reductive solution	1.4% w/v $NaBH_4$ in 0.4% w/v NaOH (flow: 3 mL/min)

3.3.3 Determination of volatile Se species

3.3.3.1 Extraction of volatile Se species

For selenium speciation analysis of volatile Se species, plant samples were prepared as follows: 8 mL of water without or with 60 mg of enzyme (protese from *Streptomyces griseus*) was mixed with 0.6 g of lyophilised sample. All of the samples were ultrasound treated at 37 °C for 30 min (VWR International West Chester, PA, New York).

3.3.3.2 Preconcentration of volatile Se species

For the volatile species preconcentration SPME fiber with 100 µm Polydimethylsiloxane (PDMS, Supelco, US) or 75 µm Carboxen-PDMS (Supelco, US) coating was used. After extraction, The SPME fiber was exposed to the headspace of each sample for 15 min. Ultrasound bath was at constant temperature, 37 °C. Afterwards, the fiber was immediately injected into GC-MS.

3.3.3.3 Separation and detection of volatile Se species

GC-MS

An HP 6890 GC–MS system (Hewlett-Packard, Waldbron, Germany) was used for analysis. The capillary column was a fused-silica DB-5 MS (30 m×0.25 mm I.D., 0.25 μm film thickness). The chromatographic conditions were as follows: the carrier gas was helium with a linear velocity of 37 cm/s, injection was in the splitless mode (240 °C) with an injection volume of 1 μL. The temperature programme was as follows: 50 °C, 4 min, first ramped at 15 °C/min to 125 °C (held for 2 min), then the temperature was ramped again at 30 °C/min to 250 °C (held for 2 min).

The mass-selective detector conditions were 280 °C for the transfer line and electron impact ionisation of 70 eV. The spectra obtained were measured in the TIC mode (50-550 m.u.) for qualitative detection and in the SIM mode for quantitative detection using characteristic ions for each compound studied.

4 Results and Discussion

4.1 Determination of water-soluble Se species

4.1.1 Enzymatic extraction of Se species from biological samples

Accurate determination of selenium compounds in samples of different origin is not straightforward, due to its low concentration in samples, low extraction efficiencies and the instability of Se compounds during the analytical procedure (Mazej et al., 2006). Before a chromatographic separation, the analytes must be extracted from the solid sample where identification and determination of the selenium species is intended. Thus, several sample extraction techniques have been evaluated in order to obtain the highest selenium extraction efficiency in samples of different origin (Capelo et al., 2004; Montes-Bayon et al., 2006).

Our first aim was to check if commercially available enzymes are pure and therefore appropriate for extraction analysis of Se species. For this purpose, we studied the Se content and its distribution between soluble and insoluble part in commercially available enzymes (powder form) of different origin and classification, such as protease, lipase, cellulase and amylase (Table 6). In some cases enzymes of different lots were used.

Table 6: Se in commercially available enzymes.

ENZYME	total Se	residue	supernatant	soluble Se
	ng Se/g of enzyme			%
protease from *Streptomyces griseus* (1)	378 ± 1	-	350 ± 16	93
protease from S*treptomyces griseus* (2)	211 ± 9	-	210 ± 18	100
protease from subtilisin Carlsberg (1)	63 ± 4	-	53 ± 2	84
protease from subtilisin Carlsberg (2)	719 ± 36	-	484 ± 14	67
lipase from porcine pancreas (1)	654 ± 21	114 ± 10	443 ± 20	68
lipase from porcine pancreas (2)	540 ± 76	93 ± 1	331 ± 36	61
lipase from wheat germ (1)	1127 ± 14	349 ± 1	813 ± 10	72
cellulase from *Aspergillus niger* (1)	< LOD	-	< LOD	
cellulase from *Aspergillus niger* (2)	< LOD	-	< LOD	
β-amylase from barley (1)	3100 ± 180	897 ± 11	2050 ± 100	66
β-amylase from barley (2)	1885 ± 82	250 ± 6	1606 ± 12	85
β-amylase from sweet potato (1)	< LOD	-	< LOD	
α-amylase from *Aspergillus oryzae* (1)	824 ± 1	257 ± 10	610 ± 29	74
α-amylase from *Aspergillus oryzae* (2)	814 ± 80	105 ± 10	610 ± 8	75
α-amylase from *Bacillus species* (1)	315 ± 22	-	-	
α-amylase from barley malt (1)	102 ± 2	25 ± 5	34 ± 3	33
α-amylase from porcine pancreas (1)	394 ± 31	21 ± 5	379 ± 20	96
α-amylase from porcine pancreas (2)	528 ± 50	66 ± 1	316 ± 5	60
amyloglucosidase from *Aspergillus niger* (1)	< LOD	-	< LOD	

Se content in water was under LOD. Total Se analysis were done in triplicate and the results given for residuum and supernatant represent an average with the absolute error of two determinations.
(1) = first lot
(2) = second lot
LOD = 20 ng Se/g enzyme (Mazej et al., 2006)
- = no residuum was present after the extraction procedure

The results showed that the Se content was relevant in several cases. The highest value was obtained for β-amylase from barley. Its Se content was found to be 3100 (first lot) and 1885 (second lot) ng per g of enzyme. We can see that the difference between the values for the two lots of this enzyme is large, and large differences between the values for different lots were also observed for protease from *Streptomyces griseus* (378 and 211 ng/g), subtilisin Carlsberg (63 and 719 ng/g), and α-amylase from porcine pancreas (394 and 528 ng/g). On the other hand, comparable results for different lots were observed for lipase from porcine pancreas (654 and 540 ng/g) and α-amylase from *Aspergillus oryzae* (824 and 814 ng/g). The values obtained for most of the enzymes under investigation were significant and had to be considered when determining Se (in the mass balance calculation) and its species in real samples. The soluble Se in enzymes represented between 60% (α-amylase from porcine pancreas (2)) and 100% (protease from *Streptomyces griseus* (2)) of the total Se, except in the case of α-amylase from barley malt (1), where it represented 33% of the total Se.

4.1.1.1 Optimization of extraction of Se species from Se-enriched plants

Since an enzyme works as a catalyst, a number of variables must be controlled in order to assure the enzyme activity. Variables affecting the enzymatic hydrolysis include pH, temperature, ionic strength, enzyme mass, reaction volume and incubation time. Several of these parameters were considered, by varying one parameter and leaving the others constant, in order to obtain the highest extraction efficiency. The extraction efficiency (soluble Se, %) was calculated in comparison with the total concentration of Se in the sample (taken as 100%) and the results were corrected for Se content in the enzyme used (Table 6). Se analysis was done in duplicate and results are given as an average with an absolute error of two determinations.

One of the main problems cited by different authors is directly related to the extraction methodology for quantitative recovery of selenium species. Different approaches such as enzymatic and basic hydrolysis have been proposed, the process requires 24 h. This methodology is extremely time-consuming, the efficiency of the process is far from being quantitative, and the risk of selenium interconversion is very high. All these drawbacks probably explain why selenium speciation results are hardly comparable when this methodology is applied by different authors in intercomparison exercises (Capelo et al., 2004).

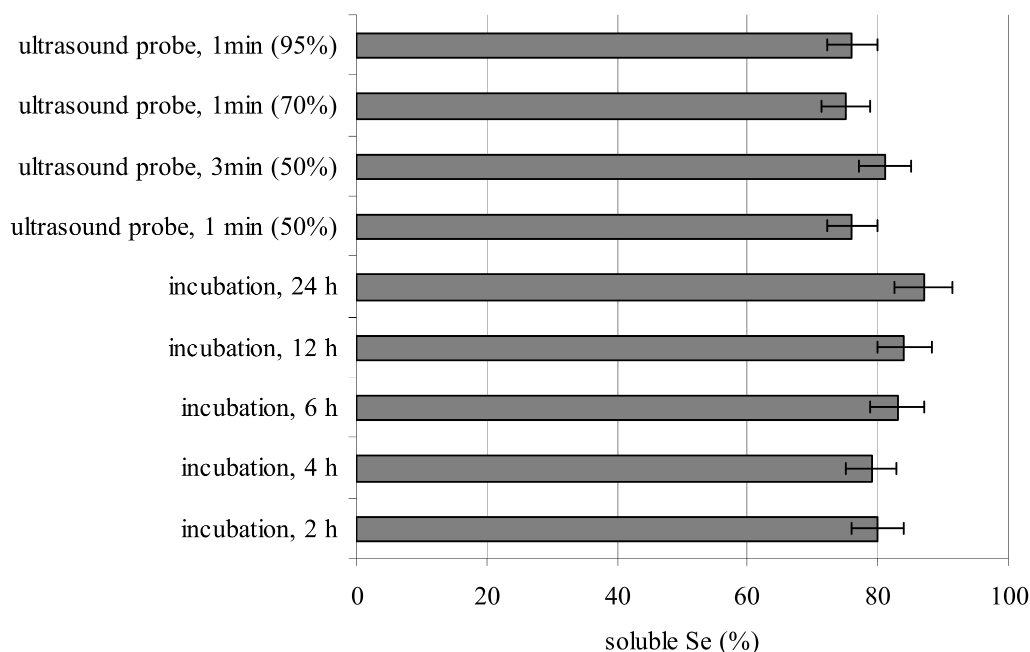


Figure 4: Optimization of enzymatic hydrolysis procedure (incubation and ultrasound probe) for NIST RM 8436.

Therefore, two different ways of hydrolysis were compared. Incubation at constant temperature and stirring (37 °C, 200 rpm) using different time of shaking (2, 4, 6, 12, 24 h) and ultrasound probe (Cole-Parmer, 300 W), using different extraction times (1, 3 min) and its intensity (50, 70, 95 %, 300 W). Sample being analysed was NIST RM 8436, Durum Wheat Flour and for hydrolysis, enzyme protease from *Streptomyces griseus* was used. The ratio between the sample and the enzyme was kept constant (10 : 1)

(Figure 4).

Time of shaking had almost no influence on the extraction efficiency of Se from the sample. By incubation at 37 °C (200 rpm) for 24 h, 7% higher extraction efficiency was achieved. We presumed that ultrasound would fragment plant cells and Se would be released more efficiently from cells and their components. However, the results obtained using ultrasound assisted extraction for 3 min at 50% of power intensity were comparable with the one obtained after 2 h incubation. At the same time, the efficiency was almost 10% lower than the one obtained after 24 h incubation.

Due to the strong correlation of extraction efficiency with the matrix used, the effect of ultrasound and incubation on the extraction yield was checked on a real sample basis. Potato was chosen for the sample material (Figure 5).

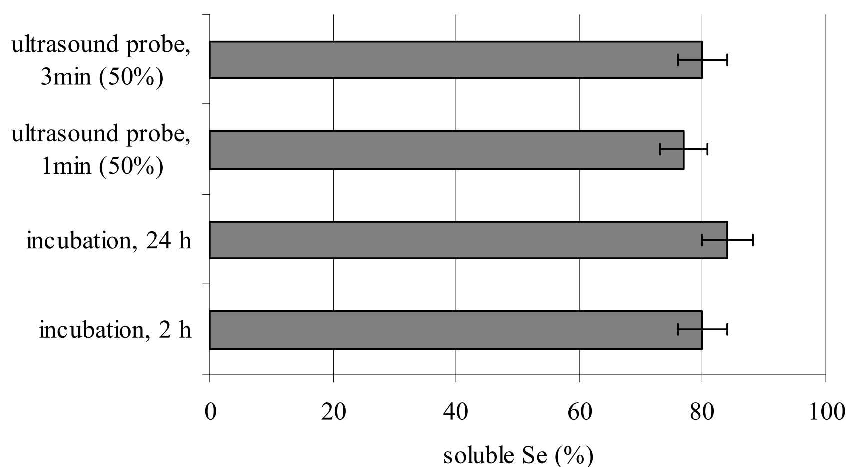


Figure 5: Extraction efficiency of Se from potato.

24 h incubation at constant stirring (200 rpm) and temperature (37°C) was shown to be the optimal extraction conditions. Nevertheless, major advantage of ultrasound probe is short extraction time, as also reported by Cabanero et al. (2005), Capelo et al. (2004), Lavilla et al. (2007), Vale et al. (2007), Montes-Bayon et al. (2006). However, incubation still seems to be predominant in the literature dealing with Se speciation analysis. Despite these results, no experiments were made in order to determine to which extent soluble peptides are broken down into free aminoacids when other incubation procedures or lower incubation times are used. Therefore, 24 h incubation at 37 °C and 200 rpm, was chosen for further work, to obtain comparable results.

Since the chemical structure of selected Se-enriched plants (potato tubers (*Solanum tuberosum* L.), cultivar Desiree; common buckwheat (*Fagopyrum esculentum*) sprouts, cultivar Darja and leafy vegetables (chicory (*Cichorium intybus* L. cv. 'Anivip' and 'Monivip'), dandelion (*Taraxacum officinale Waggner*), rocket (*Eruca sativa Mill.*) and wild rocket (*Diplotaxis tenuifolia* DC.)) is dissimilar, the enzyme for hydrolysis was needed to be carefully chosen for each plant group individually as well as for antioxidant food supplements.

Starch plant

Starch is the major component of potato tubers, accounting for 75-85% of the tuber dry weight. Other chemical components of potato are reducing sugars, proteins, vitamins, and minerals. Since starch is the predominant component in potato, enzyme hydrolysis with amylase was used to clarify its role in selenium speciation. Several amylases of different origin and classification were used (β-amylase from barley, β-amylase from sweet potato, α-amylase from porcine pancreas). α-amylase acts at random locations along the starch chain (glucose unit), and therefore tends to be faster acting with a higher efficiency than β-amylase, which works from the non-reducing end, catalyzing the hydrolysis of the second α-1,4 glycosidic bond, cleaving off two glucose units (maltose) at a time. Further, to hydrolyse peptide bonds in proteins, protease from *Streptomyces griseus* was used. Additionally, water-soluble Se compounds were extracted from potato tubers by a combination of protease and amylase or by water alone. The sample to enzyme ratio was constantly 10 against 1.

The extraction efficiency was optimized for well-watered potato (W+) (Figure 6). By using different enzymes, the extraction efficiency was between 59 and 78%, the lowest when using only α-amylase and the highest when using protease and α-amylase together, although little difference was obtained when using protease in combination with either α-amylase or β-amylase, or alone. In water extracts we found about

20% lower results (44%). From this can be seen, that the enzymatically gained part of Se was incorporated into protein and starch structures.

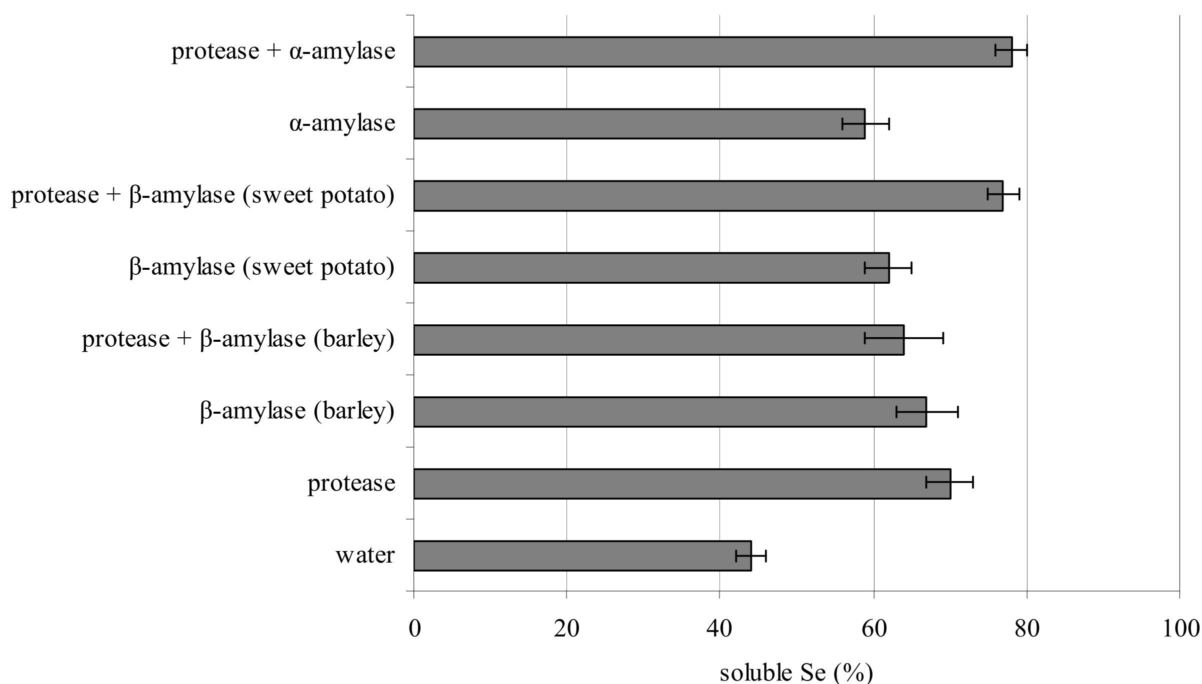


Figure 6: Soluble Se (%) obtained after different extraction media used for well-watered potato.

Protease and α-amylase together gave the highest extraction yield. It was comparable with the results obtained by using protease alone. Therefore, protease was chosen as an optimal enzyme for further analysis. The ratio between the sample and protease was 10 : 1.

Turakainen et al. (2006) reported that 57-76% of Se was soluble when water was used as an extraction media from potato tubers. On the other hand, Ferri et al. (2007) used amyloglucosidase to extract Se species from the same matrix, but no data about extraction efficiency were given.

Young plants - sprouts

Buckwheat is rich in starch, proteins, minerals and antioxidants like rutin and tannin. The extraction procedure was optimized by a) using optimal extraction media (water, phosphate buffer, 0.1, 0.2, 0.3 M HCl, the non-specific enzyme protease from S*treptomyces griseus* alone or in combination with the specific enzymes cellulase from A*spergillus niger*, α-amylase from porcine pancreas or lipase from porcine pancreas), and by b) optimizing the ratio between the sample and the enzyme. In the optimization procedure buckwheat sprouts, grown from seeds soaked in 10 mg Se as Se(IV), Se(VI) and SeMet per L, were used.

In the first part water-soluble Se was extracted from samples either by water, 25 mM phosphate buffer (pH 7.5) or hydrochloric acid (0.1, 0.2, 0.3 M). Using water media, the extraction efficiency was between 9% and 11% for Se(VI) sprouts and 4% to 12% for Se(IV) sprouts. Comparable extraction efficiencies were obtained using phosphate buffer as the extraction media, namely 8 – 13% for Se(VI) and 4 – 10% for Se(IV) treated sprouts. The same extraction efficiency was obtained for SeMet treated sprouts, 10 ± 2% for both extraction media. Using hydrochloric acid, the extraction efficiency for Se(IV) stayed comparable, 9 – 13%, for SeMet it was twice as high, 22 ± 3%, regardless of the acid concentration used. A three to four times higher efficiency was achieved for Se(VI) sprouts; 32 – 35% with 0.1M HCl; 36 – 43% with 0.2M HCl and 39 – 44% with 0.3M HCl (Figure 7).

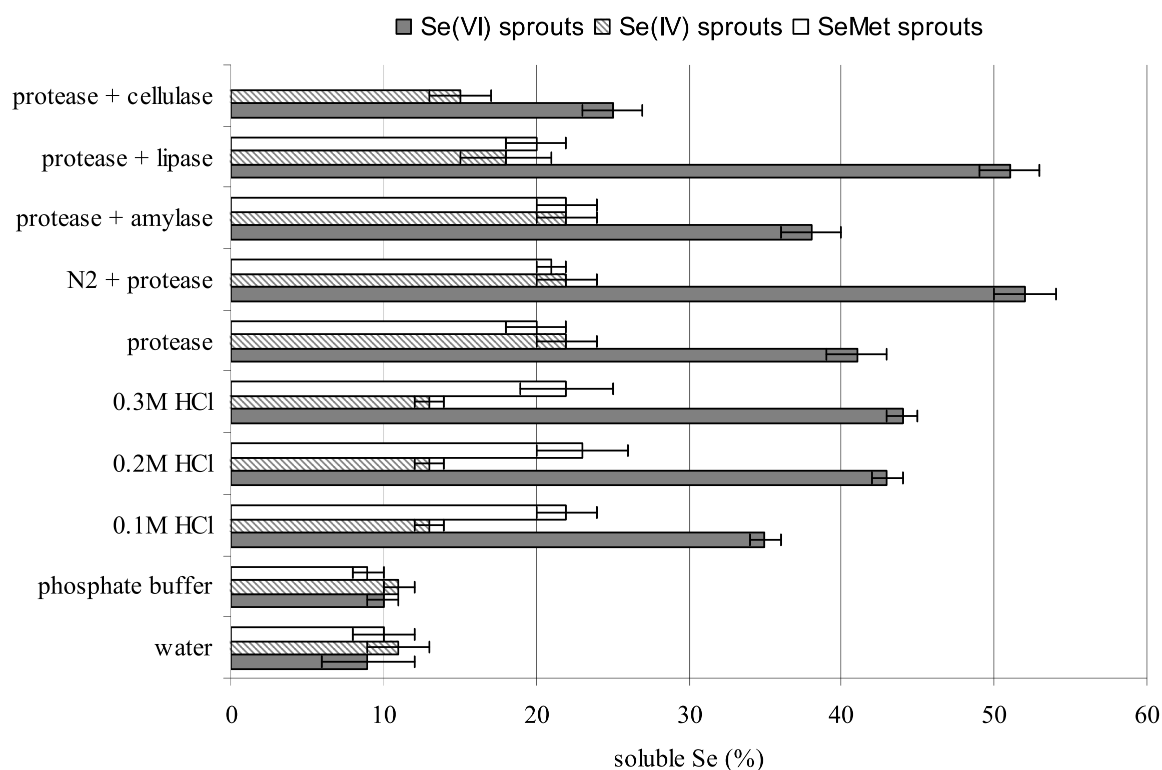


Figure 7: Soluble Se (%) obtained after different extraction media used for 10 mg/L Se(VI), Se(IV) and SeMet sprouts.

To enhance the extraction efficiency, enzymatic hydrolysis was applied. Firstly, the non-specific enzyme protease was employed. Efforts to increase the extraction efficiency included using different ratios between enzyme and sample, by breaking the cells with liquid nitrogen prior to enzymatic hydrolysis with protease and by combining the non-specific enzyme with a specific one, like cellulase or amylase or lipase.

When the ratio between enzyme and sample was 1 to 6, the extraction efficiency for Se(VI) and SeMet sprouts remained comparable to the ones obtained after acidic hydrolysis of 38 ± 2% and 20 ± 2%. In the case of Se(IV) a twice higher result was obtained, 22 ± 3% (Figure 7). The extraction efficiency remained the same after changing the enzyme - sample ratio to 1 : 10 and 1 : 18. When the ratio between enzyme and sample was 1 : 90 and 1 : 900, the results obtained were comparable with those using water media or phosphate buffer. Therefore the enzyme – sample ratio, 1 against 10, was used in further experiments.

Further, by breaking cells with liquid nitrogen before enzymatic hydrolysis with protease, we succeeded in increasing the extraction efficiency only in Se(VI) sprouts, i.e. to 52 ± 2%. In Se(IV) and SeMet sprouts the extraction efficiency (approximately 20%) was comparable to the one obtained without liquid nitrogen.

Since we were not satisfied with such a low efficiencies, we tried the combination of several specific enzymes, like cellulase, amylase or lipase with protease. The ratio between the two enzymes and sample was 1 : 1 : 10. In supernatants of buckwheat sprouts treated with Se(VI) the efficiency using protease or amylase was the same. Protease in combination with lipase gave around 10% higher result, but on the other hand, when combining protease and cellulase about 34% lower result was achieved than in combination with lipase. For enzymatic extracts of Se(IV) and SeMet sprouts the efficiency stayed the same, regardless of the enzyme combination used (Figure 7). Independent of the short growing period, from 8 to 22 days, a great part of Se remained insoluble.

Since we were not able to increase the extraction efficiency with a combination of different enzymes, we decided to use protease as the optimal enzyme for the extraction of Se species from Se-enriched buckwheat sprouts. In further analysis, 0.6 g of sample was treated with 0.06 g of protease, dissolved in 8 g of water.

Lintchinger et al. (2000) studied sunflower, wheat and alfalfa sprouts, from seeds soaked in solutions containing 0.78 – 800 mg Se(VI)/L. Water extracts of sunflower sprouts showed quantitative extraction efficiency (100%), regardless of the Se concentration used. In water extracts of wheat and alfalfa an increase of extracted Se was observed with increasing Se(VI) concentration (from 25% to 64% for wheat and from 40% to 72% for alfalfa sprouts).

Leafy vegetables

The nutritional content of vegetables varies considerably, though generally they contain little protein or fat, and varying proportions of vitamins, provitamins, dietary minerals, fiber and carbohydrates. Several extractions of Se species from the green parts of Se-exposed plant samples were made to find the optimal conditions. For the optimization procedure, leafy vegetables foliarly sprayed with 2 + 2 mg Se(VI)/L solution were taken.

In the first part, water-soluble Se was extracted from the samples by either protease from *Streptomyces griseus* alone or in combination with cellulase from *Aspergillus niger*. The ratio between sample and enzyme was constant at 10 against 1. Comparable extraction efficiencies were obtained using the non-specific enzyme alone or in combination with the specific enzyme cellulase, namely 48% for the chicory cv. ‘Anivip’, 51% for chicory cv. ‘Monivip’, 47% for dandelion, 60% for rocket and 64% for wild rocket (Figure 8).

To enhance the extraction efficiency, several consecutive enzymatic hydrolysis were performed. Water extraction was performed first to remove inorganic Se and additionally the non-specific enzyme protease was employed. Firstly alone, secondly in combination with detergent (CellLytic P, Cell Lysis; the Se content in detergent was under limit of detection) and thirdly, protease was added twice. By performing this two- and three-step hydrolysis we were able to increase the extraction efficiency for 20%, obtaining efficiency between 70% and 80% irrespectively to the plant used (Figure 8). However, the process proposed required 48 to 72 h. This methodology is time-consuming, the efficiency of the process is not quantitative, and the risk of selenium species inter-conversion is very high. Due to this, we decided to use one-step extraction with protease as the optimal procedure for extraction of Se species from Se-enriched leafy vegetables. Besides, extracts obtained after three step extraction were taken into consideration when speciation analysis were performed.

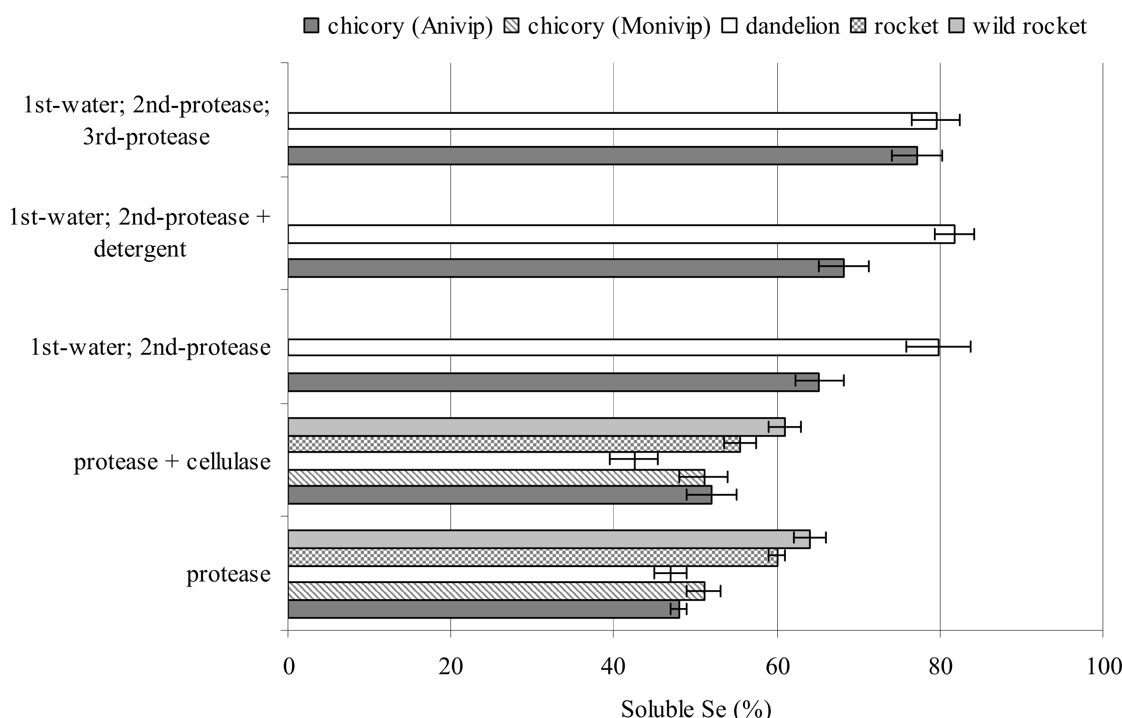


Figure 8: Soluble Se (%) obtained after different extraction media used for leafy vegetables foliarly sprayed with 2 + 2 mg Se(VI)/L.

In a study published by Kapolna et al. (2007) pronase E and protease XIV were used to hydrolyse peptide bonds in sesame seeds. The values obtained for the extraction efficiencies for Se were found to be 68% and 83% for protease XIV and pronase E, respectively. Mazej et al. (2008) extracted 45-85% of soluble Se from the green parts of chicory, lettuce, dandelion and parsley using protease XIV for enzymatic extraction. Using the same enzyme (protease XIV), Kapolna et al. (2009) extracted 75% of Se from carrot leaves.

Food supplements

The optimization of the extraction efficiency of Se from antioxidant food supplements was performed in each product selected (products A, B, C), since not all of the tablets were present as solids. Additionally,

the Se species content in these products is scarce and therefore extraction procedure is product dependent.

Firstly, the purification/separation of fats from proteins in the oil tablets (product A) was necessary. To do that, diethyl-ether was used. Several clean-up steps were performed and the solution and residue obtained were analyzed for Se content. In a one-step extraction we were not able to quantitatively remove fats, therefore we repeated the extraction. For almost quantitative elimination of fats, four extractions were required. Afterwards, 96% of total Se was present in the insoluble protein fraction, which was taken for Se speciation analysis. In the fat fraction only 2% of total Se remained present.

Further, two different types of extraction were used for Se species from tablets. The first was simple water extraction for water-soluble Se species, and the second was enzymatic hydrolysis, using protease from *Streptomyces griseus*, to release Se species bound to proteins (Figure 9).

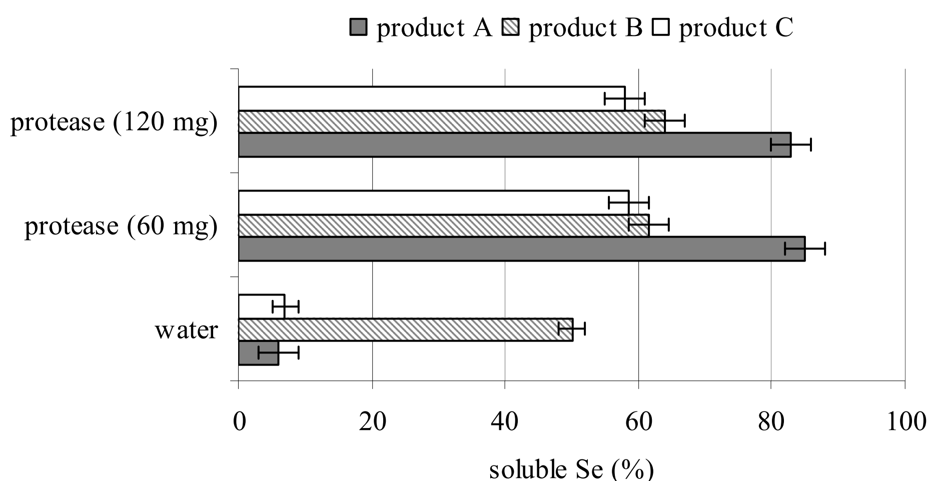


Figure 9: Soluble Se (%) obtained after different extraction media used for antioxidant food supplements.

In products A and C the efficiency was not satisfying (< 10%) when extraction of Se species was performed in water alone. So, due to the fact that Se in food supplements may be bounded to proteins the simple extraction conditions, i.e., MilliQ water as an extraction solvent and single step extraction, were also used with enzymatic hydrolysis. Only the mass of enzyme was optimised and as a result, either 0.06 or 0.12 g of the enzyme protease in 8 mL of MilliQ water was added to 0.6 g of sample. The efficiency increased to an average of 60% for products B and C and to 85% for product A (Figure 9) in the presence of enzyme, irrespectively to the mass of the enzyme.

According to the results obtained for different sample matrixes, one-step extraction seems to be enough for satisfactory extraction of soluble Se species. Further, the non-specific enzyme protease from *Streptomyces griseus* can be considered as an appropriate enzyme for extraction of Se species, regardless of the sample used. The ratio between enzyme and sample (1 : 10) is high enough for satisfactory extraction of Se from selected plant samples and food supplements.

Additionally, suppliers of this commercially available enzyme stated that the protease enzyme is highly stable in the pH range 5 to 9 and its optimum activity is at pH 7. Due to this fact, the enzyme was strictly prepared in Milli Q water, without further pH adjustments. Therefore, for further analysis, 0.6 g of sample was treated with 0.06 g of protease, dissolved in 8 g of Milli Q water. The mixture was then incubated for 24 h at constant temperature (37 °C) and stirring (200 rpm).

4.1.1.2 Mass balance in selected plant samples

In order to determine possible losses of selenium due to volatilisation during the incubation processes, the insoluble part of sample was also analyzed for Se content, after every extraction procedure (to perform a mass balance calculations between the total Se in the sample and the amount of Se in supernatant and residue). Quantitative results were obtained for food supplements, potato tubers, leafy vegetables and Se(IV) buckwheat sprouts, while in the case of Se(VI) and SeMet buckwheat sprouts, only between 65-83% of total Se in the sample was found in soluble and insoluble part. The results obtained were the same, regardless of the extraction media used. This means that Se could be adsorbed to the tube walls or could be

present in volatile form.

Unfortunately, the mass balance is not often included in speciation studies. Nevertheless, Roberge et al. (2003) reported that the mass balance for Se-enriched broccoli was between 70-100%. They suspect that there were losses of Se due to volatilisation process. On the other hand, Montes-Bayon et al. (2006) studied different extraction methodologies (0.1 M HCl, 25 mM ammonium acetate buffer (pH 5.6) and protease in aqueous solution) for the extraction of non-protein Se-amino acids such as Se-methyl selenocysteine and Se-methionine from selenium-enriched plant tissues. In the case of protease, Se recoveries ranged from 103 to 127%, indicating that recoveries higher than 100% could be due to Se contamination of the protease used for extraction.

4.1.2 HPLC-ICP-MS method development

4.1.2.1 Optimization of chromatographic separation of Se species

Due to the ionic properties of selenoaminoacids and inorganic Se forms over a wide pH range, ion exchange chromatography is mainly the method of choice for their separation. Both anionic and cationic exchange columns can be applied. However, before a separation can be performed the distribution of species regarding the solution pH should be known (Table 7). Selenium (VI) acid is strong, while selenium (IV) acid is a weak acid. In aqueous Se solution can be present as one or twice charged anions. SeMet can be present in three and $SeCys_2$ in five forms, which are pH dependent. Therefore, the pH of mobile phase plays a crucial role in separation of Se species.

Table 7: pK_a values for Se species under investigation (Zheng and Kosmus, 2000; Chatterjee et al., 2003)

Se species	pK_a
H_2SeO_3	pK_1 2.46; pK_2 7.31
H_2SeO_4	pK_2 1.92
SeMet	pK_1 2.19; pK_2 9.05
$SeCys_2$	pK_1 1.68; pK_2 2.15; pK_3 8.07; pK_4 8.94
SeMeSeCys	data not found

4.1.2.1.1 Separation on anion exchange column

For the separation of Se species Hamilton PRP-X 100 anion exchange column (4.1 mm x 250 mm x 10 µm), which allows wide range (1-13) of mobile phase pH, was selected. Anion exchange is performed on three methyl ammonium groups, which are bonded to polymer holder (copolymer of styrene and divinyl benzene).

For anion exchange chromatography, a citrate buffer was selected as the mobile phase (Bird et al., 1997). Firstly, the concentration and pH of mobile phase were optimized, leaving its flow and injected volume constant, 0.5 mL/min and 50 µL, respectively.

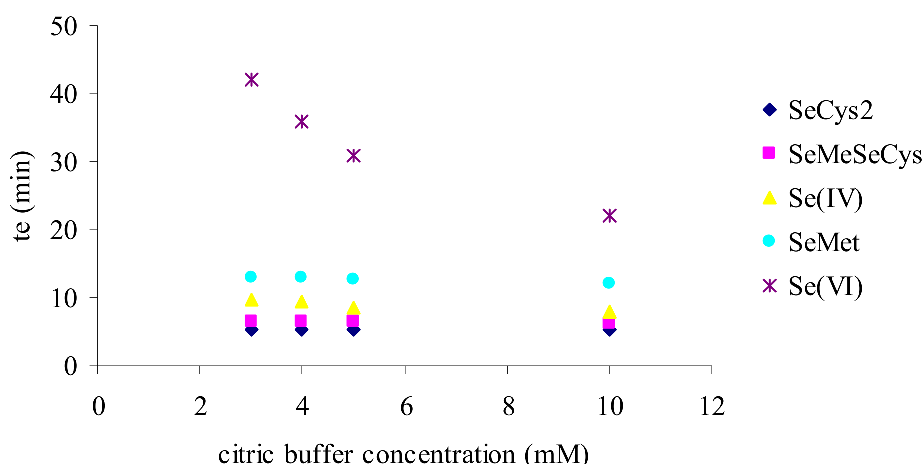


Figure 10: Citric buffer concentration dependent elution time (pH = 4.8, flow rate 0.5 mL/min)

It is seen (Figure 10) that the best separation of $SeCys_2$, SeMeSeCys, Se(IV) and SeMet was achieved using 3 mM citric buffer. By increasing the mobile phase concentration, Se species were eluted earlier, indicating overlaping of SeMeSeCys with $SeCys_2$ and Se(IV). Therefore, from this point of view, 3 mM citric buffer should be the optimal. However, by using this conditions, the retention time for Se(VI) was quite low, i.e. 42 min. That consequently increased the measurement time enormously. By increasing the molarity of the buffer to 10 mM, Se(VI) retention time decreased to 22 min, what shortened the time of analysis for 20 min. Therefore, to obtain a good baseline separation a gradient elution using 3 mM citric buffer (mobile phase A) and 10 mM citric buffer (mobile phase B) was introduced, finding the following conditions the most appropriate: 14 min gradient from 100% A to 50% A, 1 min gradient to 100% B, isocratic to 25 min, gradient for 2 min to 100% A, isocratic to 32 min.

To check possible mobile phase pH dependency of baseline separation of Se species, the pH value was optimized in the range between 3 and 5 (pH adjustment with citric acid) and pH value 4.8 was chosen as the optimal one (Figure 11).

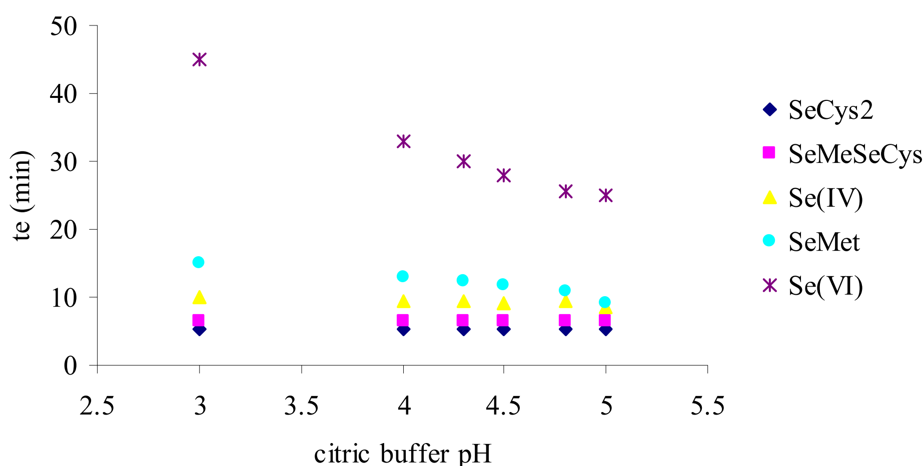


Figure 11: Elution time vs. citric buffer pH (gradient elution, flow rate 0.5 mL/min)

Additionally, we tried to increase the flow rate of the mobile phase, from 0.5 mL/min to 1.0 and 1.5 mL/min, to shorten the measuring time. But, due to the fact that $SeCys_2$, SeMeSeCys and Se(IV) elutes very close, they were overlaying. Therefore, 0.5 mL/min was chosen as an optimal flow rate. Additionally, the volume of injected standard was taken under consideration (20 µL, 50 µL, 100 µL). In accordance to the instrument response and signal repeatability, 50 µL was chosen as the optimal volume.

The effect of signal enhancement of elements with ionisation potentials in the range from 9 to 11 eV by carbon-containing compounds is a well-known phenomenon in ICP-MS (Kovačevič et al., 2005). Due to this reason, methanol (1, 2, and 3%) and acetone (1 and 2%) were added to the mobile phase in order to

obtain a better ionisation efficiency of Se (ionisation of Se in the plasma is low, approximately 30%) (Kovačevič et al., 2005). The best results were obtained using 2% MeOH in both mobile phases, A and B. That increased the efficiency for 50-100% (Figure 12), while on the other hand, the acetone addition decreased the instrument response for approximately 30%. Separation of Se species on anion exchange column, using optimal parameters, is presented in the following chromatogram (Figure 13).

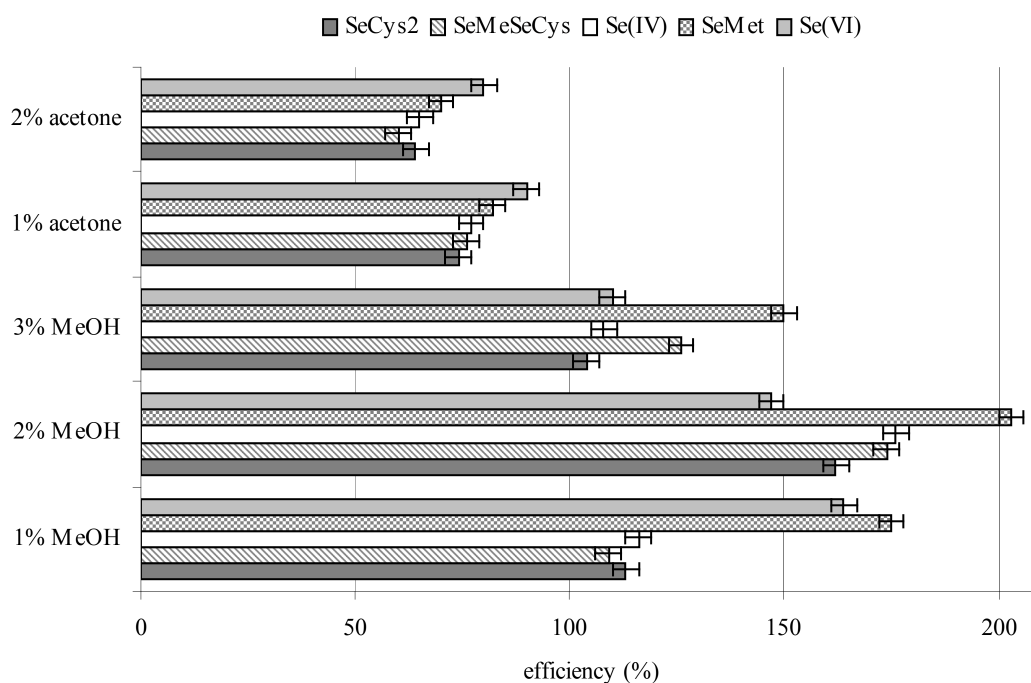


Figure 12: Organic solvent addition (MeOH, acetone) vs. responses for separate Se species relative to their responses without solvent addition (taken as 100%). Results presented are given as an average of two determinations with an absolute error.

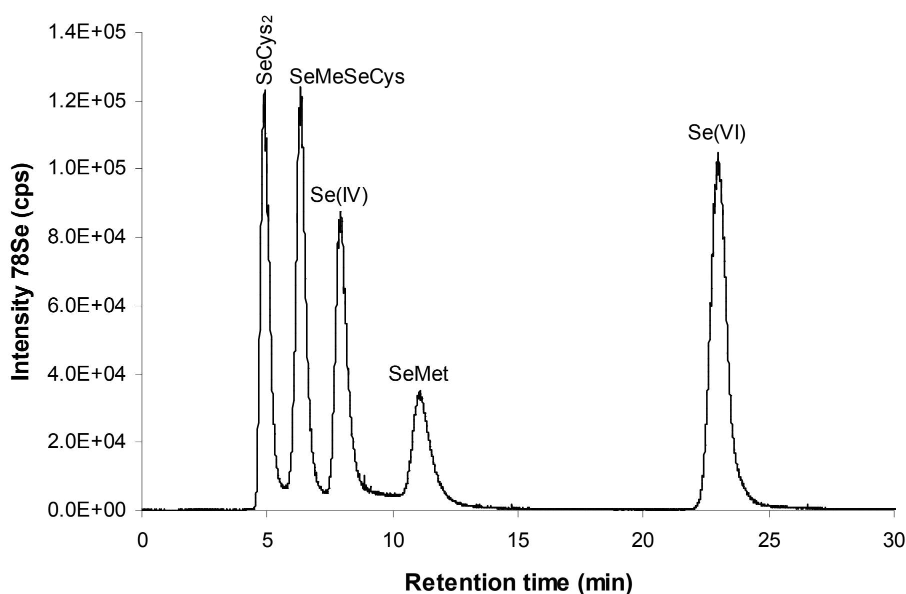


Figure 13: Chromatogram of Se species (100 ng/g) obtained with gradient elution (3 and 10 mM citric buffer, 2% MeOH, pH 4.8) on Hamilton PRP-X 100 (flow rate 0.5 mL/min, injected volume 50 µL).

Further, the analytical parameters of Se species (linearity, repeatability, detection limit, column recovery) determination with Hamilton PRP-X 100 – ICP-MS were checked. The linearity was checked in

the range between 5 and 200 ng Se per g solution and linear correlation was obtained for all the investigated standards in the proposed range, with the correlation coefficient above 0.99.

The repeatability within one day and one week was measured. The repeatability within one day was between 5 and 11% for Se species at concentration 100 ng/g (n=4) and was comparable with the one determined within one week, which was between 8 and 12% (n=10).

Further, based on the standard deviation (standards were prepared at concentration 5 ng/g, n=6), the detection and quantification limits were determined and are presented in the following table (Table 8). By using the same column in connection to ICP-MS Li et al. (1999) obtained lower detection limits (0.34 ng/mL (Se(IV)), 0.07 ng/mL (Se(VI)) and 0.18 ng/mL (SeMet)). On the other hand, by using the same technique, values obtained by Chassaigne et al. (2002) were higher in comparison to ours (7.0 ng/mL (Se(IV)), 5.5 ng/mL (Se(VI)), 6.5 ng/mL (SeMet), 5.3 ng/mL ($SeCys_2$)). Also Mazej et al. (2006) obtained higher detection limits (2.7 ng/g solution ($SeCys_2$), 3.0 ng/g (SeMeSeCys), 9.0 ng/g (SeMet), 2.3 ng/g (Se(IV)), 5.7 ng/g (Se(VI)) by using the same column in connection to UV-HG-AFS system.

Table 8: Detection and quantification limits for separate Se species (Hamilton PRP-X 100) in the measured solutions.

Se species	**LOD**		**LOQ**	
	ng/g solution	ng/g sample*	ng/g solution	ng/g sample*
$SeCys_2$	1.2	16	3.9	52
SeMeSeCys	0.9	12	2.9	39
Se(IV)	1.1	15	3.8	51
SeMet	0.9	10	2.9	39
Se(VI)	0.1	1	0.4	5

* calculation based on 0.6 g of sample in 8 mL solution

Additionally, the column recovery was tested. Peak areas obtained for separated Se species (100 ng of Se/g) after column separation were compared to peak areas obtained without a column. Ratios between peak areas were expressed as column recovery, taking peak areas obtained without a column as 100% intensity. Each species was analyzed in triplicate, and results obtained are shown in Table 9. Mazej et al. (2006) studied column recovery on an anion-exchange column (Hamilton PRP-X 100) coupled to a UV-HG-AFS system. They took peak areas for Se(IV) obtained after column separation as 100% intensity. All data were normalized to this value. Results reported for Se(IV), Se(VI), and SeMet were approximately 20% lower than the ones reported in this study. Differences between our results and results obtained by Mazej et al. (2006), using the same column, could be ascribed to a lower decomposition of Se species with UV-HG-AFS in comparisson with a plasma source. Regardless of the system used, the highest part of SeMeSeCys and SeMet remained on the column.

Table 9: Column recovery obtained by anion exchange chromatography in conjunction with ICP-MS.

Se species	**column recovery (%)**
$SeCys_2$	85.2 ± 0.6
SeMeSeCys	62.5 ± 0.9
Se(IV)	81.2 ± 0.4
SeMet	63.6 ± 0.3
Se(VI)	93.5 ± 1.2

Results are given as an average of three determinations with the appropriate standard deviation.

4.1.2.1.2 Separation on cation exchange column

As $SeCys_2$ was eluted in the void volume of the anion exchange column, separation on the cation exchange column was introduced. Further, this complementary technique served for additional conformation of Se species present in the samples. Agilent Zorbax 300-SCX (4.6 mm x 250 mm x 5 µm), which allows pH values of mobile phase in range 2-6.5, was selected. It is a polar bonded-phase column. Packing consists of an aromatic sulfonic acid moiety covalently bonded to a porous silica microsheres through Si-O-Si bonds.

The parameters affecting Se species separation were optimized in the same way as for anion exchange chromatography. For the cation exchange chromatography a pyridine solution was selected as the mobile phase (Cabanero et al., 2005; Mazej et al., 2006). Firstly, the concentration and pH of mobile phase were optimized, leaving its flow and injected volume constant, 0.5 mL/min and 50 µL, respectively. Optimization of the concentration of pyridine solution was made in the range between 2 and 7 mM (Figure 14). It is seen that the best separation of $SeCys_2$, SeMeSeCys and SeMet was achieved by using 3 mM pyridine solution.

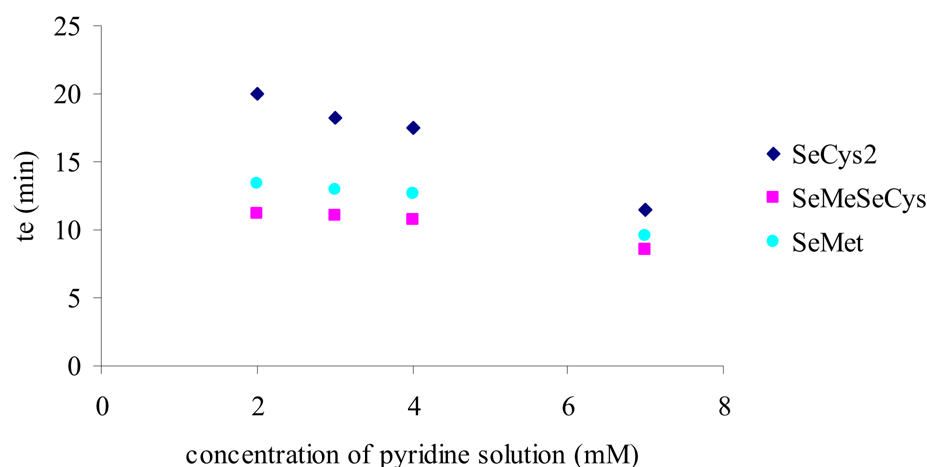


Figure 14: Elution time vs. concentration of pyridine solution (pH = 2.1, flow rate 0.5 mL/min)

Further, to check for the possible pH dependency on baseline separation of Se species, its value was optimized (pH 2.1 and pH 2.4, pH adjusted with hydrochloric acid). pH value 2.1 was chosen as being the optimal (Figure 15).

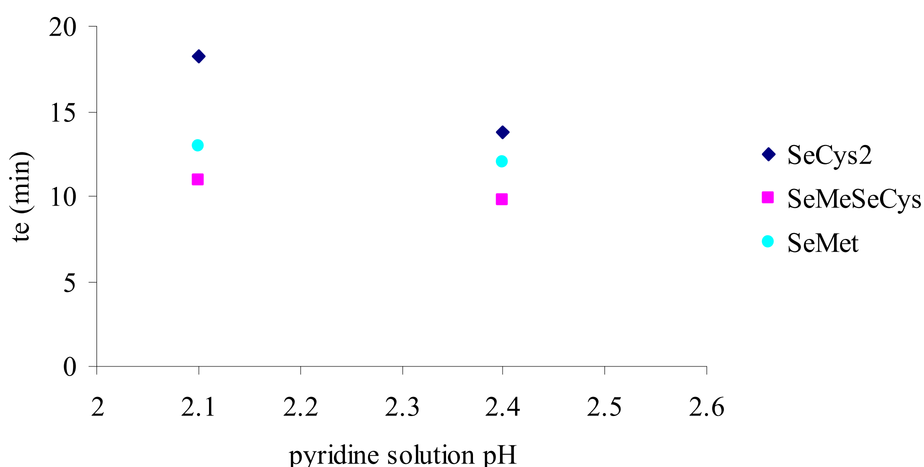


Figure 15: Elution time vs. pyridine solution pH (gradient elution, flow rate 0.5 mL/min)

Additionally, the effect of organic solvent was taken under investigation. 1, 2 and 3% MeOH was used

to obtain better ionisation efficiency, and as well as for anion exchange chromatography, 2% MeOH solution was found to be the optimal (Figure 16). Choosing 3 mM pyridine in 2% MeOH solution at pH 2.1 (flow rate 0.5 mL/min, injected volume 50 µL) we obtained the optimal separation of Se species as presented in the Figure 17.

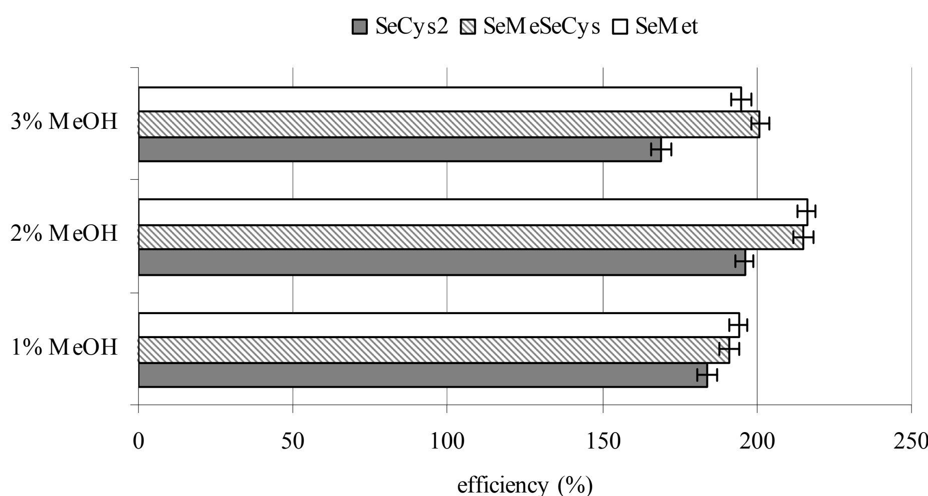


Figure 16: Organic solvent addition (MeOH) vs. response for separate Se species relative to their responses without solvent addition (taken as 100%). Results are given as an average of two determinations with an absolute error.

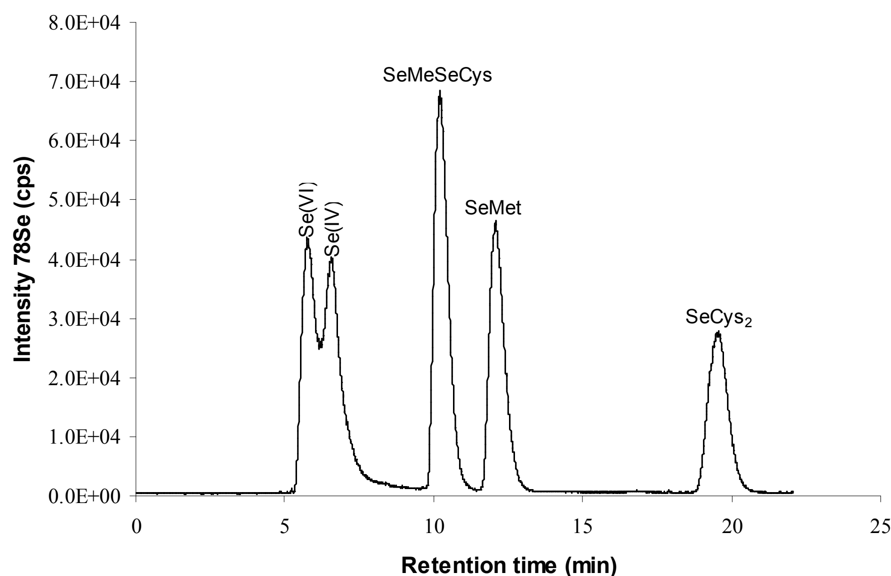


Figure 17: Chromatogram of Se species (100 ng/g) obtained with isocratic elution (3 mM pyridine solution, 2% MeOH, pH 2.1) on Zorbax 300-SCX (flow rate 0.5 mL/min, injected volume 50 µL).

Further, the analytical parameters were considered. The linearity was checked in the range between 5 and 200 ng Se per g solution and linear correlation was obtained for all the investigated standards in the proposed range, with the correlation coefficient above 0.99.

The repeatability within one day and one week was measured. The repeatability within one day was between 6 and 10% for Se species at concentration 100 ng/g (n=4) and was comparable with the one determined within one week, which was between 8 and 14% (n=10).

Based on the standard deviation (standards were prepared at concentration 5 ng/g, n=6), the detection and quantification limits were determined and are presented in the Table 10. In comparison to anion exchange column, lower detection limit was obtained for $SeCys_2$, while the values for SeMeSeCys and SeMet were comparable. Due to the fact, that the decomposition of Se species with UV-HG-AFS system is

not as good as with ICP-MS, the values obtained by Mazej et al. (2006), using Hamilton PRP-X 200 – UV-HG-AFS, were higher (4.3 ng/g solution ($SeCys_2$), 6 ng/g (SeMeSeCys), 41 ng/g (SeMet)) in comparison to ours.

Table 10: Detection limits (ng/g solution) for separate Se species (Zorbax 300-SCX) in the measured solutions.

Se species	LOD		LOQ	
	ng/g solution	ng/g sample*	ng/g solution	ng/g sample*
SeMeSeCys	0.7	10	2.5	33
SeMet	0.9	12	3.4	45
$SeCys_2$	0.2	3	0.7	9

* calculation based on 0.6 g of sample in 8 mL solution

Additionally, the column recovery was tested in the same way as on anion exchange system (Table 11). The values obtained for this system were better for SeMeSeCys, $SeCys_2$, SeMet, Se(VI) and comparable for Se(IV).

Table 11: Column recovery obtained by cation exchange chromatography in conjunction with ICP-MS

Se species	column recovery (%)
Se(VI)	86.6 ± 1.1
Se(IV)	80.9 ± 1.9
SeMeSeCys	95.3 ± 0.7
SeMet	102.5 ± 1.3
$SeCys_2$	91.6 ± 1.0

Results are given as an average of three determinations with the appropriate standard deviation.

4.1.2.2 Optimization of detection and quantification

Selenium is considered to be a difficult trace element to analyze by ICP-MS due to interferences from molecular ions and the relatively high ionisation potential (9.75 eV) causing low ionisation efficiency (approximately 30%) in the plasma. The most (^{80}Se, 49.6%) and second most (^{78}Se, 23.8%) abundant isotopes are markedly affected by polyatomic interferences from the argon plasma source, i.e., $^{40}Ar^{40}Ar^{+}$ and $^{40}Ar^{38}Ar^{+}$, respectively. Other isotopes, including ^{74}Se (0.89%), ^{76}Se (9.37%), ^{77}Se (7.63%) and ^{82}Se (8.73%) are also affected by the polyatomic interferences originating from the Ar plasma source and biomatrices, such as $^{38}Ar^{36}Ar^{+}$, $^{40}Ar^{36}Ar^{+}$, $^{38}Ar^{21}H^{+}$, $^{40}Ar^{37}Cl^{+}$, $^{40}Ar^{42}Ca^{+}$, $^{81}Br^{1}H^{+}$ and ^{82}Kr (an argon contaminant) (Pinho et al., 2005; Ogra et al., 2005). ICP-MS equipped with a reaction/collision cell has been successfully used for dissociating polyatomic interferences. Hydrogen is one of the most effective and frequently used reaction/collision gases (Ogra et al., 2005).

Therefore, to obtain the optimal operating conditions, all Se isotopes, ^{74}Se, ^{76}Se, ^{77}Se, ^{78}Se, ^{80}Se and ^{82}Se, using different integration time (0.1 s, 0.2 s, 0.3 s, 0.5 s) were monitored. Hydrogen and combination of hydrogen and helium were used as reaction/collision gas for eliminating Ar polyatomic interferences. Its flow was optimized in the range between 2-6 mL/min (H_2) and 4 mL/min (H_2) with 1.5 mL/min (He), respectively, finding hydrogen in the range between 4.0 and 4.5 mL/min the optimal signal-to-noise ratio. Since the ion intensity at *m/z* 78 (integration time 0.3 s) presented a better signal-to-noise ratio than the other Se isotopes, it was used for identification and quantification. Further, the ICP-MS measurement conditions were optimized daily using a standard solution containing 1ppb Li, Mg, Y, Ce, Co and Tl. Before HPLC coupling, the ICP-MS signal was monitored at 7, 89 and 205 *m/z* ratios, and the ion intensity was optimised. Oxide and doubly charged ions were minimised by measuring the mass ratios $^{140}Ce^{16}O/^{140}Ce$ and $^{140}Ce^{2+}/^{140}Ce^{+}$, respectively. Resolution and mass axis were optimised by monitoring m/z ^{7}Li, ^{89}Y and ^{205}Tl. Optimised conditions were accepted as appropriate for ^{78}Se monitoring.

All results were calculated using the signal for ^{78}Se (also all of the following chromatograms show the

signal for *m/z* 78). Quantification was performed by comparison with the corresponding standard compounds, where possible, in the peak area mode. Selenium compounds, for which no corresponding standard compound was available, were quantified with the nearest Se compound eluted. According to our experiences, the responses of the various available Se standards are comparable (the differences are lower than 10%). However, it cannot be completely excluded that the response for an unknown Se compound is different from that of an available standard. The concentrations for unknown Se compounds are, therefore, estimations.

4.1.3 Optimal HPLC-ICP-MS parameters for Se species determination in plants

In the following table (Table 12), optimal extraction parameters, as well as the optimal conditions of Se species separation by ion-exchange chromatography and their detection by ICP-MS are presented.

Table 12: Optimal conditions for Se species determination with HPLC-ICP-MS.

Parameter	Value
Enzymatic extraction	
protease	0.6 g of sample + 60 mg protease (check the enzyme purity) in 8 g of water, incubation 24 h at 37 °C, centrifugation 1 h at 11,000 rps, filtration of soluble part (0.45 and 0.22 µm filter), storage at -20 °C before the analysis (analysis performed within 30 days; always check the extract stability)
HPLC	
instrument: Agilent 1100	
Anion exchange chromatography:	
Hamilton PRP-X 100 column	4.1 mm x 250 mm x 10 µm
mobile phase A	3 mM citrate buffer in 2% MeOH (pH 4.8)
mobile phase B	10 mM citrate buffer in 2% MeOH (pH 4.8)
gradient	14 min gradient from 100% A to 50% A; 1 min gradient to 100% B, isocratic to 25 min; gradient for 2 min to 100% A; isocratic to 32 min
flow rate (mL/min)	0.5
injected volume (µL)	50
Cation exchange chromatography:	
Zorbax 300-SCX column	4.6 mm x 250 mm x 5 µm
mobile phase	3 mM pyridine solution in 2% MeOH (pH 2.1)
flow rate (mL/min)	0.5
injected volume (µL)	50
ICP-MS	
instrument: Agilent 7500ce	
nebulizer: Micro Mist	
plasma:	
RF power (W)	1500
outer gas flow rate (L/min)	15.0
octopole reaction cell:	
H_2 gas flow rate (mL/min)	4.0 – 4.5
measuring parameters:	
m/z monitored	^{78}Se
integration time (s)	0.3

4.1.4 Verification of the method developed

Since there are no certified reference materials available for selenium species contents in materials of plant origin, the accuracy of selenium species determination using the developed method (HPLC-ICP-MS) was checked by analyzing the standard reference material Durum Wheat Flour, NIST RM 8436, that is certified for the total Se content. Results for SeMet in this material were compared with those obtained by Wolf and Goldschmidt (2004) using GC-MS, Smrkolj et al. (2006), Mazej et al. (2006) and Vogrinčič et al. (2009), all using HPLC-UV-HG-AFS. Total Se concentrations obtained were comparable with certified values (Table 13).

Moreover, in most dietary supplements the predominant chemical form of Se is SeMet, either as synthetic L-SeMet or yeast-based SeMet. There is lack in availability of certified reference materials for Se species contents with which to evaluate measurement performance for SeMet. In response to this need, the Institute for National measurement Standards (INMS) of the National Research Council Canada (NRC) recently completed certification of a new selenized yeast material (Selenium Enriched Yeast, SELM-1), certified for SeMet, Met and total Se amount content (Mester et al., 2006). There are currently no other certified reference materials available for quality control of measurements characterizing these supplements. The challenge in determining SeMet is that it is basically an amino acid measurement. Several extraction procedures were performed, enzymatic extraction as well as hydrolysis with methanesulfonic acid, leading to comparable results (Mester et al., 2006). Nevertheless, variability between results obtained for SeMet in seven laboratories (from 1.970 to 3.587 mg/kg) is not negligible what proves how difficult it is to obtain accurate results in Se speciation analysis. Therefore, the accuracy of Se and its species determination using the method developed was checked by analysing the certified reference material SELM-1(Table 13). In all cases, a very good agreement for SeMet and total Se was found between our results and the reported or certified values (Table 13).

Table 13: Total Se and SeMet concentrations in RM NIST 8436, Durum Wheat Flour, and CRM, SELM-1.

	total Se content (µg Se/g)		SeMet* (µg Se/g)		
	Found value	Certified value	Found value	Certified value	Literature data
	HG-AFS		HPLC-ICP-MS		
Durum Wheat Flour, NIST RM 8436	1.10 ± 0.11	1.23 ± 0.09	0.60 ± 0.07		0.59 ± 0.04 (a) 0.57 ± 0.04 (b) 0.61 ± 0.07 (c) 0.69 ± 0.04 (d)
Selenium Enriched Yeast, SELM-1	2089 ± 142	2059 ± 64	1441 ± 100	1380* 3431 ± 157**	810 ± 30 (c)

The values are reported as an average of three determinations with the standard deviation.
* Se as SeMet (calculated value)
** SeMet
a Wolf and Goldschmidt, 2004
b Smrkolj et al., 2006
c Mazej et al., 2006
d Vogrinčič et al., 2009

Further, the accuracy of selenium species determination using HPLC-UV-HG-AFS was checked by the method developed (HPLC-ICP-MS), by measuring the Se species content on the real sample basis. For this purpose potato supernatants obtained after extraction with a) protease and b) a combination of protease and β-amylase from sweet potato (Table 14) and buckwheat sprout extracts obtained after extraction with a) 0.3 M HCl and b) protease (Table 15) were analysed. Differences between results obtained with both techniques were within 10%.

Table 14: Selenate and SeMet content in well watered (W+) and drought (W-) exposed potato tuber extracts obtained with HPLC-ICP-MS and HPLC-UV-HG-AFS.

	HPLC-ICP-MS		HPLC-UV-HG-AFS	
ng Se/g DM	SeMet*	Se(VI)	SeMet*	Se(VI)
protease				
W-	124 ± 5	80 ± 4	134 ± 17	82 ± 16
W+	313 ± 15	288 ± 13	264 ± 40	335 ± 28
protease + beta-amylase				
W-	100 ± 5	103 ± 5	104 ± 27	131 ± 34
W+	284 ± 15	278 ± 14	341 ± 48	351 ± 69

The values are reported as an average of three determinations with the standard deviation.
* Se as SeMet

Table 15: Selenite, selenate and SeMet content in Se(VI), Se(IV) and SeMet buckwheat sprout extracts obtained with HPLC-ICP-MS and HPLC-UV-HG-AFS.

	HPLC-ICP-MS			HPLC-UV-HG-AFS		
ng Se/g DM	Se(IV)	Se(VI)	SeMet*	Se(IV)	Se(VI)	SeMet*
0.3 M HCl						
Se(VI), 20 mg/L	24	4299	< LOD	76	4500	< LOD
Se(IV), 20 mg/L	tr	41	< LOD	< LOD	< LOD	< LOD
protease						
Se(IV), 20 mg/L	tr	47	338	tr	tr	371
SeMet, 10 mg/L	tr	13	117	< LOD	< LOD	tr

The values are reported as an average of two determinations with the absolute error below 10%.
* Se as SeMet

4.1.5 Se species in commercially available enzymes

Since the total content of Se in enzymes was relevant (see chapter 4.1.1), a speciation analysis of the supernatants was performed. The results of the Se speciation analysis are shown in Table 16. The water-soluble fraction of enzymes contained detectable amounts of selenite, selenate, SeMet and some unknown compounds (Table 16, Figure 18). Se compounds were confirmed by the standard addition method (Figure 19).

Table 16: Se species in soluble fraction of commercially available enzymes.

ENZYME	Se species (ng Se/g enzyme)			
	Se(IV)	SeMet*	Se(VI)	u.c.
protease from *Streptomyces griseus* (1)	243 ± 10	66 ± 5	52 ± 5	-
protease from *Streptomyces griseus* (2)	79 ± 10	100 ± 5	47 ± 1	-
protease from subtilisin Carlsberg (1)	-	-	-	-
protease from subtilisin Carlsberg (2)	-	-	-	15 ± 1 (4.8 min); 324 ± 12 (15.1 min)
lipase from porcine pancreas (1)	21 ± 5	-	-	571 ± 30 (4.8 min)
lipase from porcine pancreas (2)	-	tr	-	25 ± 3 (4.8 min); tr (17.3 min)
lipase from wheat germ (1)	87 ± 9	389 ± 20	19 ± 1	-
cellulase from *Aspergillus niger* (1)	-	-	-	-
cellulase from *Aspergillus niger* (2)	-	-	-	-
β-amylase from barley (1)	30 ± 3	442 ± 4	19 ± 1	51 ± 2 (4.8 min)
β-amylase from barley (2)	65 ± 5	625 ± 4	34 ± 1	-
β-amylase from sweet potato (1)	-	-	-	-
α-amylase from *Aspergillus oryzae* (1)	-	144 ± 8	-	35 ± 3 (4.8 min)
α-amylase from *Aspergillus oryzae* (2)	-	261 ± 18	-	9 ± 1 (4.8 min)
α-amylase from barley malt (1)	-	tr	-	-
α-amylase from porcine pancreas (1)	-	tr	-	60 ± 2 (4.8 min)
α-amylase from porcine pancreas (2)	-	tr	-	65 ± 6 (4.8 min)
amyloglucosidase from *Aspergillus niger* (1)	-	-	-	-

Results for Se species content are given as an average with absolute error of two determinations.
u.c. = unknown compound (unknown Se compound with retention time 4.8 min is calculated as $SeCys_2$, unknown Se compound with retention time 15.1 min is calculated as SeMet)
- = < LOD
* Se as SeMet

The Se species identified represent a small fraction of the total Se in the enzyme. For instance, only 17% of the total Se was identified for β-amylase from barley. On the other hand, for both lots of protease from *Streptomyces griseus*, about 100% of the total Se was identified as species. Upon comparing the results for Se compounds from different lots of the same enzyme, not all of them were found to be comparable, similar to the results obtained for the analysis for total Se content.

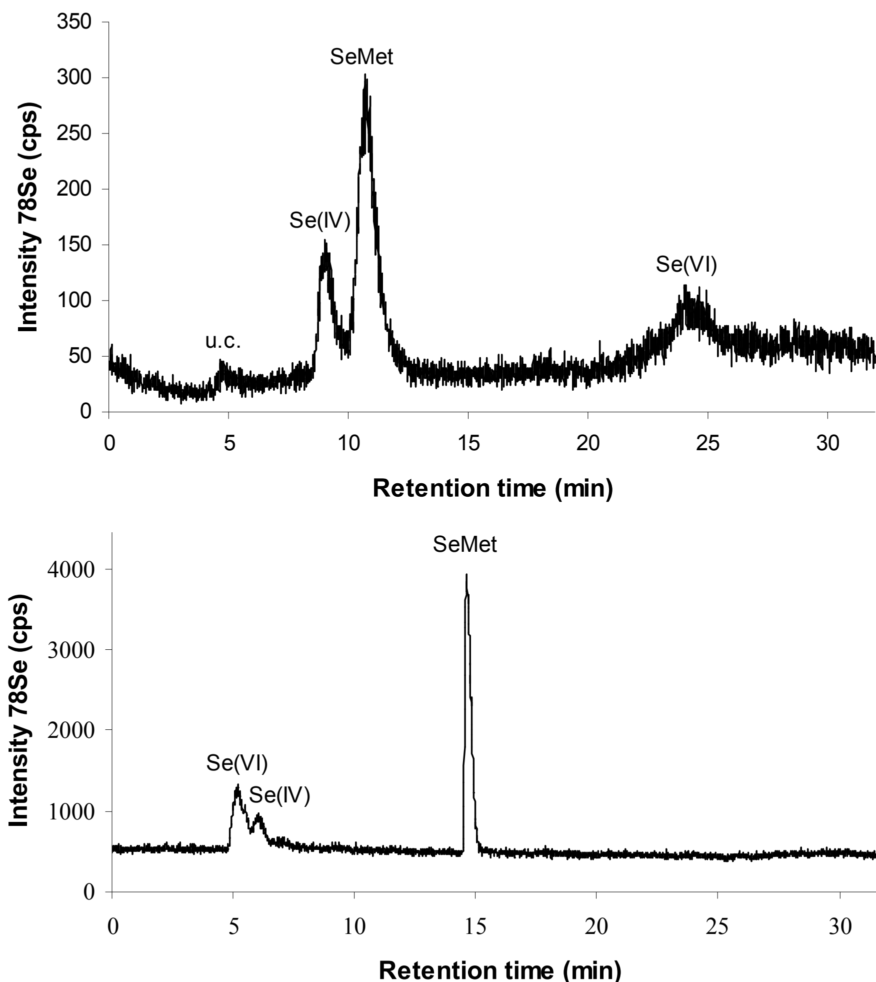


Figure 18: Se species present in commercially available enzyme (beta amylase from barley), obtained after separation on Hamilton PRP-X 100 (above) or Zorbax SCX-300 (below) and detection by ICP-MS.

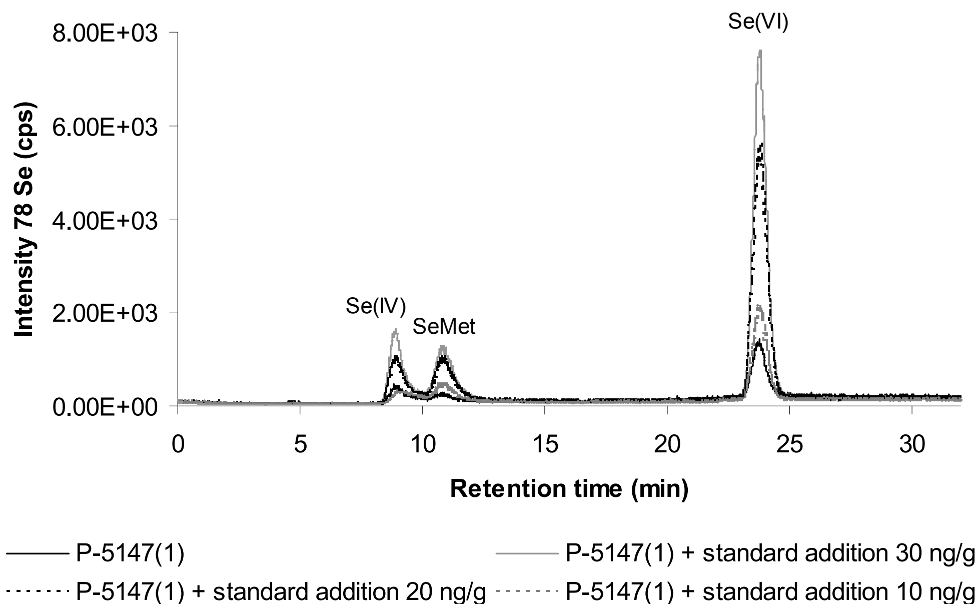


Figure 19: Standard addition method for enzyme protease from *Streptomyces griseus* obtained by anion exchange chromatography and ICP-MS detection.

Moreover, possible effects of the enzymes on each other were studied. While the non-specific enzyme protease is most commonly used in combination with different specific enzymes for enzymatic hydrolysis, interactions between protease from *Streptomyces griseus* and (a) amylase (from porcine pancreas or from barley), (b) cellulase from *Aspergillus nieger*, or (c) lipase from porcine pancreas were evaluated. No differences in the Se species present and their concentrations were observed. Evidently the enzymes do not influence each other when they are present in the same water solution.

Further, we tried to purify the enzyme that contained the highest Se content, β-amylase from barley. A 3,000 Da cut-off filter (Amicon, Concentrator, Centricon YM 3) was used for enzyme purification. 1.5 mL of enzyme supernatant was centrifuged at 4 °C at 11,000 rpm for 10 min. The residue was dissolved in 1.5 mL of water. Several clean-up steps were performed, and the filtrate and residue obtained were analysed for Se species content. In a one-step filtration, we managed to remove 73% of an unknown compound, 82% of the selenate, 86% of the SeMet and 93% of the selenite with respect to the contents of these species in β-amylase solution. We repeated the filtration with 1.5 mL water three times. After four filtrations, the residue on the filter was dissolved in 1.5 mL of water and injected into the HPLC-ICP-MS, and no Se species were found. For quantitative elimination of Se impurities, four filtrations were required. The Se species reported were present in the filtrate obtained. These results show that the Se species determined in the filtrate are present as impurities and are not bounded to the enzyme.

To make a point, most of the enzymes studied contained detectable amounts of selenium. In some cases, we found large variations between the levels in different lots of the same enzyme. The presence of selenium species in commercially available enzymes could be due to the preparation procedure used for the enzyme, since they could be present as degradation products. Further, no variations in the contents of Se and its species were observed when two enzymes were placed in the same solution. To achieve the quantitative elimination of enzyme impurities, several filtrations with a cut-off filter were required. Therefore, when determining selenium species present in sample supernatants at low levels, more attention should be devoted to enzyme purity in relation to selenium compounds when an enzyme is used for hydrolysis. If they are not corrected for Se species from the enzyme, the results obtained for such samples will be overestimated.

4.1.6 Stability of Se species

One of the main problems related to the extraction methodology is not only ensuring a satisfactory recovery of Se species, but obtaining a satisfactory recovery while preventing possible Se species inter-conversion. Accurate speciation is necessary for determining the biochemical role of Se because different forms of Se can have very different biological effect (Rayman, 2000; Ellis et al., 2003; Pedrero et al., 2008).

Extraction is probably the most crucial step in Se speciation analysis. The stability of Se species in the matrix considered is rarely reported in the literature. The matrix may affect the analyte, therefore we must consider the reactions that can take place between the Se species and the matrix components. During extraction, liberated matrix components can react with extracted Se compounds. Hence, the stability of Se species in water and enzymatic solutions in the presence of the most common phenolic compounds in plants (tannins, flavonoids) was investigated. Additionally, its stability was checked in the presence of several other antioxidants, like coenzyme Q10, beta-carotene and vitamin E, as being the most common ingredients in antioxidant food supplements.

Further, we wanted to be sure that no species transformation ocurs during the sample extract storage at minus 20 °C (after the incubation and before the analysis). Therefore, the extracts were analysed immediately after incubation and after 30 days of storage. No differences occurred in Se species present as well as its concentrations were comparable. Due to these results we tried to analyse the sample extracts as soon as possible, but within 30 days after the incubation was performed.

4.1.6.1 Stability of Se species in the presence of rutin and tannin

The aim was to investigate the effect of the most common phenolic substances in plant parts, namely tannin and the flavonoid rutin, on the concentration and/or transformation of several Se species (SeMet, $SeCys_2$, SeMeSeCys, Se(VI), Se(IV)) during sample preparation (24 h water and enzymatic incubation at 37 °C) and storage (4 days at 4 °C).

Since several recent studies have focused on buckwheat as a valuable food material, particularly with respect to its seeds, the ratio between rutin and tannin was taken as present in buckwheat seeds (1:100,

w:w). Each Se species (Se(IV), Se(VI), SeMet, $SeCys_2$, SeMeSeCys, 100 ng Se/g solution) in 8 g of water was mixed with A) rutin (0.2 mg/g solution), B) tannin (20 mg/g solution), C) rutin and tannin (same concentrations), D) rutin and protease (60 mg), E) tannin and protease (same amounts and ratio), F) rutin, tannin and protease (same amounts and ratio). Additionally, to study the possible influence of the ratio between the antioxidants on Se species stability, two additional ratios were taken: rutin and tannin 1:1 (G: water solution; J: enzymatic solution, w, w) and rutin and tannin 1:50 (H: water solution; I: enzymatic solution, w, w). Aqueous and enzymatic hydrolysis was performed, using a concentration of Se species of 100 ng Se/g solution. In all cases, the mixture was shaken for 24 h at 37 °C. After this extraction procedure, the extract was centrifuged at 11,000 rpm for 60 min at 4 °C. The supernatant was filtered through 0.45 and 0.22 µm Millex GV filters and subjected to Se speciation analysis by HPLC-ICP-MS. Solutions were kept in PTFE tubes (Eppendorf AG, Hamburg, Germany). The stability of Se standards was compared with standard solutions prepared in water.

Signals for SeMet, $SeCys_2$, SeMeSeCys and Se(VI) were found to be stable in the presence of both antioxidants, regardless of whether they were mixed with water or enzymatic solution. However, some differences occurred on performing these experiments with inorganic Se, as Se(IV) (Table 17). Firstly, the flavonol rutin and tannin were added separately to a water solution containing of 100 ng/g Se(IV). A 7% decrease was obtained in the presence of rutin and a 16% decrease in Se(IV) response in the presence of tannin. When phenolics were added together (ratio 1:100, w:w) to the Se(IV) standard, a 60% decrease in Se(IV) response was observed after 24 h incubation at 37 °C. Moreover, 24 h after incubation was completed (samples were kept at 4 °C) only 10% of Se(IV) remained. Additionally, when we reduced the amount of tannin, this effect no longer occurred (Table 17).

Table 17: Percentage of Se(IV) remaining after 24 h at 37 °C; solutions were analysed immediately (0 h) or kept at 4 °C for 24 h and 4 days.

	% Se(IV) response remain		
	0 h	**24 h**	**4 days**
water solution			
Se(IV) and rutin (A)	93.0 ± 2.0	87.2 ± 0.1	95.2 ± 0.1
Se(IV) and tannin (B)	83.6 ± 2.6	84.4 ± 1.0	17.0 ± 2.0
Se(IV), rutin and tannin (1:100) (C)	40.4 ± 3.9	10.4 ± 1.6	10.5 ± 0.4
Se(IV), rutin and tannin (1:50) (H)	95.0 ± 0.1	n.d.	98.0 ± 1.0
Se(IV), rutin and tannin (1:1) (G)	97.2 ± 1.0	n.d.	93.3 ± 0.1
enzymatic solution			
Se(IV) and rutin (D)	99.0 ± 1.0	92.2 ± 0.1	92.9 ± 0.1
Se(IV) and tannin (E)	86.3 ± 0.9	79.3 ± 4.3	74.1 ± 0.9
Se(IV), rutin and tannin (1:100) (F)	80.2 ± 2.8	74.4 ± 2.1	71.5 ± 1.5
Se(IV), rutin and tannin (1:50) (I)	93.9 ± 1.5	n.d.	95.8 ± 0.3
Se(IV), rutin and tannin (1:1) (J)	95.3 ± 0.7	n.d.	97.0 ± 1.0

Results reported were obtained on anion exchange column and are given as the average of two determinations along with the absolute error.
n.d. – not determined

Since the enzyme plays an important role in speciation analysis, further experiments were performed in its presence. The non-specific enzyme protease, which induces proteolysis, was used. Further, tannins possess unwanted properties since they inhibit enzymes and in larger quantities have a negative influence on amino acid digestibility (Luthar, 1992). They may be located in all plant parts, but very rarely in monomer form since tannin forms complexes with specific proteins and sugars. After the incubation a lower decrease in Se(IV) response was observed in solutions where rutin was added than where tannin or a combination of both phenolics was added (ratio 1:100, w:w). In enzymatic extracts containing Se(IV), rutin and tannin (1:100, w:w), a 20–30% decrease in Se(IV) was observed after 24 h incubation at 37 °C and then stayed stable for 4 days at 4 °C. A possible explanation is that when the enzyme was added to the Se(IV) solution tested, tannin and protease could react and there would not be enough tannin left to react with Se(IV). This could explain the fact that when tannin was added in lower amounts no changes in Se(IV) signal were observed (Table 17). In the presence of rutin, the response for Se(IV) stayed stable, regardless

of whether performing a water or enzymatic extraction. This shows that rutin by itself does not affect Se(IV) stability but only in the presence of tannin. It can function as a catalyst, accelerating the reaction.

In addition, no new Se species were observed in the chromatograms obtained after anion and cation exchange chromatography, regardless of whether analysing water or enzymatic extracts. It is most likely that Se(IV) was reduced to Se(0) or that an insoluble complex was formed, which could not be detected by the separation and detection system used. To confirm this, we increased the content of Se(IV) in the water solution from 100 ng Se/g to 0.05 mg Se/g and 0.5 mg Se/g solution, the ratio between rutin and tannin remaining 1:100, w:w, but we increased its concentration for two orders of magnitude. No precipitation was observed in a one week period when using 0.05 mg Se/g solution. On the contrary, when the concentration of Se(IV) was as high as 0.5 mg Se/g solution, a red and grey deposit was observed after leaving the mixture at room temperature for one day. The precipitate obtained was only soluble in 100% fuming HNO_3 (Merck, p.a.) indicating formation of Se(0) or an insoluble complex between the antioxidants and Se(IV).

4.1.6.2 Stability of Se species in the presence of coenzyme Q10, beta-carotene and vitamin E

The easy access to supplements, also available in drugstores, makes their use uncontrolled and potentially dangerous. Coenzyme Q10 and selenium are the most topical antioxidants, therefore we decided to check whether the Se species are stable in the presence of coenzyme Q10, beta-carotene and vitamin E, since their benefits to human health are strongly correlated with the chemical form consumed.

One of the possible reactions affecting the stability of supernatants during extraction and storage is reduction of Se species, mainly inorganic Se. According to the literature, selenite is easily reduced to Se(0) by ascorbic acid (Lipinski, 2005; Gosetti et al., 2007). In the present study, the possibility of reactions taking place among the different components present in the antioxidant food supplement C (coenzyme Q10, beta-carotene and vitamin E) and inorganic Se was studied.

An enzymatic solution of Se(IV) and Se(VI) with a mass fraction of Se around 100 ng/g was used in a test experiment with a) coenzyme Q10, b) beta-carotene, c) vitamin E, d) coenzyme Q10, beta-carotene and vitamin E, in the ratio present in supplement C (coenzyme Q10 : Se : vitamin E : beta-carotene = 10 : 10 : 5 : 1, w/w). After mixing inorganic Se with the other ingredients and hydrolysis, Se speciation analysis showed that about 3% of Se(IV) was oxidised to Se(VI), regardless of the ingredient combination used. Results obtained for Se(VI) showed no transformation.

Further, thermal stability of inorganic Se in the presence of coenzyme Q10 was investigated. Se(IV) or Se(VI) and coenzyme Q10 were dissolved in a solution of ethanol and water (20 : 1, v/v) and heated at 60 °C and/or 80 °C for 10 min. The solutions were evaporated to dryness at room temperature. Hydrolysis was performed without and with the enzyme protease and the supernatant analysed for Se species present. The results showed that about 3% of Se(IV) was oxidised to Se(VI) (Figure 20), regardless of the temperature used.

Moreover, to see if there was any change in Se composition we left the mixtures overnight at room temperature and repeated the analysis. No changes were observed within 24 h. Therefore, the main ingredients in the products do not affect the content and transformation of inorganic Se species.

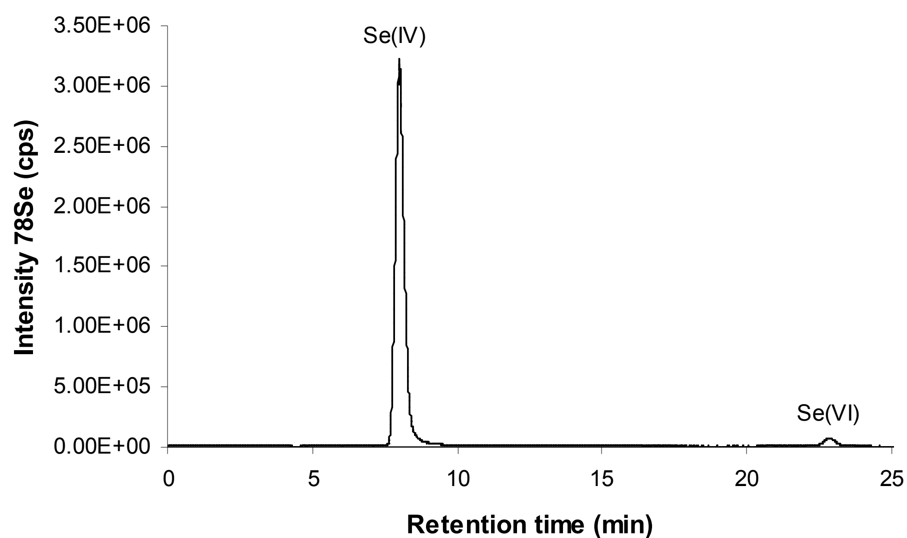


Figure 20: Chromatogram of a mixture of Se(IV) and coenzyme Q10, dissolved in ethanol, heated for 10 min at 80 °C, evaporated to dryness and then incubated. Separation of Se species was made on an anion exchange column (Hamilton PRP-X 100) with ICP-MS as the detection system.

4.2 Determination of volatile Se species

Among the most abundant organic species in environmental and biological samples, methylated species such as DMSe or DMDSe, are frequently found. Due to this reason, the importance of mass balance calculations should be emphasised when performing speciation analysis. This is the key factor in determining the species volatility, during the sample preparation. We have to be aware that the possibility of Se species volatilisation, during the extraction process, is high. Therefore, a method for the determination of volatile organo seleno compounds (DMSe and DMDSe) has been developed using a solid phase micro extraction with gas chromatography-mass spectrometry (SPME-GC-MS).

Se(VI) buckwheat sprouts (soaked in 10 mg Se(VI)/L) were taken as a sample material, due to the fact that the mass balance calculations in this material were far from being quantitative (65-83%), regardless of the extraction parameters used as well as of the Se content present (see chapter 4.1.1.2).

4.2.1 Extraction of Se species from Se(VI) buckwheat sprouts

Firstly, we decided to decrease the time of extraction, since the sample preparation used for determination of water-soluble Se species included 24 h incubation. Ultrasound treatment was used to assure that. Results obtained after different time (30 min, 1 h, 1.5 h and 2 h) of ultrasound treatment were compared with the ones obtained after 2 and 24 h incubation. Milli Q water was taken as an extraction solvent due to the fact that the mass balance was not extraction dependent. The temperature was set to be constant, 37 °C. Additionally, a combination of incubation and ultrasound were considered, taking 2 h incubation with additional 30 min of ultrasound treatment or 24 h incubation with additional 30 min of ultrasound treatment. Se(VI) buckwheat sprouts (10 mg/L) were taken as a sample material and results obtained are presented below (Figure 21).

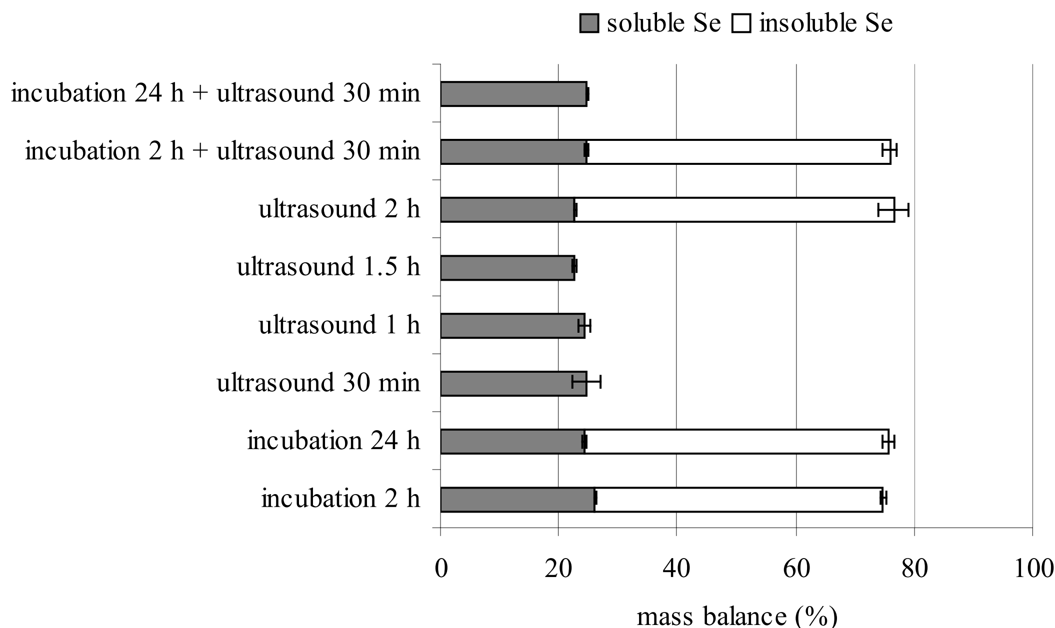


Figure 21: Comparison of extraction efficiency (mass balance regarding total Se) obtained using different way of extraction.

The values obtained, using different extraction parameters, were found to be comparable. The extraction efficiency was between 22.5%, after 1.5 h and 2 h of ultrasound treatment and 26.0% after 2 h of incubation. Additionally, the mass balance calculations were found to be constant, approximately 75%, regardless of the extraction procedure used. However, the results obtained were within 4%, indicating that the differences between the treatments were negligable. Therefore, 30 min of ultrasound treatment was taken as the most appropriate for further treatment.

However, to be sure that there is no difference in comparison with the incubation used for the determination of soluble Se species, speciation analysis, using HPLC-ICP-MS, was performed. Supernatants obtained after 24 h incubation and after 30 min of ultrasound treatment were analysed and compared (Figure 22). No differences in Se species present and its concentrations were found.

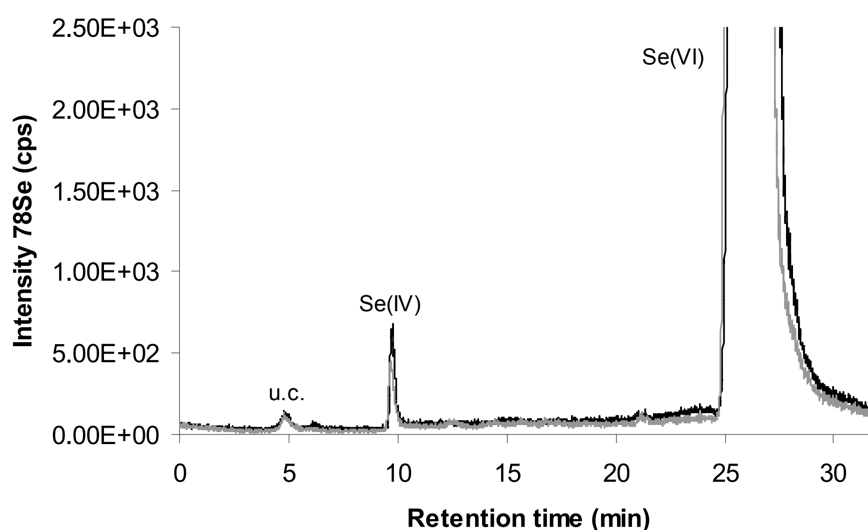


Figure 22: Chromatograms of buckwheat Se(VI) sprouts water extracts obtained with 24 h incubation (black colour) and with 30 min of ultrasound treatment (grey colour). Hamilton PRP-X 100 – ICP-MS was used as a separation and detection system of soluble Se species.

4.2.2 SPME-GC-MS method development

Volatilisation of DMSe and DMDSe is one of the most important processes for removing Se from Se-contaminated environments. Therefore, a possibility of Se species volatilisation during the plant growth and extraction process, exist. A very important property of volatile Se compounds is vapour pressure. DMDSe has a lower vapour pressure (0.38 kPa) than DMSe (32 kPa). Therefore, DMDSe would have a relatively longer retention time than DMSe where they are formed (Zhang and Frankenberger, 2002).

4.2.2.1 Optimization of chromatographic separation and detection

The temperature program, injection temperature and the gas flow rate were optimised in order to obtain a good baseline separation. Due to the fact that DMSe is very volatile, it eluted within the first 4 min, with the solvent. Therefore, we were not able to optimise these parameters directly (standard dissolved in MeOH and injected directly). Pretreatment with SPME (headspace sampling) was needed for the optimization of separation and detection. In the case of SPME, there was no solvent present and consequently, there was no need to use solvent delay function. Therefore, we were able to measure the whole chromatogram and optimise the separation of both Se standards.

The SPME parameters, most often reported in the literature (Haberhauer-Troyer et al., 1999; Dietz et al., 2004; Campilo et al., 2005) were taken. PDMS, as being the most widely used fiber coating, was taken. The extraction was performed as optimised (30 min of ultrasound treatment at 37 °C). Further, the fiber was exposed to the volatile phase for 20 min (ultrasound treatment at 37 °C), to be sure that the exuilibrium between volatile part and fiber was achieved.

To begin with, parameters published by Meija et al. (2002) were taken. The temperature program was as follows: beginning at 40 °C for 4 min, than ramp 15 °C/min to 125 °C, afterwards 30 °C/min to 250 °C and hold the final temperature for 5 min. The injector temperature was set to be 240 °C, the transfer line temperature was 280 °C and the He flow rate was 1.0 mL/min. We obtained a good baseline separation ($t_{R(DMSe)}$=2.2 min, $t_{R(DMDSe)}$=9.7 min), but we were not satisfied with the DMSe peak shape (tailing).

Therefore, we tried to improve it by increasing the beginning temperature to 50 °C and by lowering the gas flow rate to 0.5 mL/min for the first minute and than increase it to the 1.5 mL/min. The other parameters were set to be constant. We obtained a better peak shape, and still a good baseline separation ($t_{R(DMSe)}$=2.0 min, $t_{R(DMDSe)}$=5.5 min).

Further, we increased the beginning temperature to 60 °C and leave the gas flow rate at 0.5 mL/min for 1.5 min and than increase it to the 1.5 mL/min. However, the results were comparable, and 50 °C was chosen as the optimal beginning temperature. The flow rate was set to be 0.5 mL/min for the first minute and a half and then we increased it to 1.5 mL/min.

Since, the measurement time was as long as 17.5 min and the last Se standard eluted around 6 min, we tried to decrease it. The following T program was introduced: 50 °C for 4 min, ramp 15 °C/min to 125 °C, hold for 2 min and ramp 30 °C/min to 250 °C (to remove all the components remained on the column), hold for 2 min, leaving the rest of the parameters as reported earlier. The measurement time at these conditions was 15.17 min. These parameters were chosen to be the optimal one ($t_{R(DMSe)}$=2.4 min, $t_{R(DMDSe)}$=5.7 min, Figure 23).

To increase the sensitivity, selected ion monitoring was introduced, taking *m/z* 80, 95 and 110 for DMSe (Figure 24) (the first 3 min) and *m/z* 80, 110 and 190 for DMDSe (Figure 25) (3-15 min).

Additionally, to check if there are some traces of the standards left on the fiber, we repeated the analysis, without taking the fiber out of the injector. In the first run, around 3% of the standards were still detected and in the second run its content was below the detection limit. Therefore, to remove the memory effect, the injector temperature was increased to 260 °C, after the measurement, for 5, 3 or 2 min. The time, as short as 2 min, was high enough to remove all the impurities from the fiber. Therefore, this was done after every measurement.

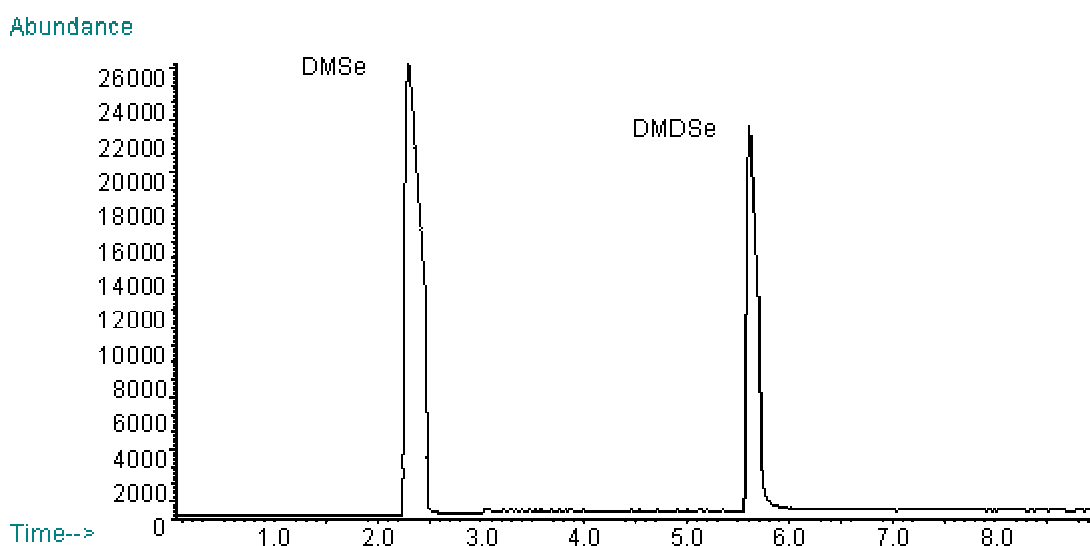


Figure 23: Chromatogram of volatile Se species, DMSe (5 μg/g solution) and DMDSe (0.5 μg/g solution), using optimal GC-MS operating conditions.

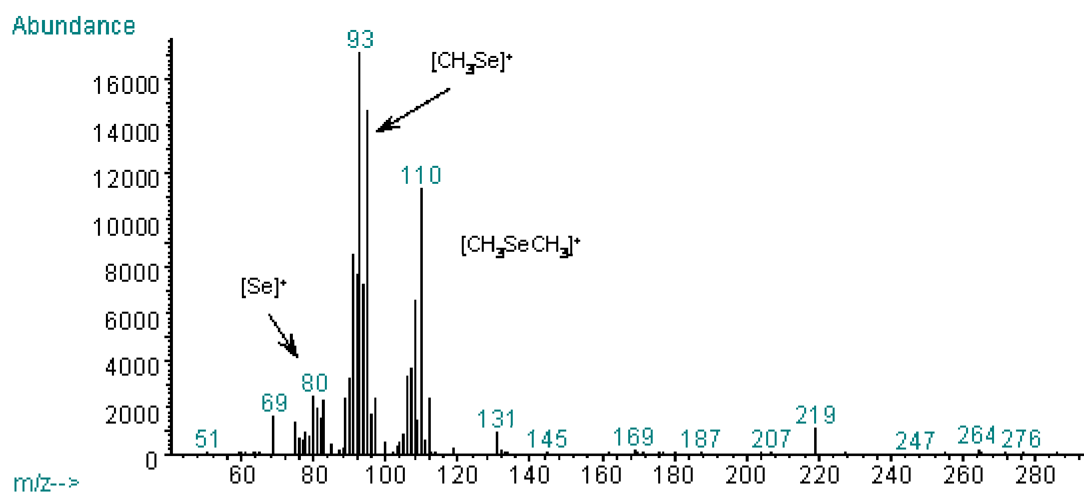


Figure 24: Mass spectra of DMSe.

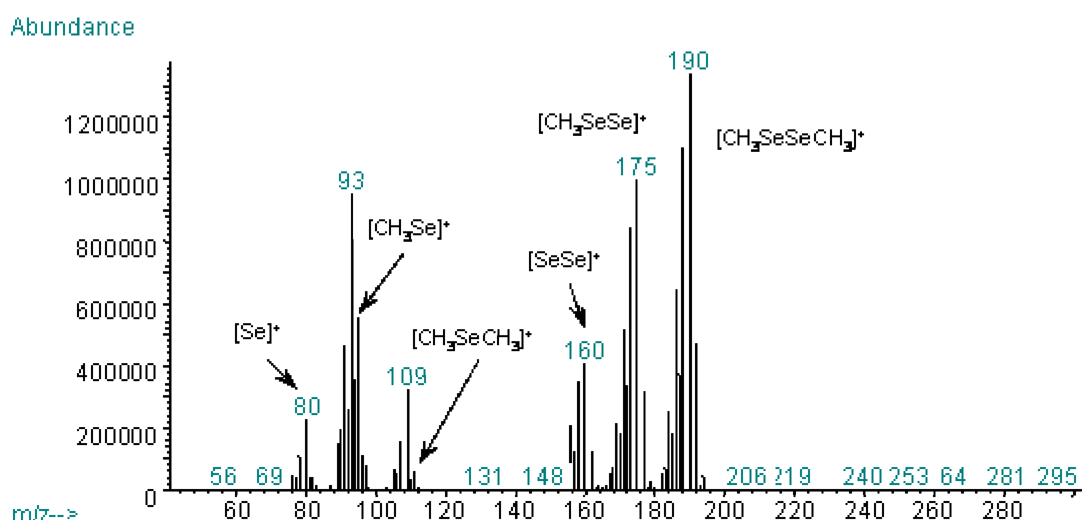


Figure 25: Mass spectra of DMDSe.

4.2.2.2 Optimization of SPME parameters

Further, the preconcentration step, using solid phase micro extraction, was optimised. Fibers, namely PDMS and Carboxen-PDMS, were selected based on the polarity and volatility of target compounds.

PDMS

To obtain a good signal-to-noise ratio and repeatability, the adsorption time (5, 10, 15, 20, 30 min) was optimised. The extraction parameters were set to be constant (30 min ultrasound treatment at 37 °C). The concentrations of Se standards, namely DMSe and DMDSe were 500 ng/g solution and 50 ng/g solution, respectively.

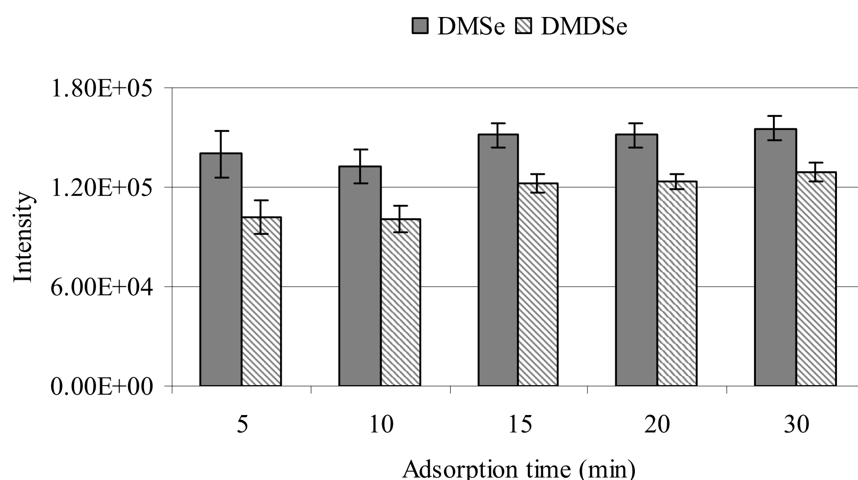


Figure 26: Intensity of DMSe and DMDSe vs. adsorption time.

15 minutes was chosen to be the optimal adsorption time (Figure 26). The absolute error between two parallels was 5% and no increase in instrument intensity was observed by increasing the time. What is more, the ultrasound bath was set to be 37 °C, but by increasing the adsorption time, the temperature started to grow, ending at 42 °C after 30 min of extraction and additional 30 min of adsorption of volatile Se species to the fiber. Due to the fact that ultrasound bath did not have a proper temperature regulation system, 15 min was chosen to be the optimal adsorption time.

The linearity was checked in the range between 10 and 500 ng/g for DMSe and between 1 and 50 ng/g solution for DMDSe and linear correlation was obtained for both the standards investigated in the range proposed, with the correlation coefficient above 0.98. The repeatability within one day was measured and was as low as 4.4% for DMSe (n=5) and 4.9% for DMDSe (n=5).

Carboxen-PDMS

Firstly, the adsorption time (10, 15, 20 min) was optimised. The extraction parameters were set to be constant (30 min ultrasound treatment at 37 °C). The concentrations of Se standards, namely DMSe and DMDSe were 0.93 ng/g solution and 5.0 ng/g solution, respectively.

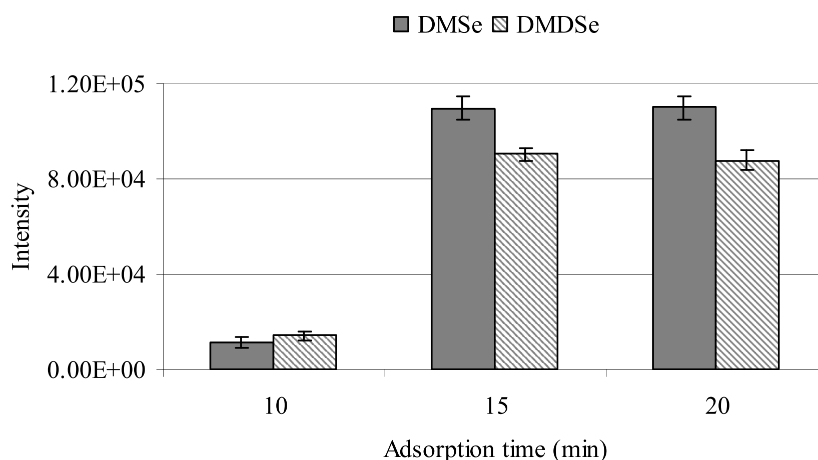


Figure 27: Intensity of DMSe and DMDSe vs. adsorption time.

Comparable to the results obtained with PDMS, 15 minutes was chosen to be the optimal adsorption time for Carboxen-PDMS fiber. The absolute error between two parallels was 4% and no increase in instrument intensity was observed by increasing the time (Figure 27). The comparison of the performance of the 100 µm PDMS and the 75 µm Carboxen-PDMS fiber coating demonstrates the superiority of the latter in terms of sensitivity and repeatability as also reported by Haberhauer-Troyer et al. (1999). Due to this reason, Carboxen-PDMS was chosen over PDMS.

The linearity was checked in the range between 0.0625 and 3.35 ng/g for DMSe and between 0.5 and 19 ng/g solution for DMDSe and linear correlation was obtained for both the standards investigated in the

range proposed, with the correlation coefficient above 0.99. The repeatability within one day was measured and was as low as 4.7% for DMSe (n=5) and 3.0% for DMDSe (n=5). The detection limits were determined based on the lowest visible signal (performed in triplicate) and were as follows, 0.0625 ng/g solution for DMSe and 0.5 ng/g solution for DMDSe. Campillo et al. (2005) obtained linear signal in the range 5-100 ng/L and 10-80 ng/L for DMSe and DMDSe, respectively. Their detection limits were 0.8 ng/L for DMSe and 1.1 ng/L for DMDSe. Purge-and-trap preconcentration was used in connection to GC-AED. By using SPME-GC-ICP-MS Duan et al. (2009) obtained detection limits, 760 ng/L for DMSe and 1330 ng/mL for DMDSe, while on the other hand Meija et al. (2002) obtained detection limits as low as 1 ng/L for DMSe and 7 ng/mL for DMDSe by using the same technique.

4.2.3 The purity of water and vial covers used for SPME

The Milli Q water, used for the extraction, contained a huge number of hydrocarbons making the analysis difficult. In order to decrease the effect of impurities from the solvent matrix to the species identification to the minimum, ultra pure water (J.T. Baker, Baker analysed LC-MS reagent) was used.

Additionally, to check if the vial covers have any effect on the species determination, several of them (white silicone septa (Tan PTFE, Agilent), black butyl molded septa (Gray PTFE, Agilent), BD Vacutainer LH, 8D Vacuntainer Z) were tested. With the exception of white silicone septa, which formed some impurities but not at the place of both standards investigated, DMSe and DMDSe, all of the other covers used contained some impurities on the same retention time as DMSe eluted, making them unsuitable to use.

4.2.4 Stability of volatile Se species

The Se species prepared in MeOH were found to be stable within one month, while on the other hand, the standards prepared in water had to be prepared on a weakly basis, regardless of the concentration used.

Since it is known that Se can be incorporated into proteins, aminoacids and we are aware of high plant Cys content and we did not find any published data dealing with the subject we decided to check what the influence of Cys on the Se species stability can be. 10% Cys standard (L-Cysteinium chloride (monohydrate), Merck, > 99%) water solution, with standards (DMSe and DMDSe) at concentration approximately 2 ng/g was prepared. The solution was treated with ultrasound for 30 min at 37 °C with additional 15 min for standards preconcentration. Three replicates gave comparable results. The concentration of DMDSe was below the detection limit of the method, while on the other hand the response for DMSe lowered for approximately 10% (Figure 28). It is obvious that the matrix component affect the speciation analysis, therefore we have to be careful dealing different sample matrixes.

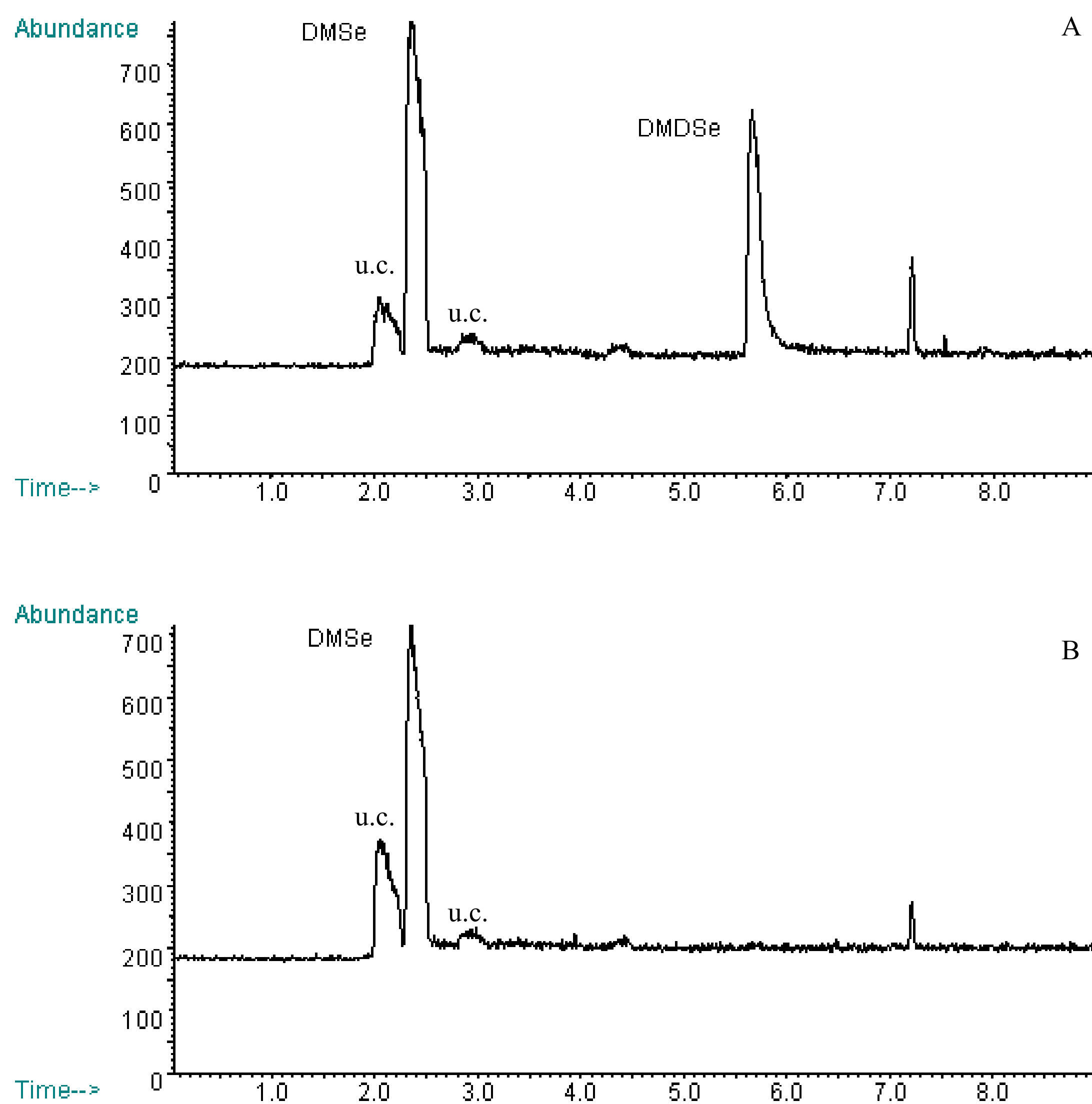


Figure 28: Stability of DMSe and DMDSe in water (A) and 10% Cys solution (B). Unknown compounds originate from a white silicone septa used.

4.2.5 Optimal SPME-GC-MS parameters for volatile Se species determination in plants

In the following table (Table 18), optimal extraction parameters, as well as the optimal conditions of Se species separation and their detection by GC-MS are presented.

Table 18: Optimal conditions for Se species determination with SPME-GC-MS.

Parameter	Value
Extraction	
water (or enzymatic)	0.6 g of sample without or with 60 mg of protease in 8 g of water (J.T. Baker), ultrasound treatment 30 min at 37 °C
SPME	
fiber	Carboxen-PDMS
vial cover	white silicone septa (Tan PTFE)
adsorption time (min)	15
GC-MS	
instrument: HP 6890 GC-MS	
column:	
DB-5 MS	30 m x 25 mm x 0.25 μm
injection T	240 °C
transfer line T	280 °C
temperature program	50 °C, 4 min, 15 °C/min to 125 °C, hold for 2 min, 30 °C/min to 250 °C, hold for 2 min
He flow rate	0.5 mL/min to 1.5 min, afterwards 1.5 mL/min
fiber conditioning	2 min at 260 °C
measuring parameters:	
m/z monitored	0-3 min: 80, 95, 110; 3-15 min: 80, 110, 190

4.3 Application of the methods developed

4.3.1 Potato tubers (*Solanum tuberosum* L.)

Global worming, which causes fluctuations of precipitation distribution, could increase the risk of plants being exposed repeatedly to drought. Drought stress can also affect the growth of plant organs and lowers productivity. The possible effects of Se foliar spraying and drought were studied in potato (*Solanum tuberosum* L.), cultivar Desiree.

The total Se content was found to be 16 ± 10 ng/g in drought exposed and 117 ± 4 ng/g in well-watered potato tubers, 347 ± 17 ng/g in drought-exposed Se enriched potato (W-) and 1101 ± 46 ng/g in well-watered Se treated potato (W+). The water content was on average 85% for drought exposed and 87% for well watered potato tubers. Results for total selenium content in control and Se enriched samples show that the selenium content in well-watered potato foliarly treated with Se(VI) solution was higher than in those exposed to drought.

Se content in Se-enriched potato, especially in drought exposed one, was low. It is known that performing speciation analysis of Se at such low concentrations is a difficult task. Additionally, the Se content in enzymes used for hydrolysis was found to be quite high. Due to these reasons, we wanted to check how relevant the influence of enzyme impurities on Se species determination can be, before Se speciation analysis in potato was performed.

4.3.1.1 The effect of enzyme impurities on Se species determination in potato extracts

Ferri et al. (2007) investigated the distribution of selenium in selenium-enriched potato, using amyloglucosidase from mould of the *Rhizopus* genus (12,000 units/g), in order to clarify the role of starch in selenium speciation in this matrix. In our experiment, β-amylase from barley was used to extract Se species from drought-exposed potato tubers and to estimate the effect of enzyme impurities on Se species determination.

Supernatants were prepared and analysed as follows: (a) supernatant of control drought-exposed potato tubers obtained after water extraction (Figure 29 a); (b) supernatant of β-amylase from barley in water (Figure 29 b); (c) supernatant of control drought-exposed potato tubers obtained after extraction with β-amylase from barley (Figure 29 c); (d) supernatant of drought-exposed Se-treated potato obtained after extraction with β-amylase from barley (Figure 29 d). No differences in the Se species and their contents were observed between enzyme supernatants prepared in water or in the considered matrix, except for some differences in retention times for some Se species due to the matrix effect. In the supernatant of the control drought-exposed potato sample prepared in water (no enzyme added), no detectable Se species were observed. We can gauge the influence of Se impurities present in commercially available enzymes on the results obtained by considering Figure 29. The result obtained for the SeMet content in potato supernatant is about 40% higher if the SeMet concentration obtained for the enzyme is not subtracted. Moreover, two other Se species (an unknown compound and Se(IV)) would be attributed to the potato sample, when in fact they originate from the enzyme. Therefore, the results must be corrected for these values, especially when determining samples with low contents of Se species.

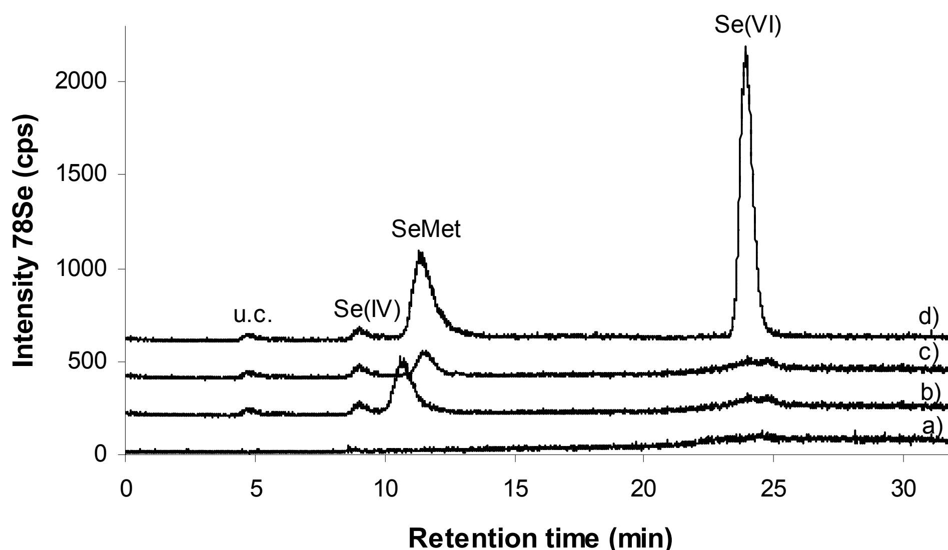


Figure 29: Chromatogram showing Se species present in a) supernatant of control drought-exposed potato tubers obtained after water extraction, b) supernatant of β-amylase from barley in water c) supernatant of control drought-exposed potato sample obtained after extraction with β-amylase from barley and d) supernatant of Se enriched drought-exposed potato sample obtained after extraction with β-amylase from barley. Se species were separated and detected by a Hamilton PRP-X 100 column and ICP-MS. To form a better picture, the baselines of chromatograms b, c and d were raised by 200cps (b), 400cps (c) and 600cps (d).

The enzyme supernatant was also tested for autohydrolysis. This could be influenced by the sample matrix or the time of storage (stability). First, the influence of the sample matrix was studied. Supernatants of (a) enzyme in water and (b) control drought-exposed potato tubers obtained by enzymatic hydrolysis were used. The same Se species and concentrations were observed for enzyme in potato supernatants as in the water solution. Secondly, the stabilities of the Se species and their concentrations were studied over three days. No differences were observed within this interval. Therefore, we can conclude that in the considered matrix, and within a three-day period, there is practically no autohydrolysis of the enzyme (β-amylase from barley), or that its effect is negligible.

4.3.1.2 Se species in Se-enriched potato

After separation of Se species on the HPLC anion exchange column we found Se(VI) and SeMet, which were confirmed on the cation exchange column (Figure 30). In chromatograms obtained for potato supernatants (Figure 30) some Se species at trace level were observed, corresponding to impurities from the enzyme used.

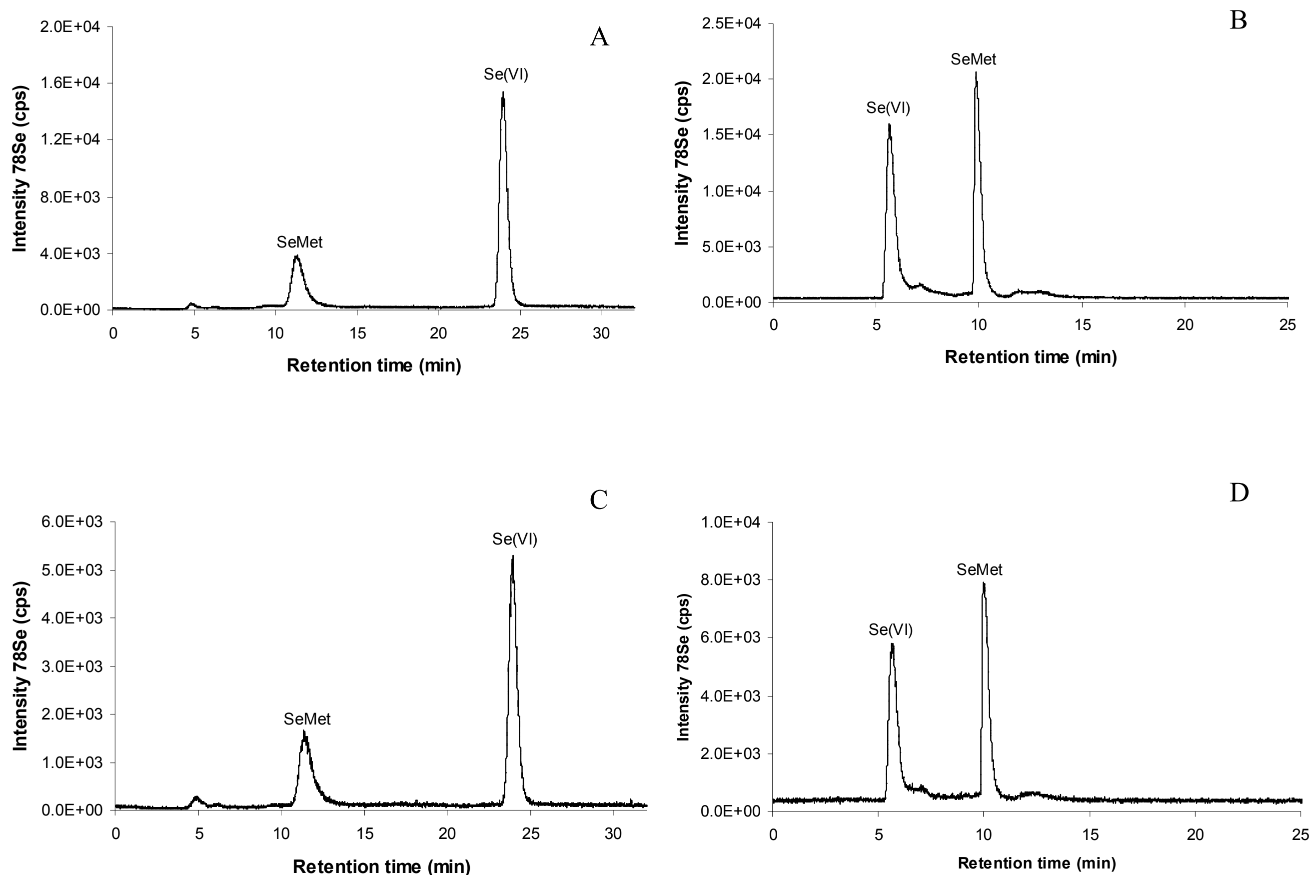


Figure 30: Se species from a well-watered potato sample (A, B) and exposed to drought (C, D), both treated with Se and extracted with protease, using HPLC-ICP-MS detection after anion (A, C) and cation (B, D) exchange.

Table 19 shows that concentrations of Se(VI) are independent of the extraction used. In drought exposed potato the values were around 98 ng/g and in water treated samples around 308 ng/g. If we look at SeMet concentrations we can see that protease released around 30% of SeMet according to the total Se content of the sample, regardless of the growing conditions. Also amylase released some SeMet but the values were lower, and by combining both enzymes, around 40% of SeMet was expected to be released, but the results obtained were similar to those using only protease, 30%. We can conclude that, inorganic selenium (selenate) applied as a foliar spray, was converted in the plant to selenomethionine. On average 30% of the Se content was in the form of SeMet, regardless of the growing conditions (drought or watered potato) and the enzyme used for extraction (Table 19).

While only about 80% of soluble Se was determined, column recovery was tested in the considered matrix. Firstly supernatant was injected through the column and secondly without the column. The ratio obtained was approximately 80%, indicating that about 20% of the soluble selenium remained on the column. However, this does not affect calculated Se species content in sample, due to the fact that standards were prepared in the same matrix solution.

Literature data on Se species in potato are very scarce, and therefore comparison with our results is very difficult. Ferri et al. (2007) reported that in potatoes, selenium is mainly stored in the non-protein fraction, probably as inorganic Se, even if organic forms cannot be excluded. Our results showed that the sum of the different forms of Se species represents about 80% of the soluble selenium, regardless of which type of amylase in combination with protease was used. Approximately half of it is present in the protein fraction (as SeMet) and the other half in the non-protein fraction (as selenate).

Turakainen et al. (2006) reported that 49-65% of total Se was present in the protein fraction in potato tubers (*Solanum tuberosum* L. cv. Satu). In our study the same Se form (selenate) was used to obtain selenium-enriched potato (cv. Darja), but only about 30% of the total Se in tubers was found to be present in the protein fraction. This difference could be due to the different potato cultivar used.

Table 19: Soluble and residue Se content and Se species content, namely Se(VI) and SeMet (ng/g of sample and % of species according to total Se) determined in potato tubers, obtained after different hydrolysis.

Extraction	Growing condition	Soluble Se ng/g DM (%)	Residue ng/g DM (%)	Se(VI) ng/g DM	%	SeMet* ng/g DM	%	Sum %
	W-	239 ± 10 (69)	128 ± 6 (37)	89 ± 4	26	<LOD		26
water	W+	484 ± 25 (44)	473 ± 20 (43)	321 ± 15	29	<LOD		29
	W-	291 ± 8 (84)	86 ± 4 (25)	80 ± 4	23	124 ± 5	36	59
protease	W+	771 ± 10 (70)	198 ± 5 (18)	288 ± 13	26	313 ± 15	28	54
	W-	274 ± 15 (79)	125 ± 25 (36)	87 ± 5	25	73 ± 4	21	46
β-amylase from barley	W+	738 ± 25 (67)	297 ± 10 (27)	313 ± 15	28	113 ± 5	10	38
	W-	274 ± 9 (79)	125 ± 13 (36)	100 ± 5	29	115 ± 5	33	62
protease + β-amylase from barley	W+	705 ± 33 (64)	319 ± 11 (29)	321 ± 16	29	351 ± 14	32	61
	W-	253 ± 7 (73)	145 ± 12 (42)	98 ± 5	28	44 ± 4	13	41
β-amylase from sweet potato	W+	683 ± 22 (62)	517 ± 5 (47)	286 ± 14	26	<LOD		26
	W-	316 ± 11 (91)	83 ± 9 (24)	103 ± 5	30	100 ± 5	29	59
protease + β-amylase from sweet potato	W+	848 ± 55 (77)	253 ± 8 (23)	278 ± 14	25	284 ± 15	26	51
	W-	215 ± 12 (62)	149 ± 8 (43)	110 ± 6	32	34 ± 4	10	42
α-amylase	W+	650 ± 2 (59)	517 ± 9 (47)	325 ± 15	30	< LOD		30
	W-	264 ± 22 (76)	135 ± 6 (39)	115 ± 6	33	122 ± 6	35	68
protease + α-amylase	W+	859 ± 55 (78)	330 ± 23 (30)	332 ± 15	30	285 ± 14	26	56

Results are given in form: average of three measurements plus/minus standard deviation. The Se content in enzymes was substracted.
*Se as SeMet

4.3.2 Buckwheat (*Fagopyrum esculentum* Moench)

In the first part, the stability of water-soluble Se species in separate buckwheat parts (in the extracts of the control group of buckwheat sprouts, seeds, stems and leaves) was evaluated and at the same time we checked what the effect of Se species stability on the speciation analysis of Se-enriched buckwheat sprouts as well as of other Se-enriched buckwheat parts (seeds, stems and leaves) can be.

In the second part, the Se and its species content in buckwheat sprouts were investigated. The content of each Se species in buckwheat seeds, stems and leaves was reported elsewhere (Vogrinčič et al., 2009). Firstly, water-soluble Se species content in buckwheat sprouts were investigated. Additionally, the possibility of volatilisation, during the extraction process was taken under investigation. This was done in accordance with the mass balance calculations. The sum of soluble and insoluble part in Se(VI) buckwheat sprouts was not quantitative in comparison with the total Se content. Firstly, matrix effect on the stability of volatile Se species was checked and then speciation analysis in Se(VI) buckwheat sprouts was performed. Additionally, the method was used to see if DMSe and DMDSe are formed during the growth process of buckwheat sprouts. Nevertheless, the influence of Se addition on the composition of several other elements, which are important to plant physiology (S, Mn, Fe, Cu, Zn, Mo, Cd) were considered.

4.3.2.1 Stability of soluble Se species in the extracts of buckwheat sprouts, seeds, stems and leaves

Flavonols, especially rutin as well as quercetin and quercitrin, are frequently found sub-groups of flavonoids in buckwheat. In addition to flavonoids, buckwheat also contains tannins (Luthar, 1992). Different growing conditions, various cultivars of buckwheat (genetic factors) and different plant parts may have different contents of tannin and rutin (Luthar, 1992; Luthar, 1999). In all plants studied, the highest amount of rutin was reported to be in flowers, 36-59 g/kg DM (Kreft et al., 1999). More rutin was found in stems (0.7-1.2 g/kg, (Kreft et al., 1999)) than in leaves (0.1-0.7 g/kg, (Kreft et al., 1999)), and the least rutin was found in seeds (0.1-0.2 g/kg, (Holasova et al., 2002; Fabjan et al., 2003; Kreft et al., 2006)). Also buckwheat sprouts have a high rutin content, 5-22 g/kg (Kim et al., 2008). Most tannin is accumulated in the leaves (130 g/kg, (Ožbolt et al., 2008)) and a comparable amount was found in the stems (18 g/kg, (Ožbolt et al., 2008)) and seeds (5-45 g/kg, average 20 g/kg (Eggum, 1981; Luthar, 1992)).

Firstly, the Se species stability was checked in the extracts of control buckwheat parts. The Se content in the control group of selected samples was low (buckwheat sprouts 50 ng Se/g DM, buckwheat leaves 24 ng Se/g DM, stems 15 ng Se/g DM, seeds 7 ng Se/g DM) and the Se species present in the extracts obtained were below the detection limit of the method.

To do this, four experiments were performed. 0.6 g of dry sample was treated with A) 8 g of water; B) 8 g of water containing Se standards (100 ng Se/g); C) 8 g of water containing 60 mg of protease and D) 8 g of water containing 60 mg of protease and Se standards (100 ng Se/g). After extraction procedure and before Se speciation, Se standards (100 ng Se/g) were added to supernatant A) and C). Supernatants/solutions were kept in PTFE tubes and the stability of Se standards was compared with standard solutions prepared in water.

The results showed that Se species added in the extracts were stable in extracts of buckwheat sprouts (Figure 31), buckwheat leaves and stems, regardless of the fact that rutin and tannin are present in all buckwheat parts. The stability of Se species in the extracts obtained was monitored during storage at 4 °C for four days. If compared with Se standards prepared in water, some differences in retention times of separate Se species in the sample extracts were observed. These differences are important for qualitative evaluation of Se species and can be eliminated by adding Se standards to the sample (standard addition method).

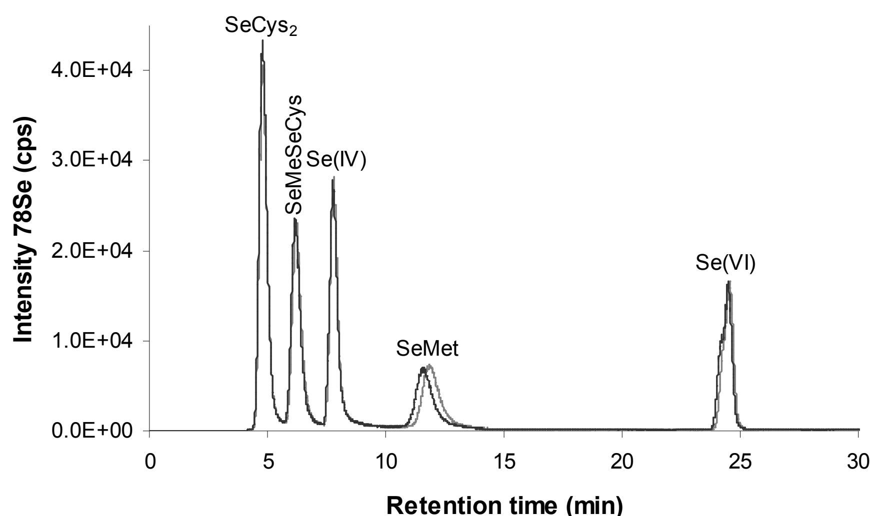


Figure 31: Chromatograms obtained on a Hamilton PRP-X 100 – ICP-MS system for the enzymatic extract of buckwheat sprouts with Se standards added after the incubation (time of measurements: immediately and 4 days after incubation).

Since the fact that rutin and tannin are present in all buckwheat parts, Se speciation analysis of buckwheat seed extracts showed a decrease in Se(IV) response (Figure 32, Table 20). We assume that the ratio between rutin and tannin in separate buckwheat parts could influence the stability of Se species. The difference in ratio between rutin and tannin in buckwheat seeds (1:100) is greater than in stems and in leaves, where it is approximately 1:1 (Ožbolt et al., 2008). After water extraction of buckwheat seeds only 31% of Se(IV) remained. Furthermore, after 4 days at 4 °C only 7% Se(IV) was present in the extracts. Similar result was obtained when Se standards were added to the extract after hydrolysis. When enzymatic hydrolysis was performed, a quantitative response was obtained after the incubation. Further, the response of Se(IV) started to decrease and 54-59% of Se(IV) remained after 4 days at 4 °C. On the contrary, the responses for other Se species (SeMet, $SeCys_2$, SeMeSeCys and Se(VI)) were stable (data not shown). The enzyme effect seems to be similar to the experiments performed *in-vitro*. Very probably, tannin and rutin, both present in seeds, have an effect on the stability of inorganic Se. What is more, the enzyme protease decreases reactions between Se(IV) and matrix components (Table 20).

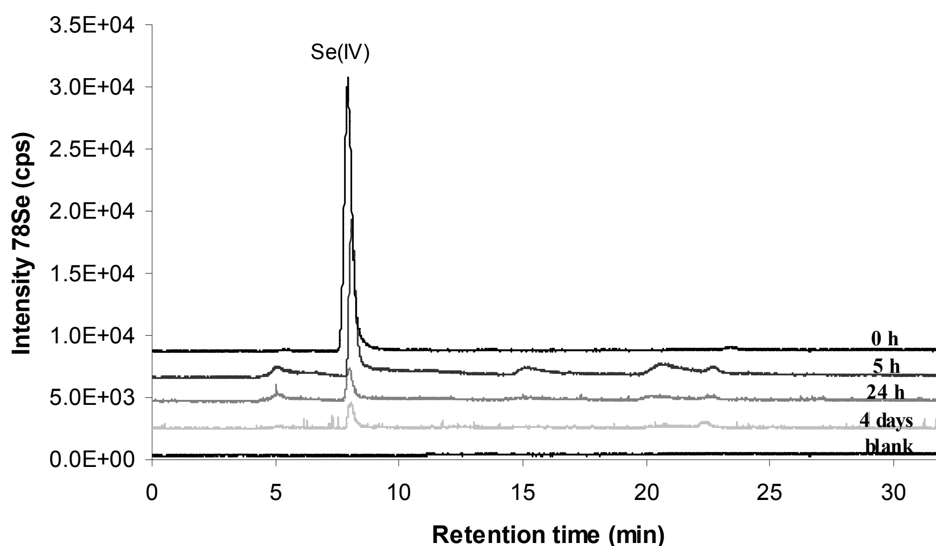


Figure 32: Chromatograms obtained on a Hamilton PRP-X 100 – ICP-MS system for the water extract of buckwheat seeds without (blank) and with Se(IV) standard added after the incubation (time of measurements: 0 h, 5 h, 24 h and 4 days of storage). To improve clarity, the baselines of chromatograms obtained imediately, 5 h, 24 h and 4 days after the incubation were raised by 2000 cps (4 days), 4000 cps (24 h), 6000 cps (5 h), 8000 cps (0 h).

Table 20: Percentage of Se(IV) remaininig after 24 h at 37 °C; analysed immediately after incubation (0 h) or kept at 4 °C for 5h, 24 h and 4 days. Se(IV) was added to the extracts of control buckwheat seeds before or after the incubation.

	% Se(IV) response remain			
	0 h	**5 h**	**24 h**	**4 days**
water extract				
Se(IV) added after the incubation (A)	100.0 ± 2.0	46.0 ± 2.0	10.1 ± 1.1	6.5 ± 0.5
Se(IV) added before the incubation (B)	30.1 ± 1.1	25.3 ± 3.2	10.3 ± 0.8	6.9 ± 0.9
enzymatic extract				
Se(IV) added after the incubation (C)	100.4 ± 0.4	92.3 ± 2.3	62.0 ± 2.0	59.3 ± 1.3
Se(IV) added before the incubation (D)	100.8 ± 1.8	n.d.	60.0 ± 1.0	54.1 ± 0.9

Results reported were obtained on anion exchange column and are given as the average of two determinations along with the absolute error.
n.d. = not determined

We assume that Se(IV) reacted with matrix components and that this compound could not be separated on the column used or be eluted with the citric buffer. Additionally, Se(IV) could be reduced to Se(0). To confirm this, analysis of total Se was performed in the extracts obtained; this gave quantitative results. Further, by injecting standards prepared in water after the extracts (on the same column), no memory effect was observed. However, some new Se species were observed on performing separation by ion-exchange chromatography and ICP-MS detection. Broad new peaks can be clearly seen in the chromatogram obtained after 5 hours (Figure 32). Unfortunately, we were not able to identified them. Under the same operation conditions we did not obtained comparable results by injecting the same extracts after 24 hours and 4 days. It is possible that these compounds were transferred. In these cases, the unknown Se species were under the limit of detection. Roberge et al. (2003) observed that standards spiked into the broccoli extracts were often transformed or lost. They suggested that components present in the broccoli caused pH- and buffer-dependent changes in the composition of the organo-selenium compounds or sorbed to the broccoli matrix, but specific compounds that could affect Se compounds were not reported. Otherwise, the stability of Se species in a plant matrix is rarely reported in the literature. Authors' are mainly concerned with the extract stability during storage and pay no attention to possible interactions between the analyte and matrix components during the extraction process. Thus, extracts of several plants that do not contain tannin, like pumpkin seeds (Smrkolj et al., 2005) and potato (Cuderman et al., 2008) were reported to be stable.

Since it was shown that the matrix effects Se species stability enormously, we wanted to check what the influence on the determination of Se species in Se-enriched buckwheat can be. Due to the fact that Se species were stable in the extracts of buckwheat sprouts, stems and leaves, Se-enriched buckwheat seeds were taken. The Se species present in Se-enriched buckwheat seeds were analysed previously (Vogrinčič et al., 2009). SeMet was found to be the main Se species. But the content of Se(IV) in extracts of Se-enriched buckwheat seeds was under the detection limit of the method (Figure 33). This could indicate that Se(IV) may be present in Se-enriched buckwheat seeds, but since it is not stable in the extracts obtained, we were not able to determine it. In contrast, the possibility that selenite is not present in the buckwheat seeds at all, because of the presence of polyphenols in the seeds, exists. In this case speciation analysis would yield the correct result (i.e. that there is no selenite), although selenite would degrade upon extraction. Kitaguchi et al. (2008) detected Se(IV) in enzymatic extracts of buckwheat seeds in relatively low quantity, less than 1% of the total Se. However, the total Se content in their investigation was approximately 58-fold higher in comparison to ours. Therefore, it is clear that reactions in the extraction process may affect speciation and may result in misidentifications and inaccurate values if analyses are reported as quantitative. We have to keep in mind that if the results are not fit for the purpose, they will lead to wrong conclusions.

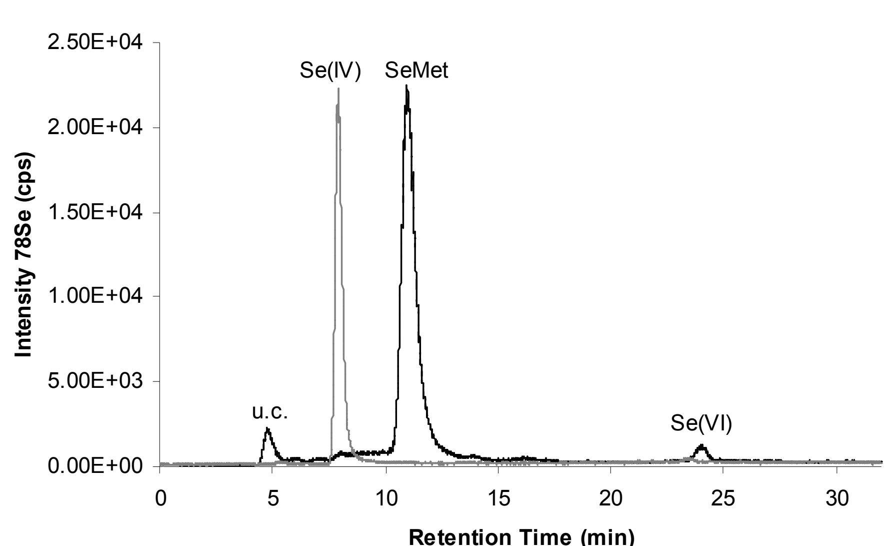


Figure 33: Chromatograms of standard Se(IV) in water (grey colour) and of the extract of Se-enriched buckwheat seeds (black colour) after enzymatic hydrolysis (Hamilton PRP-X 100 – ICP-MS).

To compare with the results performed *in-vitro.* A strong decrease was shown in Se(IV) response, when phenolics were added together in the ratio 1:100, higher in water (60%) and lower in enzyme (20%) solution. In addition, the response for Se(IV) was not stable over 4 days at 4 °C. Therefore, the results obtained for buckwheat seeds were comparable with those performed *in-vitro*. It is most likely that Se(IV) was reduced to Se(0) or that an insoluble complex was formed, which is no longer detectable by the separation and detection system used. Signals for other Se species (SeMet, $SeCys_2$, SeMeSeCys, Se(VI)) were stable.

4.3.2.2 Buckwheat sprouts

The transformation of Se in common buckwheat sprouts grown from seeds soaked in various Se solutions (Se-methionine (10 mg Se/L), selenate (5, 10, 20 mg Se/L) or selenite (5, 10, 20 mg Se/L) was investigated. During the plant growth no toxic signs, like leaf necrosis, drying or plant death were noticed. No differences were observed in the height of Se(VI), Se(IV) and SeMet sprouts for different concentration ranges and environmental (temperature, light) conditions. Dry weight was a little lower when seeds were soaked in Se(VI) and Se(IV) solution corresponded to those soaked in water (treated at the same time). Comparing buckwheat sprouts whose seeds were soaked in different Se solutions at the same concentration, the dry weights were as follows: Se(VI) > Se(IV) > SeMet, in average 10%. Average masses of buckwheat sprouts were practically not dependent on the Se(VI) and Se(IV) concentration and were comparable with the control group (approximately 0.1 g).

The Se content was raised by increasing Se concentration in the soaking solution. The dependence between Se(VI) concentration and Se content obtained is almost linear (Table 21). Lintchinger et al. (2000) treated seeds of clover and wheat with Se(VI) solution (seeds were soaked for 12 h) and in the comparable concentration range, 0.78 – 20 mg Se(VI)/L, they also observed a linear response between the Se content obtained in sprouts and the added Se concentration. Buckwheat sprouts treated with 5 and 10 mg Se(IV)/L took up a comparable amount of Se, while sprouts treated with 20 mg Se(IV)/L took up twice as much. When we compared the ability of seeds to take up Se from soaking solutions containing different Se species at the same concentration, the following order was obtained: Se(VI) (53-73%) > SeMet (31%) > Se(IV) (12-27%). On analysing Se(VI) soaking solutions no Se(VI) transformations or losses were observed. Zayed et al. (1998) studied uptake of Se(VI), Se(IV) and SeMet in rice, sugarbeet, broccoli and Indian mustard sprouts. Se was added to the nutrient solution after germination. The highest Se content was observed in rice, broccoli and Indian mustard sprouts treated with Se(VI) and sugarbeet sprouts treated with SeMet. The lowest Se level was determined in sprouts treated with Se(IV). The same order was also observed by De Souza et al. (1998) who treated Indian mustard with Se(VI), SeMet and Se(IV).

Table 21: Se content in Se(VI), Se(IV) and SeMet buckwheat sprouts.

Experiment	Soaking solution	Concentration (mg Se/L)	Se content (µg/g DM)
i)	water	0	0.050 ± 0.001
	Se(VI)	5	4.03 ± 0.11
		10	9.67 ± 0.11
		20	15.15 ± 0.32
ii)	water	0	0.07 ± 0.002
	Se(IV)	5	2.39 ± 0.22
		10	2.59 ± 0.11
		20	4.67 ± 0.32
iii)	water	0	0.078 ± 0.004
	Se(VI)	10	7.08 ± 0.18
	Se(IV)	10	1.87 ± 0.02
	SeMet	10	3.24 ± 0.06

Results are given in form: average of three measurements plus/minus standard deviation.

4.3.2.2.1 Water-soluble Se species in Se-enriched buckwheat sprouts

Since comparable extraction efficiency was obtained on hydrolysis with 0.3 M HCl or protease (see chapter 4.1.1.1), we decided to perform speciation analysis in both mentioned supernatants (Table 22). Performing speciation analysis of soluble Se species, around 30% of total Se for Se(VI) sprouts, 5-15% of total Se for Se(IV) sprouts and 5-7% of total Se for SeMet sprouts were identified as Se species.

Extracts of Se(VI) sprouts, obtained after hydrolysis with hydrochloric acid, contained the highest amount of Se(VI), 28-30% of total Se, independent of the Se concentration used as the soaking solution. Se(IV) was present in traces. Lintschinger et al. (2000) soaked sunflower, wheat and alfalfa seeds in Se(VI) solution. The Se content within the sprouts remained as Se(VI). Moreover, Slekovec and Goessler (2005) reported Se(VI) to be the major Se species in vegetative parts of onion, garlic, radish and cabbage, foliarly treated with 10 mg Se(VI)/m^2 or 20 mg Se(VI)/m^2. In Se(IV) supernatants we were not able to detect any Se species after separation on an anion exchange column using the UV-HG-AFS system. With ICP-MS detection we were able to detect Se(VI) and some Se(IV) in trace amounts. On the cation exchange column an unknown Se species occurred, with a retention time of 9.5 min (Figure 34). In contrast, Sugihara et al. (2004) reported that chemical analysis of selenium in the HCl extract of buckwheat sprouts treated with Se(IV) (10 mg Se(IV)/L) contained SeMeSeCys as the major Se species. The difference could be explained by the different mode of cultivation (Table 23). Sugihara et al. (2004) treated seeds hydroponically while in the present paper seeds were soaked in various Se solutions for 4 h. Additionally plant defence mechanisms, shown by the presence of SeMeSeCys in the sprouts (Terry et al., 2000), could develop during a longer exposure period to high Se concentration.

Table 22: Soluble and residue Se content and its species present in buckwheat sprout supernatant obtained after extraction with 0.3 M HCl and protease XIV, using UV-HG-AFS or ICP-MS (*) as a detection system. Results were corrected for the Se content in protease (Cuderman and Stibilj, 2009).

Experiment	Soaking solution	Concentration mg Se/L	Soluble Se µg Se/g DM (%[d])	Residue µg Se/g DM (%[d])	Se(IV) ng Se/g DM (%[d])	Se(VI)	SeMet[a]	X[b]	Y[c]	Sum %
Extraction with 0.3M HCl										
i)	Se(VI)	5	1.60 ± 0.04 (40)		40 (1.0)	1129 (28.0)	< LOD	< LOD	< LOD	29.0
		10	4.30 ± 0.11 (44)		97 (1.0)	2708 (28.0)	< LOD	< LOD	< LOD	29.0
		20	5.93 ± 0.27 (39)	6.10 ± 0.50 (40)	76 (0.5)	4500 (29.7)	< LOD	< LOD	< LOD	30.2
		20*			24 (0.2)	4299 (28.4)	< LOD	< LOD	< LOD	28.6
ii)	Se(IV)	5	0.24 ± 0.01 (10)		< LOD	< LOD	< LOD	120 (4.6)	< LOD	4.6
		10	0.33 ± 0.01 (13)		< LOD	< LOD	< LOD	155 (3.3)	< LOD	3.3
		20	0.47 ± 0.02 (10)	4.70 ± 0.20 (100)	< LOD	< LOD	< LOD	374 (5.3)	< LOD	5.3
		20*			tr	41 (0.9)	< LOD	228 (4.9)	< LOD	5.8
iii)	Se(VI)	10	3.32 ± 0.05 (47)		71 (1.0)	2053 (29.0)	< LOD	< LOD	< LOD	30.0
	Se(IV)	10	0.23 ± 0.01 (12)		< LOD	< LOD	< LOD	150 (4.6)	< LOD	4.6
	SeMet	10	0.73 ± 0.02 (23)	1.43 ± 0.08 (45)	< LOD	< LOD	< LOD	< LOD	164 (5.1)	5.1
Extraction with protease XIV										
i)	Se(VI)	5*	1.61 ± 0.04 (40)	1.69 ± 0.04 (42)	tr	1004 (24.9)	143 (3.5)	< LOD	< LOD	28.4
		10*	4.07 ± 0.13 (42)	3.97 ± 0.06 (41)	tr	2657 (27.5)	230 (2.4)	< LOD	< LOD	29.9
		20*	5.41 ± 0.40 (36)	6.76 ± 0.14 (45)	tr	4000 (26.4)	439 (2.9)	< LOD	< LOD	29.3
ii)	Se(IV)	5	0.50 ± 0.03 (21)		< LOD	tr	130 (5.4)	123 (4.7)	< LOD	10.1
		10	0.57 ± 0.02 (22)		tr	tr	204 (7.9)	234 (5.0)	< LOD	12.9
		20	0.94 ± 0.06 (20)	2.62 ± 0.14 (80)	tr	tr	371 (7.9)	375 (5.3)	< LOD	13.2
		20*			tr	47 (1.0)	338 (7.2)	208 (4.5)	< LOD	12.7
iii)	Se(VI)	10*	2.88 ± 0.41 (41)	1.83 ± 0.45 (26)	tr	1675 (23.7)	263 (3.7)	< LOD	< LOD	27.4
	Se(IV)	10	0.36 ± 0.06 (19)	1.40 ± 0.09 (74)	tr	tr	143 (7.6)	150 (4.6)	< LOD	12.2
	SeMet	10	0.55 ± 0.06 (17)	1.59 ± 0.08 (49)	< LOD	< LOD	tr	< LOD	105 (3.2)	3.2
		10*			tr	13 (0.4)	117 (3.6)	< LOD	92 (2.8)	6.8

[a] Se as SeMet
[b] unknown Se species with retention time of 9.5 min, obtained on Zorbax 300-SCX, estimated as SeMeSeCys
[c] unknown Se species with retention time of 20 min, obtained on Zorbax 300-SCX, estimated as $SeCys_2$
[d] percentage of Se species according total Se
Results reported are given as the average of two determinations along with the absolute error.

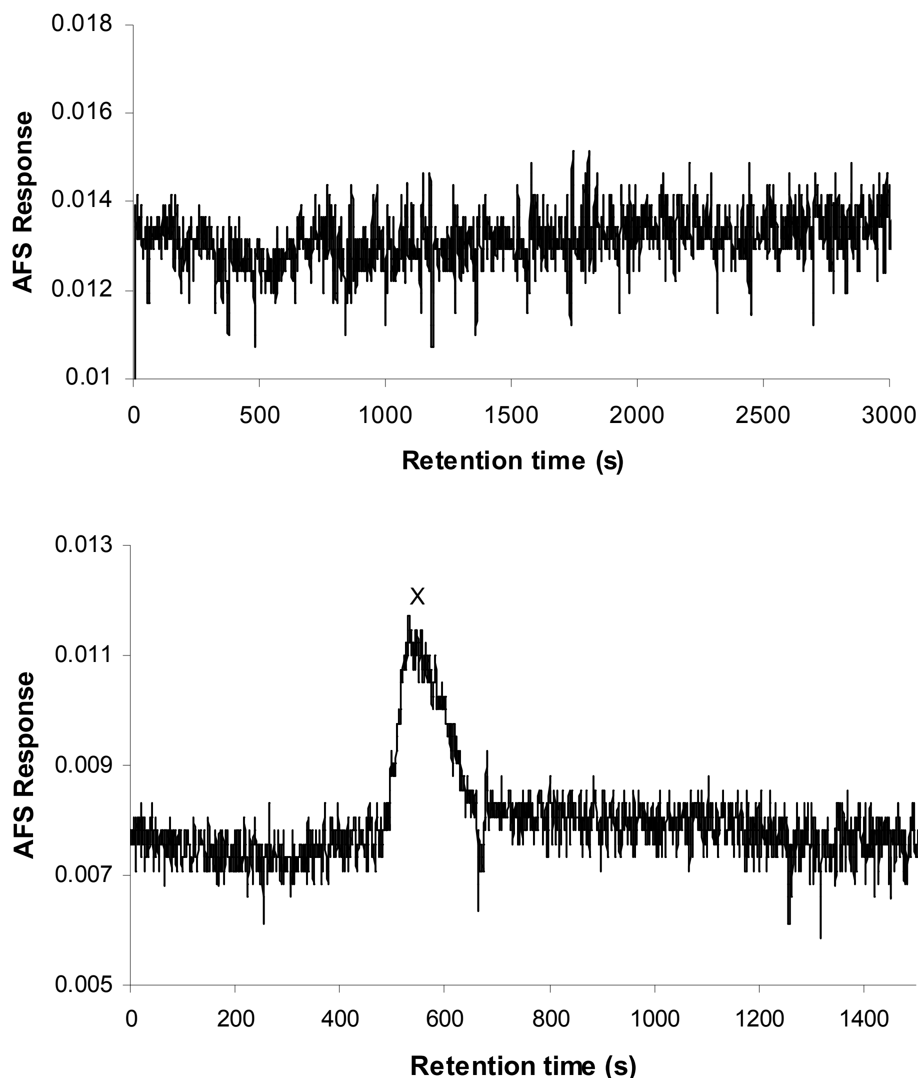


Figure 34: Chromatogram of acid sprout extracts of seeds soaked in 10 mg/L Se(IV) obtained after separation on a Hamilton PRP-X 100 column (above) and a Zorbax 300-SCX (below) column with UV-HG-AFS detection.

Further, by doing speciation analysis of extracts obtained after hydrolysis with the enzyme protease, almost the same results were obtained for identified and unknown Se species (Table 22, Figure 35). Se(IV), Se(VI) and unknown compounds were present in comparable concentration ranges, regardless of the extraction used (hydrochloric acid or protease). The most important Se species observed in each chromatogram was SeMet, and was present at 2-4% for SeMet, 5-8% for Se(IV) and 2-4% for Se(VI) buckwheat sprouts. SeMet was present as a low part of the total Se in buckwheat sprouts, while Kitaguchi et al. (2008) and Smrkolj et al. (2006) (Table 23) reported that the main Se species present in seeds of Se-enriched buckwheat, treated with Se(VI), was SeMet. Enzymatic and alkali extractions were made by Kataguchi et al. (2008) and SeMet was the only species that differed from those in alkali extracts (Se(IV), Se(VI) and SeMeSeCys). From the data reported by Kitaguchi et al. (2008) and our results it is evident that SeMet is present in proteins. From the experiment where seeds were soaked in 10 mg Se(IV), Se(VI) or SeMet/L, we can conclude that the form of Se in soaking solution has a great influence on the Se species plant uptake (Table 22). And further, no differences occurred in the Se species present under different cultivation conditions (temperature, daylight).

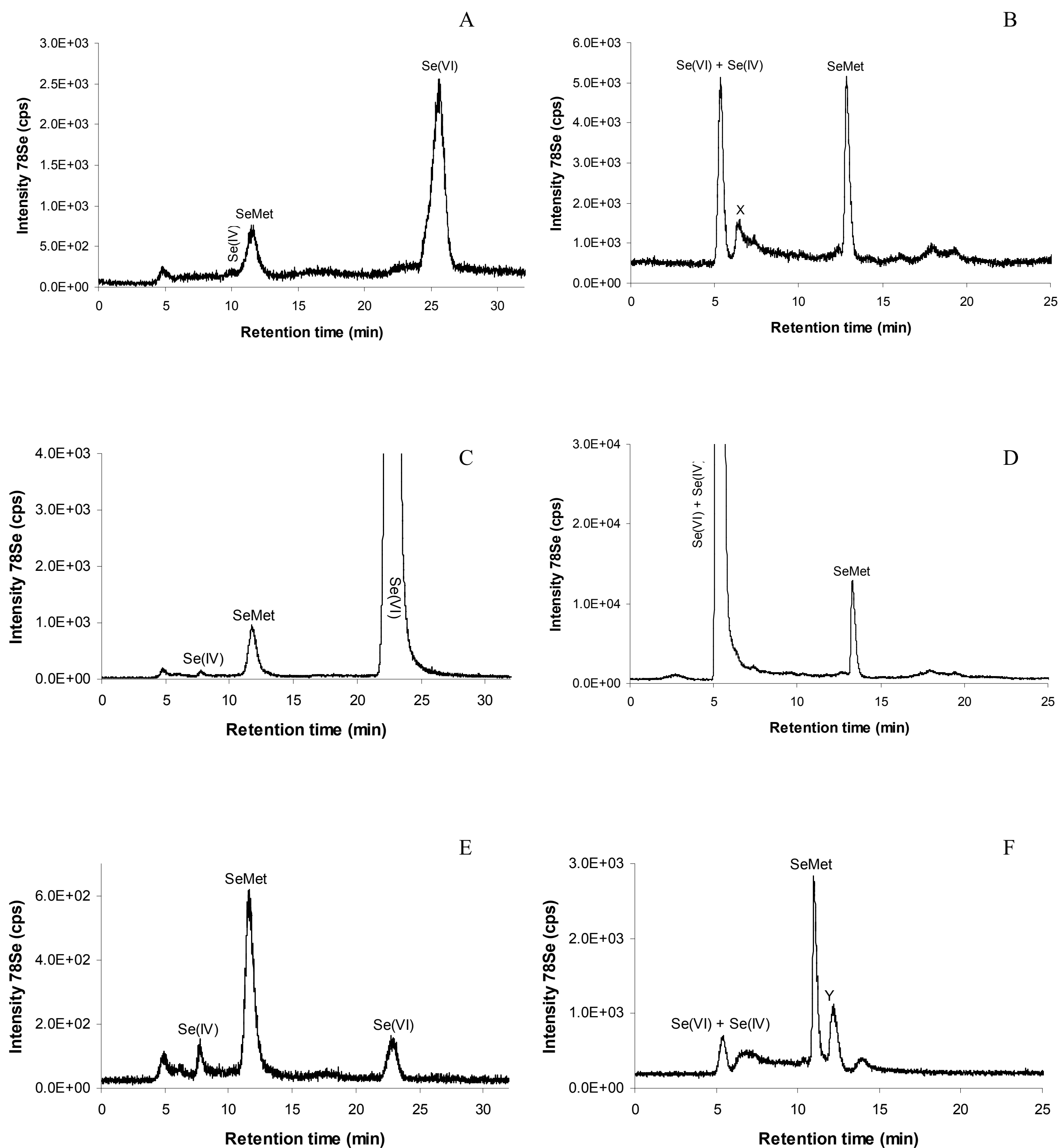


Figure 35: Chromatogram of enzymatic sprouts extracts from seeds soaked in 20 mg Se(IV)/L (A, B), 20 mg Se(VI)/L (C, D) and 10 mg SeMet/L (E, F) obtained after separation on Hamilton PRP X-100 (A, C, E) and Zorbax 300-SCX (B, D, F) columns connected to an ICP-MS system.

Table 23: Literature comparison of Se and its species content in Se-enriched buckwheat seeds and sprouts, treated under different conditions.

		Treatment conditions (conc. of solution used)	Analytical procedure	Se content (μg/g)	Se species (% of total Se)	Reference
buckwheat	seeds	foliar spraying (15 mg Se/L, Na_2SeO_4)	enzymatic extraction with protease; HPLC-UV-HG-AFS detection	2.9	SeMet (88%)	Smrkolj et al., 2006
		foliar spraying (500 mg Se/m^2, $BaSeO_4$; $BaSeO_3$)	extraction with driselase and protease or NaOH; HPLC-ICP-MS detection	170.4	SeMet (43%), Se(IV) (1%), Se(VI) (5%), SeMeSeCys (5%)	Kitaguchi et al., 2008
	sprouts	hydroponically (10 mg Se/L, Na_2SeO_3; 7 days)	extraction with HCl; HPLC-ICP-MS detection	8.5	SeMeSeCys (58%), unknown Se compound (14%)	Sugihara et al., 2004
		soaking for 4 h (10 mg Se/L, SeMet); growing period 11 days	extraction with HCl and protease; HPLC-ICP-MS detection	3.2	SeMet (3.6%), unknown Se compound (2.8%), Se(IV) and Se(VI) (traces)	this study
		soaking for 4 h (5, 10, 20 mg Se/L, Na_2SeO_3); growing period 8 days		2.4(5); 2.6(10); 4.7(20)	SeMet (5.4 - 7.9%), unknown Se compound (4.5 - 5.3%), Se(IV) and Se(VI) (traces)	
		soaking for 4 h (5, 10, 20 mg Se/L, Na_2SeO_4); growing period 22 days		4.0(5); 9.7(10); 15.2(20)	Se(VI) (23.7 - 29.7%), SeMet (2.4 - 3.7%), Se(IV) (traces)	

4.3.2.2.2 Volatile Se species in Se(VI) buckwheat sprouts

Due to the fact that mass balance calculations of Se(VI) buckwheat sprouts were far from being quantitative (losses were within 17 and 35%, Table 22), we decided to perform Se speciation analysis of volatile Se species, in order to see if loses correspond to Se volatilisation. DMSe and DMDSe were chosen as Se standards, since they are the most frequently found volatile species in plants.

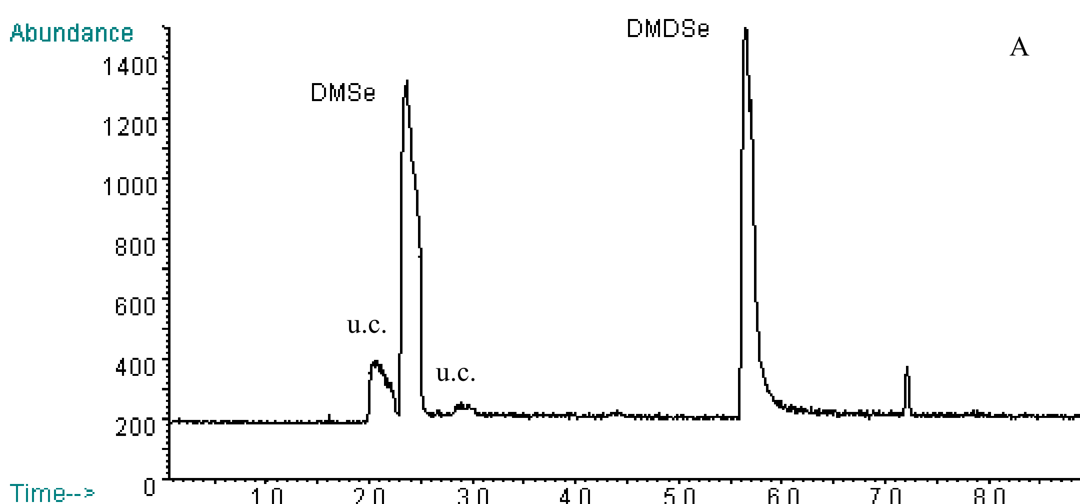
Firstly, we checked the stability of the standards in the matrix considered. Then the speciation analysis was performed and further, the method developed was used to indicate if there is some volatilisation present during the plant growth.

4.3.2.2.2.1 Stability of DMSe and DMDSe in Se(VI) buckwheat sprouts

The stability of Se standards selected (DMSe and DMDSe) in the matrix proposed (Se(VI) buckwheat sprouts) was considered. The experiment was performed in a following way. Firstly, DMSe and DMDSe were separately prepared in water in two concentrations per each species, 0.09 and 0.93 ng Se/g solution for DMSe and 0.5 and 5.0 ng Se/g solution for DMDSe. Each solution was treated with ultrasound for 30 min at 37 °C and additional 15 min for adsorption of Se species on the Carboxen-PDMS fiber and then injected into the GC-MS. Further, Se(VI) buckwheat sprouts (powder form), were added to the water solution of DMSe and DMDSe, keeping the concentrations in the same range. The following procedure was performed in the same way as for water solutions. Based on the ratios between peak area for Se standards in the sample and water solutions obtained for each individual standard, efficiency was calculated.

The yield obtained for DMSe was good. At lower concentration was it 85.4 ± 5.8% and at higher concentration was 99.9 ± 5.0%. In the chromatograms obtained under SIM and TIC conditions no new Se species were found, indicating that DMSe is stable when being added to the matrix water solution (Figure 36).

On the other hand, when DMDSe was added to the matrix solution, no chromatographic peak, corresponding to DMDSe, at retention time 5.7 min, was obtained, regardless of the concentration used (Figure 36). By performing analysis under SIM or TIC conditions, no new Se species was found, corresponding to degradation product of DMDSe. That indicates that even if DMDSe is formed during the extraction process, we will not be able to identified it with SPME-GC-MS. The same effect was observed when DMDSe was added to 10% Cys solution (see chapter 4.2.4).



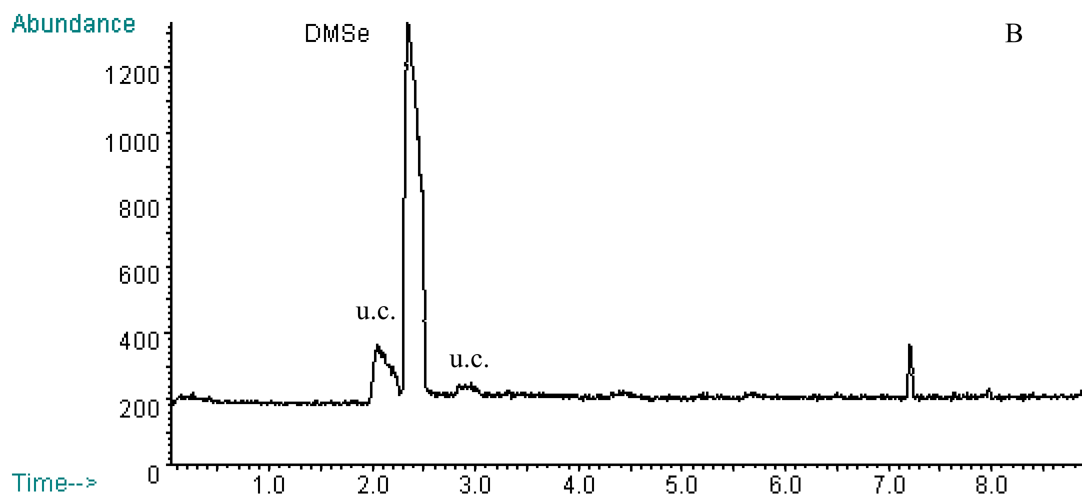


Figure 36: Stability of DMSe and DMDSe in water (A) and in buckwheat sprouts (B). Unknown compounds originate from a white silicone septa used.

Further, we wanted to find out what had happened with the standard during the process. By searching through the literature, dealing with volatile Se speciation analysis, to the author's knowledge, no such effect has been reported. However, Amoako et al. (2009) reported an interesting effect, dealing Se speciation analysis in dietary supplements. They assume that yeast-based SeMet and Se-(methyl)selenocysteine degrade to form DMDSe, which reacts with cysteine or cystine, if present, to form S-(methylseleno)cysteine. In the absence of cysteine or cystine, as in yeast-free Se supplements, selenodiglutathione may react with dimethyldiselenide to form S-(methylseleno)cysteine (Amoako et al., 2009). DMDSe has been reported as the principal volatile Se species in the headspace of selenized yeast (Dietz et al., 2004). To confirm the Amoako's thesis, HPLC-ICP-MS analysis of soluble part were performed. Selenium and sulphur were measured simultaneously.

To begin with, water and DMDSe dissolved in water, were analysed. The Se and S species content were below the limit of detection in the chromatograms obtained. In addition, soluble part of sample matrix with added DMDSe and 10% Cys solution with added DMDSe, after ultrasound treatment, were analysed. Zorbax 300-SCX and Hamilton PRP-X 100 were used for species separation. Since the main species present in the chromatograms obtained with Hamilton PRP-X 100 eluted with the void volume of the column, Zorbax 300-SCX was chosen for further analysis. The analysis of 10% Cys solution with DMDSe, gave a broad Se chromatographic peak, which could correspond to a complex or unfinished reaction process. One chromatographic peak was obtained in the S chromatogram, eluted near the void volume (Figure 37).

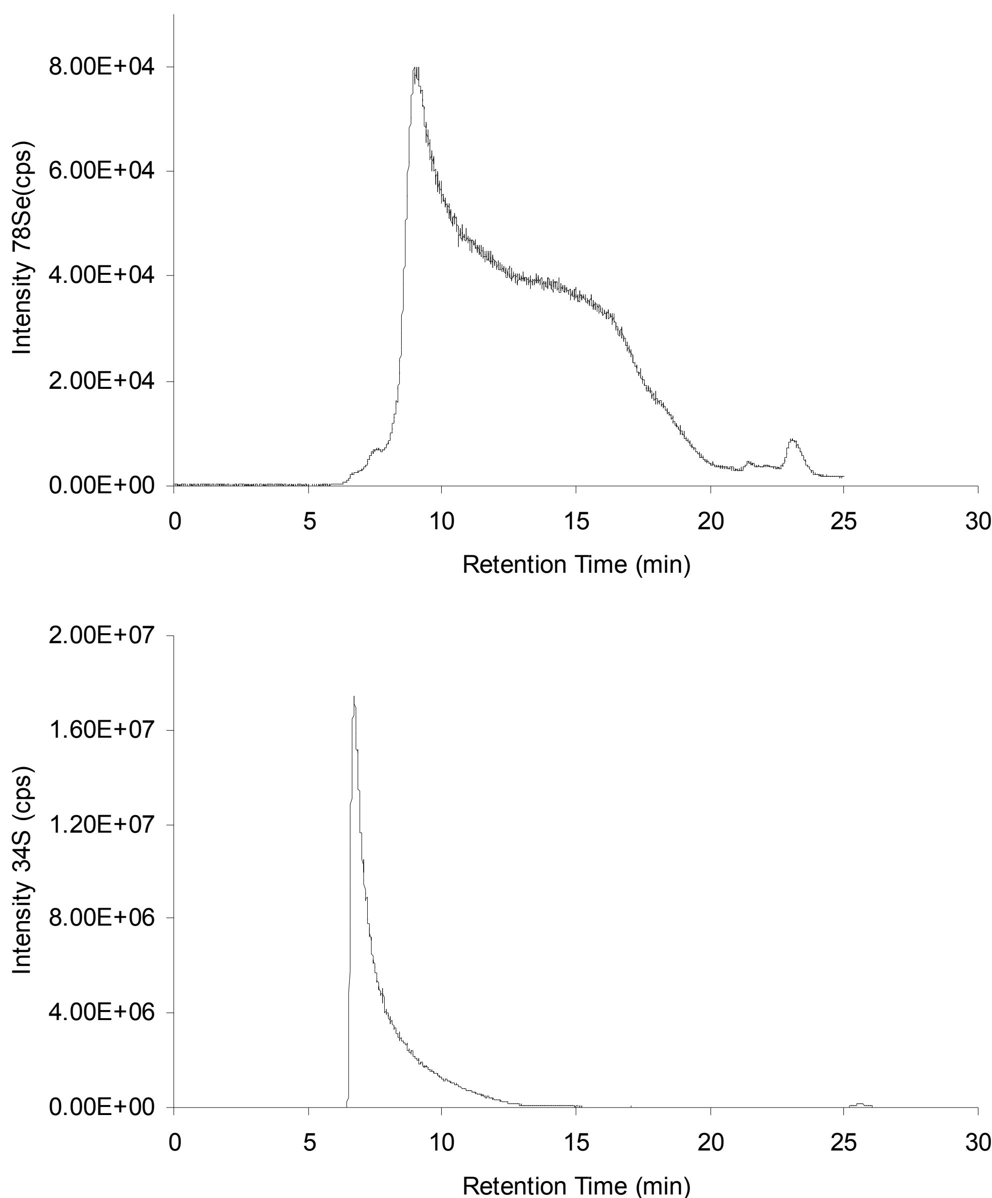


Figure 37: DMDSe in 10% Cys solution. Chromatograms showing ^{78}Se (upper curve) and ^{34}S (lower curve) obtained after separation on Zorbax SCX-300 and ICP-MS detection.

By analysing soluble part of sample (Figure 38), two major chromatographic peaks were observed in the Se chromatogram obtained. One of them corresponded to $SeCys_2$ and the second one we were not able to confirm with the standards used in our speciation studies, namely SeMet, SeMeSeCys, Se(IV), Se(VI). This unknown Se species had a retention time of 8.7 min, and at the same place there was a chromatographic peak corresponded to sulphur species. That indicates that there is a great possibility that the unknown species consist of Se and S. Additionally, $SeCys_2$ elutes near that peak (t_R=10.7 min), therefore it is possible that unknown species consist of S, Se and SeCys or $SeCys_2$ and could correspond to S-(methylseleno)cysteine, as reported by Amoako et al. (2009). However, further studies are necessary to confirm this thesis. Additionally, based on the results obtained it would be hard to say or draw any correlations between results obtained in 10% Cys solution and the one obtained in the matrix.

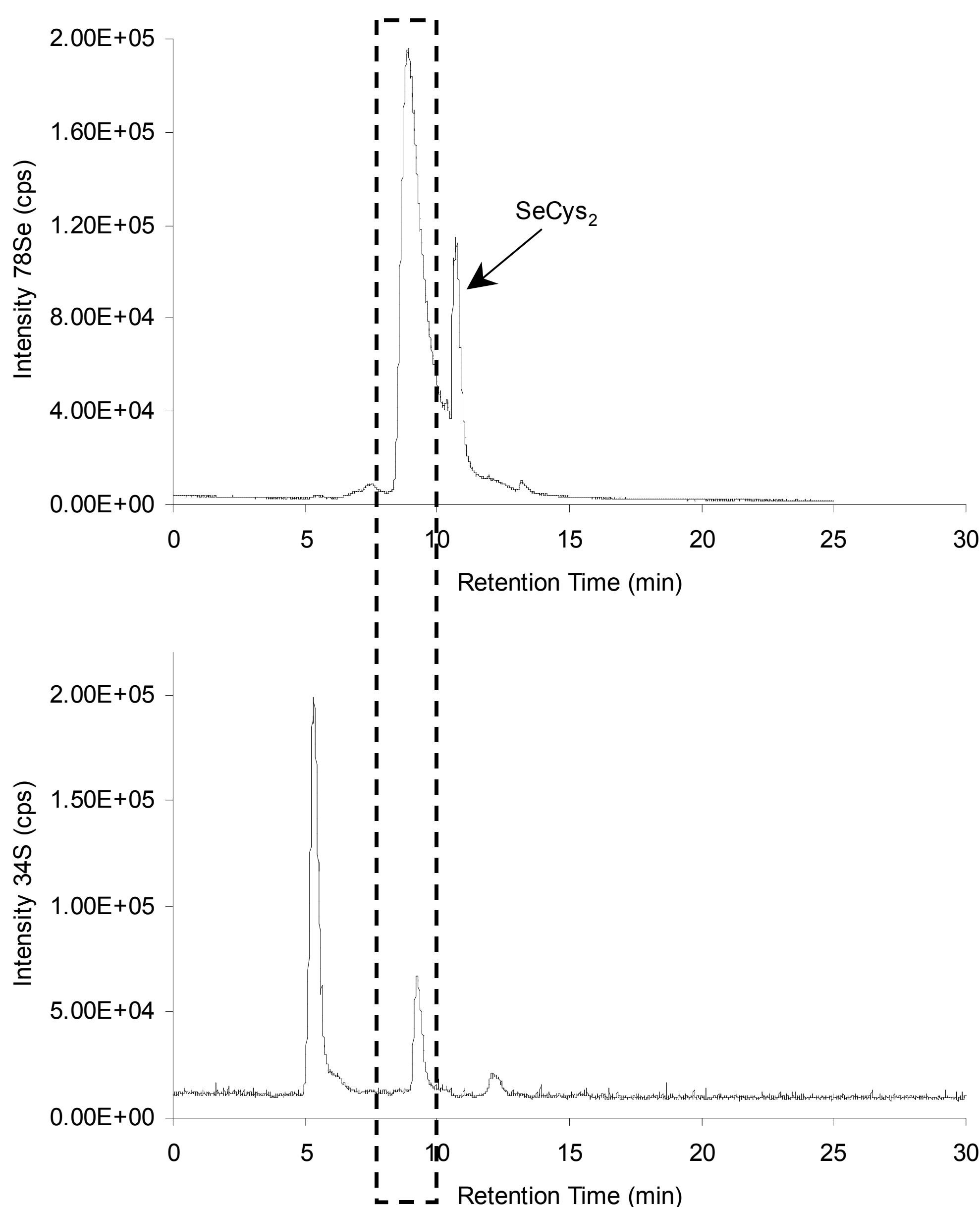


Figure 38: DMDSe in Se(VI) buckwheat sprouts. Chromatograms showing ^{78}Se (upper curve) and ^{34}S (lower curve) obtained after separation on Zorbax SCX-300 and ICP-MS detection.

4.3.2.2.2.2 Volatile Se species in Se(VI) buckwheat sprouts

Regardless the fact, that we were not able to determine DMDSe in Se(VI) buckwheat sprouts, speciation analysis of volatile Se species were performed. The possibility of DMDSe present in our sample was negligible, due to the fact that no unknown Se species was obtained when water or enzymatic extracts of Se(VI) buckwheat sprouts were analysed (Figure 35 (C, D)). In accordance to DMDSe stability, an unknown peak should be present with retention time 8.7 min, after separation on Zorbax 300-SCX and ICP-MS detection (see Figure 38).

By performing headspace Carboxen-PDMS-GC-MS sample analysis, as expected, the concentration of DMDSe was below the detection limit of the method. On the other hand, a chromatographic peak with the retention time corresponded to DMSe, occurred. Its presence was confirmed with the standard addition method (Figure 39). The presence of DMSe was confirmed, but its concentration was low. The concentrations of DMSe in Se(VI) buckwheat sprouts were as follows: 0.84 ± 0.01 ng Se/g sample, 1.06 ± 0.05 ng Se/g sample and 1.32 ± 0.04 ng Se/g sample for 5 mg Se(VI)/L, 10 mg Se(VI)/L and 20 mg

Se(VI)/L buckwheat sprouts, respectively.

DMSe was present in traces. If the volatilisation would take place during the sample preparation procedure, the results should be higher for three orders of magnitude. Then, the problem of mass balance calculation would be solved. This means that Se could be adsorbed on tube walls or some reaction between Se species and microorganisms could ocurred (Neumann et al., 2003), but this is, however, speculation.

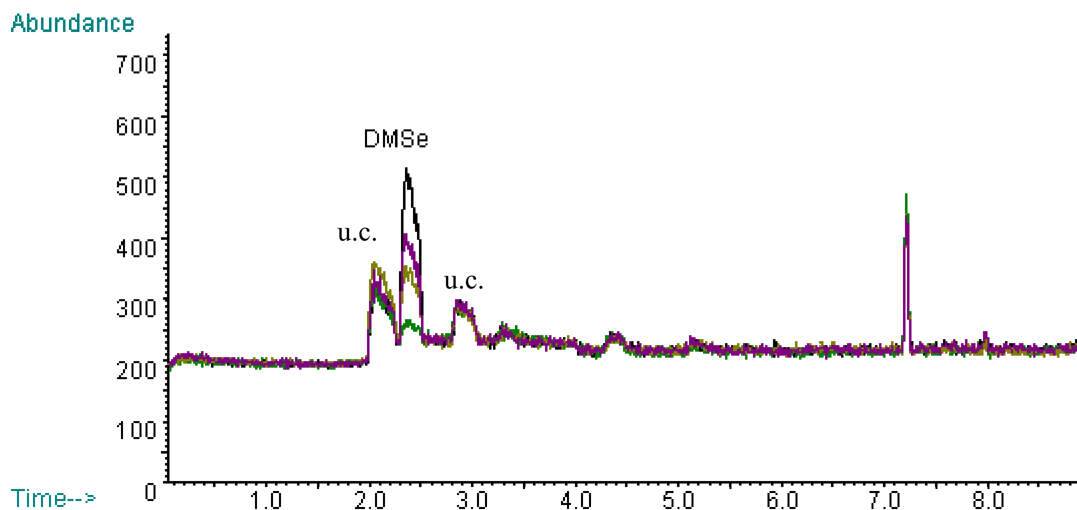


Figure 39: 10 mg/L Se(VI) buckwheat sprouts. DMSe was confirmed with standard addition method. Unknown compounds originate from a white silicone septa used.

The other possibility is the formation of Se complex that we were not able to degrade with the usual degradation step (too low temperature) and HG-AFS detection. According to this, microwave digestion and ICP-MS, which both allows higher temperatures, were used instead. The mass balance obtained was quantitative. The Se content in supernatant were comparable with the one obtained by HG-AFS, 40%, while in the case of residue analysed, 20% higher result were obtained, approximately 60% according to total Se. Apparently, by using the decomposition in a closed system (using HNO_3, H_2SO_4 and HF) the temperature was not high enough to degrade residue obtained after hydrolysis/extraction.

4.3.2.2.2.3 Volatilisation during the plant growth

GC-MS coupled with solid phase micro-extraction can provide a simple, extremely selective and sensitive technique for the analysis of volatile Se compounds in the headspace of growing plants. In this part, the technique was used to evaluate the volatilisation of Se in buckwheat sprouts, which seeds were soaked in 140 mg Se(VI)/L solution. It is known that Se is mainly volatilised as DMSe, which is 500-700 times less toxic than its inorganic forms. This is beneficial in comparison with the biovolatilisation of volatile organic compounds, which are released to the atmosphere without any changes. Volatilisation studies of Se from plant tissues may provide a mechanism of Se detoxification (Meija et al., 2002).

Buckwheat seeds were soaked in 140 mg Se(VI)/L solution for 4 h. Afterwards the seeds were transferred to 9 glass vials (4 seeds per vial). 13 days later we closed the vials (at that time, buckwheat sprouts had two leaves). After 2 h, 12 h, 24 h, 2 days, 6 days and 8 days, Se speciation analysis of the volatile part were performed. PDMS fiber was chosen in this case, because the effect from vial covers impurities was negligible. Fibers were exposed to headspace of closed plants for 30 min and then GC-MS analysis was performed. First 12 h no signs of volatile Se species was present, while after 24 h some traces of DMSe (< 10 ng/g) were observed as well as after 2, 6 and 8 days. DMDSe was under the detection limit of the method for as long as 2 days. After 6 days we were able to detect traces of DMDSe (< 2 ng/g) what is in contrary with the proposed plant metabolism (see Figure 2).

Results indicate that buckwheat sprouts, as being part of the group of Se non-accumulator plant species, do not have a high ability to transform inorganic Se to volatile forms, like DMSe and DMDSe. Buckwheat sprouts plant physiology does not involve volatilisation in higher amounts under the conditions described. On the other hand, *B. juncea* seedlings grown in closed vials and treated with Se was shown to be appropriate plant for transformation of Se(VI), Se(IV), SeMet into volatile Se species, where DMSe and

DMDSe were detected as the primary volatile Se components in the headspace (Meija et al., 2002).

4.3.2.2.3 The effect of Se addition on the concentration of essential and toxic elements in Se(VI) buckwheat sprouts

Biofortification has been defined as the process of increasing the bioavailable concentrations of essential elements in edible portions of plants through agricultural intervention or genetic selection (White and Broadley, 2005). There are currently several works, which show that fertilization with Se raises the content of this trace element in plants. However, none of these studies have thoroughly analysed the possible effect of the Se application on the essential nutrients for plant growth and development, such as S, Mn, Fe, Cu, Zn, Mo. Thus, we analysed the effect of Se addition on the S, Mn, Fe, Cu, Zn, Mo content in plant and additionally toxic element, Cd, was measured.

Our results indicate that the application of selenate slightly affected the concentration of S, Mn, Fe, Zn (Table 24). S content was found to be lower for 12%, regarding to control group.

Table 24: Content of Se, S, Mn, Fe, Cu, Zn, Mo and Cd in control and Se enriched buckwheat sprouts.

	Se	S	Mn	Fe	Cu	Zn	Mo	Cd
Soaking solution	μg Se/g DM							
Se(VI) - 0 mg Se/L	0.06 ± 0.01	4123.3 ± 247.0	36.5 ± 1.1	60.1 ± 2.1	11.2 ± 0.9	49.5 ± 0.9	0.25 ± 0.01	0.043 ± 0.004
Se(VI) - 20 mg Se/L	15.2 ± 0.3	3639.9 ± 55.9	31.8 ± 0.2	56.7 ± 0.1	11.1 ± 0.1	46.2 ± 0.2	0.23 ± 0.01	0.040 ± 0.004

Results reported are given as the average of two determinations along with the absolute error.

Possible effects of Se application on S metabolism and plant growth are still not clear. Given that both elements (Se and S) present similar chemical properties (Barak and Goldman, 1997), the study of their possible interaction is needed for the development, effectiveness, and improvement of biofortification programmes that use Se.

Despite that the literature on this subject is minimal, different researces (Barak and Goldman, 1997; Kopsell and Randle, 1997; Rios et al., 2008) have indicated that selenate would be absorbed by the same transporters as SO_4^{2-} and thus the two ions have an antagonistic relationship, provoking a decline in the S concentration in plants. In addition, according to studies by Anderson (1993), Se metabolism follows the same pathway as S, since, due to the chemical similarity of the two molecules, most enzymes involved in S metabolism can catalyse the analogous reaction with the corresponding Se substrates, and the end products of the assimilation, such as selenocysteine or selenoproteins, interfere in both the uptake as well as the metabolism of SO_4^{2-}. White et al. (2004) supported these results by suggesting that Se competes with S in the biochemical process of protein incorporation.

These statements can be supported by the results obtained in our study. With increasing Se concentration in buckwheat sprouts, S content decreased, indicating a synergism and antagonistic relationship between Se and S. In future, more attention should be devoted to Se effect on essential elements, besides its speciation in Se-enriched plant.

4.3.3 Leafy vegetables

Se-enriched leafy vegetables were grown in greenhouse of the Biotechnical Faculty, Ljubljana. During plant growth no toxic signs, like a garlic smell, red spots on the roots, black spots on the leaves, drying or plant death were noticed, regardless of the concentration of Se used. No differences were observed in the height and number of leaves per plant for concentration ranges 1+1 and 2+2 mg Se/L. Plants foliarly sprayed with 5+5, 10+0, 10+10 mg Se/L showed an increase in the mass of fresh leaves per plant. The 10+50 mg Se/L solutions of Na_2SeO_4 negatively affected plant growth.

The average content of dry matter in leaves was 10.82-13.32% for chicory cv. 'Anivip', 11.60-14.80% for chicory cv. 'Monivip', 11.82-13.82% for dandelion, 9.51-12.98% for rocket and 9.05-10.82% for wild

rocket. There were also noticeable differences in root growth. Plants without Se addition had a higher root fresh mass in comparison with the Se-enriched plants, irrespective of the concentration. With a decreased root and an increased leaf fresh weight in response to Na_2SeO_4 enrichment, a higher leaf-root ratio can be observed in the majority of treatments (Žnidarčič, 2009).

All the plants used in the study contained low amount of Se, 0.03-0.06 µg/g on a dry matter basis, when cultivated under the usual conditions. The accumulation of Se increased with increasing Se concentration in the spraying solution (Table 25). The dependence between Se(VI) concentration and Se content obtained was almost linear to the concentration of 10+0 mg Se/L, and onward an exponential increase was observed. When we compare the ability of leafy vegetables to absorb Se(VI) from the spraying solution containing various concentrations, small differences were observed at the lowest concentrations used, 1+1 mg Se/L and 2+2 mg Se/L. The contents were between 0.34 and 0.51 µg Se/g for 1+1 mg Se/L and 0.93–1.26 µg Se/g for leafy vegetables sprayed with 2+2 mg Se/L. At higher concentrations differences among Se content in the plants were observed. Salad rocket and wild rocket had the ability to absorb twice as much (3.72 µg Se/g; 2.59 µg Se/g) as other plants (1.51–1.96 µg Se/g) at the spraying concentration of 5+5 mg Se/L. At the highest foliar concentration used (10+50 mg Se/L), salad rocket had the ability to absorb the most Se, 102.38 µg Se/g. The content was comparable with that obtained for dandelion (97.42 µg Se/g), twice as high as the Se content in chicory (cv. 'Monivip' 61.42 µg Se/g; cv. 'Anivip' 63.15 µg Se/g) and ten times higher than that obtained for wild rocket (13.04 µg Se/g). Chicory cv. 'Anivip' and 'Monivip' took up comparable amounts of Se, regardless of the Se(VI) concentration in the spraying solution. On the other hand, great differences were observed in the Se content obtained for rocket and wild rocket in plants sprayed with 10+0, 10+10 and 10+50 mg Se/L. Mazej et al. (2006) cultivated the chicory cv. 'Anivip' aeroponically for 5, 10 and 41 days. The Se content in leaves was 88 µg Se/g DM after 5 days, 131 µg Se/g after 10 days and up to 480 µg Se/g after 41 days. The Se content in chicory leaves of the cv. 'Monivip' after 41 days of aeroponic cultivation was 460 µg Se/g and in dandelion leaves 49 µg Se/g (Mazej et al., 2008). In comparison, the accumulated Se values were higher in the cultivations made by Mazej et al. (2006 and 2008) than obtained in this study using foliar treatment, with the exception of dandelion, where twice as high values were obtained when the highest spraying solution of 10+50 mgSe/L was used.

Table 25: Total Se content in lyophilised green plant parts.

	Total Se content (µg Se/g DM)				
Foliar solution (mg Se/L)	Chicory cv. 'Anivip'	Chicory cv. 'Monivip'	Dandelion	Rocket	Wild rocket
0	0.06 ± 0.01	0.03 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01
1 + 1	0.35 ± 0.01	0.41 ± 0.02	0.34 ± 0.01	0.48 ± 0.01	0.51 ± 0.01
2 + 2	1.14 ± 0.01	1.22 ± 0.01	0.93 ± 0.01	1.26 ± 0.01	1.08 ± 0.02
5 + 5	1.96 ± 0.04	1.58 ± 0.06	1.51 ± 0.09	3.72 ± 0.08	2.59 ± 0.08
10 + 0	2.22 ± 0.06	2.20 ± 0.09	6.71 ± 0.06	14.31 ± 0.07	4.26 ± 0.04
10 + 10	19.84 ± 0.28	26.72 ± 0.31	12.57 ± 0.06	31.61 ± 0.37	9.67 ± 0.14
10 + 50	63.15 ± 0.18	61.42 ± 0.32	97.42 ± 0.31	102.38 ± 0.52	13.04 ± 0.17

Results reported are given as the average of three determinations along with the standard deviation.

4.3.3.1 Se species in Se enriched leafy vegetables

Extracts of leafy vegetables foliarly sprayed with 2+2 and 5+5 mg Se(VI)/L solution contained Se(VI), SeMet and an unknown compound eluted with the void volume of the anion exchange column (Table 26, Figure 40). By injecting the same extract on the cation exchange column, no response for $SeCys_2$ was observed. This unknown compound was present at low concentrations, below 3% of the total content of Se in the sample. Se(VI) and SeMet were present in higher amounts. Se(VI) represented 21-27% of total Se in chicory (cv. 'Anivip' and cv. 'Monivip') and rocket, 12-13% of total Se in wild rocket and 5% of total Se for dandelion. SeMet represents around 20% in chicory (cv. 'Anivip' and cv. 'Monivip') and around 10% in rocket and wild rocket, regardless of whether the plants were sprayed with 2+2 or 5+5 mg Se/L. In the case of dandelion, a 15% more SeMet was obtained at the higher concentration of 5+5 mg Se/L. At the highest concentration used, 10+50 mg Se(VI)/L, Se(VI) represented a higher amount of total Se, between 42% in rocket and 58% in dandelion, while on the other hand, transformation to SeMet was low, 2% in dandelion and wild rocket, 3% in rocket and 4% in chicory (cv. 'Anivip' and cv. 'Monivip'). To the authors' knowledge no research has been made on plant samples that would indicate the same effect. By increasing the selenate concentration the formation/synthesis of SeMet was decreasing. Additionally, traces of SeMeSeCys were observed (< 1% of total Se) (Table 26, Figure 40). Identified Se species represented quantitative recovery of soluble Se, excepting rocket and wild rocket foliar sprayed with 2+2 and 5+5 mg Se/L. In this plants approximately half of the soluble Se remained unidentified. This could be explained by formation of the dipeptide γ-glutamyl-SeMeSeCys as a protective mechanism, similar to that in Se accumulating plants (Terry et al., 2000). By increasing the Se content (10+50 mg Se/L) plants did not transform as much inorganic Se as the ones treated with a lower Se concentration, probably due to the storage of Se(VI) in vacuoles as a protective mechanism.

By performing analysis of the extracts obtained after different types of hydrolysis (see section 4.1.1.1) by HPLC-ICP-MS, the same Se species and concentrations were observed as when performing analysis by HPLC-UV-HG-AFS. Since lower detection limits can be achieved with the ICP-MS detection system, traces of SeMeSeCys and Se(IV) were observed after three-step hydrolysis in plants foliarly sprayed with 2+2 mg Se/L (Figure 41). The presence of SeMeSeCys was confirmed on the cation exchange column and was also determined in leafy vegetables. In selected vegetables Mazej et al. (2008) determined SeMeSeCys, SeMet, inorganic Se and $SeCys_2$.

Some interesting data in Table 26, such as the higher ability of chicory and dandelion to transform inorganic Se to SeMet than rocket, could be explained by differences in the metabolism of the selected plants belonging to different families. Chicory and dandelion are members of the same *Asteraceae* family and rocket of the *Brassicaceae* family. The chemical form of Se present in the culture medium influences the speciation of Se in the plant. In the leaves of plants foliarly sprayed with selenate supplemented media, independently of the plant (chicory, rocket or dandelion), a similar metabolism resulting in the chemical form of Se present in the plant was observed. The Se content was identified in the form of selenoaminoacids, particularly SeMet and SeMeSeCys, beside inorganic Se. The low Se(VI) conversion in plants sprayed with 10+50 mg Se/L (storage of Se(VI) in vacuoles) and the presence of SeMeSeCys in these plants, indicates plant defence mechanisms, as already shown (Terry et al., 2000). Pedrero et al. (2006) found SeMeSeCys in radish after 40 days of hydroponically treatment with 1 mg/L solution of Na_2SeO_3. Smrkolj et al. (2007) identified 30% of the total Se in bean seeds foliarly sprayed with 10 mg Se/L to be present in the form of SeMeSeCys. Sugihara et al. (2004) cultivated sprouts of several edible plants (10 families and 28 species) hydroponically in a high selenium environment (10 mg/L of Se as selenite). They reported SeMeSeCys to be the main Se species in sprouts. In addition SeMet, Se(IV), γ-glutamyl-Se-methylselenocysteine and an unknown Se compound were detected in several high selenium sprouts.

Table 26: Se content in water soluble extracts and mass balance after enzymatic hydrolysis of lyophilised green parts of foliarly sprayed plants and Se species in extracts after enzymatic hydrolysis determined by HPLC-UV-HG-AFS (µg Se/g sample and % of Se with respect to the total content in the sample).

		Enzymatic hydrolisis		Se species								
Foliar solution	Plant	Soluble Se	Residue	Se(VI)		SeMet*		SeMeSeCys		X		Sum (%)
(mg Se/L)		µg/g DM (%)	µg/g DM (%)	µg/g DM	%	µg/g DM	%	µg/g DM	%	µg/g DM	%	
2 + 2	Chicory cv. 'Anivip'	0.53 ± 0.01 (48)	0.57 ± 0.01 (53)	0.28	25	0.24	21	< LOD		< LOD		46
	Chicory cv. 'Monivip'	0.58 ± 0.01 (51)	0.58 ± 0.05 (52)	0.29	24	0.27	22	< LOD		< LOD		46
	Dandelion	0.42 ± 0.01 (47)	0.53 ± 0.02 (59)	0.04	5	0.24	25	< LOD		< LOD		30
	Rocket	0.75 ± 0.01 (60)	0.53 ± 0.03 (44)	0.29	23	0.16	13	< LOD		< LOD		36
	Wild rocket	0.71 ± 0.01 (64)	0.35 ± 0.04 (33)	0.13	12	0.09	8	< LOD		0.02	2	22
5 + 5	Chicory cv. 'Anivip'	1.01 ± 0.05 (52)	1.11 ± 0.10 (56)	0.48	24	0.37	19	< LOD		0.01	< 1	43
	Chicory cv. 'Monivip'	0.80 ± 0.01 (51)	0.77 ± 0.01 (49)	0.42	27	0.41	26	< LOD		0.02	1	54
	Dandelion	0.67 ± 0.07 (44)	0.97 ± 0.01 (56)	0.07	5	0.61	40	< LOD		0.03	2	47
	Rocket	1.94 ± 0.07 (52)	1.67 ± 0.01 (45)	0.80	21	0.33	9	< LOD		0.02	< 1	30
	Wild rocket	1.51 ± 0.05 (58)	0.87 ± 0.03 (34)	0.35	13	0.18	7	< LOD		0.07	3	23
10 + 50	Chicory cv. 'Anivip'	31.88 ± 5.14 (51)	36.51 ± 0.51 (57)	28.23	45	2.62	4	0.31	< 1	0.26	< 1	49
	Chicory cv. 'Monivip'	33.48 ± 3.71 (54)	30.99 ± 2.60 (51)	26.29	43	2.60	4	0.36	< 1	0.36	< 1	47
	Dandelion	51.10 ± 0.10 (52)	52.34 ± 4.10 (54)	57.04	58	1.93	2	0.38	< 1	0.37	< 1	60
	Rocket	75.58 ± 3.9 (69)	34.35 ± 1.10 (38)	42.99	42	2.93	3	0.61	< 1	0.79	< 1	45
	Wild rocket	7.05 ±1.28 (54)	5.62 ± 0.63 (43)	6.00	46	0.25	2	0.06	< 1	0.11	< 1	48

X, unknown Se species, eluted with void volume on anion exchange column; * Se as SeMet
Results reported were obtained on anion exchange column and are given as the average of two determinations.

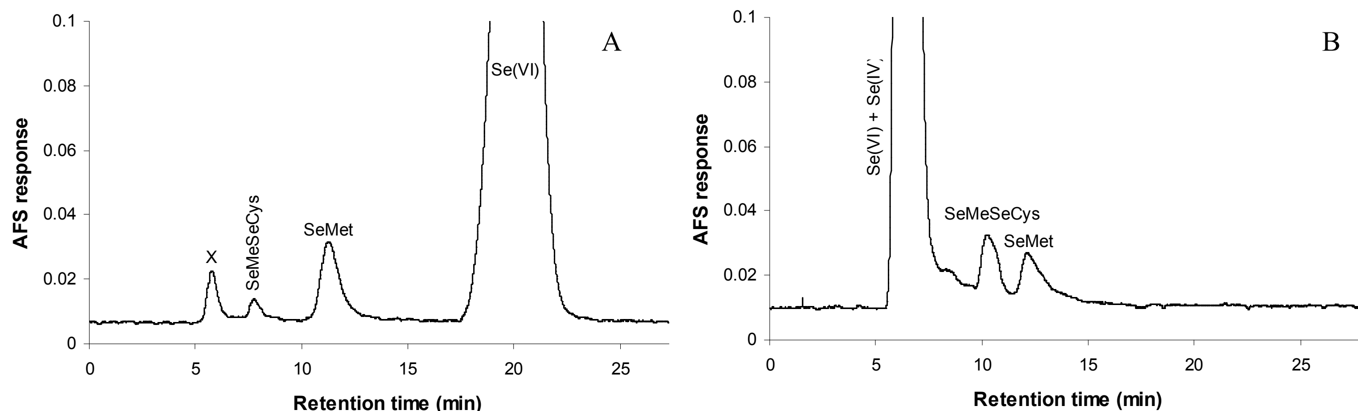


Figure 40: Anion (A) and cation (B) exchange chromatograms of chicory cv. 'Anivip' leaf extracts (10+50 mg Se/L) after enzymatic hydrolysis, obtained by HPLC-UV-HG-AFS.

Figure 41: Three-step extraction of Se from wild rocket (2+2 mg Se/L). Se species in the first extract (A) after water hydrolysis and second (B) and the third extract (C) after enzymatic (protease) hydrolysis obtained by separation on anion exchange column and ICP-MS detection. Se species (SeMeSeCys, SeMet) after the third extraction were confirmed on the cation exchange column (D).

4.3.3.2 The effect of Se addition on the concentration of essential and toxic elements in leafy vegetables

The possible effect of Se application on the content of essential and toxic elements in leafy vegetables, such as S, Mn, Fe, Cu, Zn, Mo and Cd was analysed. Selected elements in control plants and Se-enriched plants foliarly sprayed with 10+50 mg Se(VI) per litre were analysed, and the results obtained are reported in Table 27.

Rios et al. (2008) reported that there are possible effects of Se application on S metabolism, given that both elements present similar chemical properties and thus the two ions (SeO_4^{2-} and SO_4^{2-}) have an antagonistic relationship, provoking a decline in the S concentration in plants with increased Se concentration. Our results confirmed this theory in three plant species. S content decreased with increasing Se content in Se-enriched chicory (cv. 'Anivip' and 'Monivip') and dandelion, indicating a synergism and antagonistic relationship between Se and S. In contrast, by analysing S content in salad rocket and wild rocket an inverse relation was observed. A similar effect was observed by analysing Fe and Cu. The Fe content was approximately 30-times lower in Se-enriched plants in comparison to control group.

Cu has an important function as enzyme component (oxidase enzymes) and in the production of vitamin A. It is critical in the role of photosynthesis, protein and carbohydrate metabolism, and respiration. Cu is essential in the formation of chlorophyll and involved in the use of Fe by plant. Fe is required in the formation of chlorophyll and acts as an activator of many biochemical processes (found in ferrodoxin and enzymes such as peroxidase, oxidation-reduction processes). Excess Zn, Mn, Cu or Mo encourages Fe deficiency (Marschner, 2002). Additionally, Cu-Se interactions are observed mainly as inhibited Cu uptake with increased Se levels (Kabata-Pendias, 2001). For chicory (cv. 'Anivip' and 'Monivip') and dandelion this could be the reason for Cu lowering. Moreover, the Fe-Cu antagonism could play a major role in this case.

No interdependence was observed by comparing control and Se-enriched values obtained for Mn and Cd. Mn important functions in plant are as follows: it is an enzyme activator, it assist Fe in chlorophyll formation, assimilates CO_2 in photosynthesis, it is essential for P and Mg uptake. Mn is prominent in chloroplast membrane and important in N metabolism and assimilation (Kabata-Pendias, 2001; Marschner, 2002). Excess Fe, Cu or Zn may reduce Mn absorption. Several elements are known to interact with Cd in both the element uptake by plants and in biochemical roles. Several interactions are commonly observed (Cd-Zn, Cd-Cu, Cd-Fe, Cd-Se etc.), but findings appear contradictory. Cd is known as being toxic element and not essential in plant metabolism (Kabata-Pendias, 2001).

In contrast, a linear correlation between Zn and Se as well as between Se and Mo was obtained for all samples investigated. It is known that Zn is a component or regulator cofactor of enzymes (dehydrogenase, proteinase and peptidase enzymes). It also promotes starch formation. Excess Zn encourages Fe deficiency. Cu, Fe and Mn inhibit Zn uptake. Mo is essential in transformation of nitrate nitrogen into amino acid. Also, Mo excess encourages Fe deficiency. Excess Cu and S reduces Mo uptake. One possibility for increasing Zn and Mo content could be in the formation of water-insoluble Zn-Se and Mo-Se complexes (Kabata-Pendias, 2001; Marschner, 2002), through which intoxicity process can occur.

On the other hand, by analysing buckwheat sprouts, grown from seeds soaked in Se(VI) solution, the effect of Se addition on the essential and toxic elements content was not so clear. That could be attributed to lower Se content in sprouts (15 μg/g) than in leafy vegetables (> 24 μg/g).

The literature dealing the effect on distribution of essential metals according to Se accumulation in plants is scarce. However, Pedrero et al. (2006) reported that radish exposed to 1 mg/L Se(VI) or Se(IV) showed a slight decrease in the translocation of the Mn, Mo and Cu, when compared to control plants. Depending on the element, the decrease was more or less noticeable, but a tendency to reduce the translocation was observed when Se was supplied as Se(IV). No correlation was observed for Cu and Zn. It was demonstrated that the chemical form of Se can influence the uptake and translocation of essential metals in radish plants, which could be the cause of the growth reduction of those plants grown in selenite media, in which the translocations were slightly poor (Pedrero et al., 2006).

Table 27: Content of Se, S, Mn, Fe, Cu, Zn, Mo and Cd in control and Se enriched leafy vegetables.

	Se		S		Mn		Fe		Cu		Zn		Mo		Cd	
c(µg/g DM)	0	10+50	0	10+50	0	10+50	0	10+50	0	10+50	0	10+50	0	10+50	0	10+50
Chicory cv. 'Anivip'	0.08 ± 0.01	74.8 ± 0.5	14.3 ± 1.3	8.7 ± 0.4	106.4 ± 1.1	142.8 ± 1.6	3249.9 ± 4.8	109.4 ± 28.0	6.2 ± 0.2	5.2 ± 1.0	26.4 ± 0.7	73.3 ± 1.1	4.6 ± 0.1	25.9 ± 0.1	0.30 ± 0.01	0.46 ± 0.01
Chicory cv. 'Monivip'	0.03 ± 0.01	71.5 ± 2.4	15.7 ± 1.0	8.6 ± 0.4	110.3 ± 2.1	82.7 ± 1.0	3251.4 ± 5.9	82.1 ± 0.5	6.6 ± 0.5	2.7 ± 0.5	27.0 ± 0.5	79.5 ± 7.3	4.7 ± 0.3	10.4 ± 0.1	0.30 ± 0.01	0.39 ± 0.02
Dandelion	0.12 ± 0.02	117.1 ± 0.8	15.8 ± 0.1	4.3 ± 0.1	115.9 ± 1.0	102.0 ± 2.3	3395.6 ± 32.1	71.6 ± 1.6	6.3 ± 0.1	2.8 ± 0.2	27.4 ± 0.8	37.4 ± 2.7	5.6 ± 0.6	4.6 ± 0.1	0.30 ± 0.01	0.23 ± 0.01
Rocket	0.07 ± 0.01	113.0 ± 0.1	12.9 ± 0.2	17.7 ± 0.2	24.6 ± 0.5	164.3 ± 1.1	50.6 ± 0.1	87.5 ± 0.7	2.0 ± 0.3	3.2 ± 0.2	37.2 ± 3.2	66.5 ± 0.1	44.6 ± 1.7	53.6 ± 0.4	0.12 ± 0.01	0.44 ± 0.02
Wild rocket	0.07 ± 0.01	23.9 ± 1.1	11.2 ± 0.3	14.5 ± 0.4	26.3 ± 1.6	107.3 ± 10.7	73.5 ± 1.0	120.5 ± 9.9	3.8 ± 0.5	4.1 ± 0.8	29.9 ± 1.8	70.9 ± 6.2	42.5 ± 2.1	128.9 ± 11.6	0.06 ± 0.01	0.39 ± 0.04

Results reported are given as the average of two determinations along with the absolute error.

4.3.4 Antioxidant food supplements

The easy access to supplements, also available in drugstores, makes their use uncontrolled and potentially dangerous. Coenzyme Q10 and selenium are the most topical antioxidants, therefore we decided to check whether the methods developed are appropriate to determine Se and its species content in these antioxidant food supplements. We wanted to see, if Se content in these products, which nowadays are flooding the market, are in agreement with the advertised values and chemical forms, since their benefits to human health are correlated with the chemical form consumed.

Three products were selected, namely product A, B, C. Se values obtained for products A and B were in agreement with the advertised values (Table 28), the average Se content was a little below the declared value but within experimental error (4% below the advertised value for product A and 6% below the advertised value for product B). The value of Se for obtained product C far exceeded the declared value: instead of 10 μg Se per tablet, 69 μg of Se per tablet was found. Thus the Se content in this product exceeded the advertised value sevenfold and represents a potential risk of Se poisoning. To the authors' knowledge, to date there have been no studies made on the Se content in food supplements containing antioxidants. Research was restricted mainly to multi-micronutrient supplements containing vitamins and minerals (B'Hymer et al., 2000; Stibilj et al., 2005). In recent years there have been several cases of Se poisoning. Clark et al. (1996) reported a case of intoxication caused by a nutritional supplement. Although the advertised value of Se per six tablets was 5 μg, laboratory analysis demonstrated that the amount of Se per tablet was between 500 and 1000 times the declared amount. Moreover, thirteen people were poisoned with tablets containing a 180-fold higher Se content than advertised (Sunde et al., 2006).

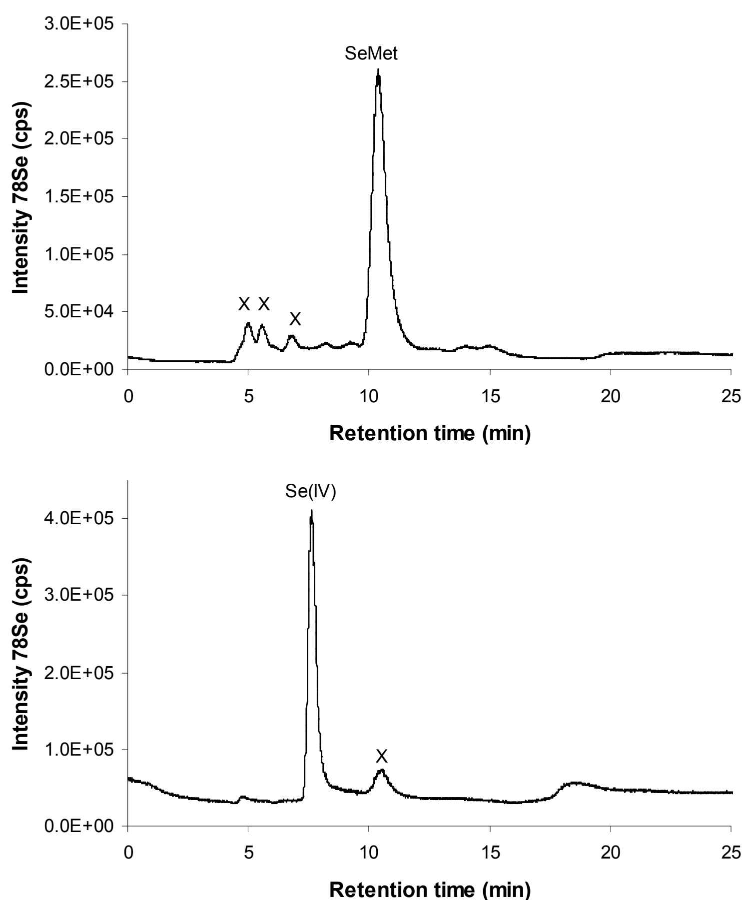


Figure 42: Chromatograms of enzymatic food supplement extracts (above sample A, below sample B) obtained after separation on an anion exchange column (Hamilton PRP-X 100) using ICP-MS as the detection system.

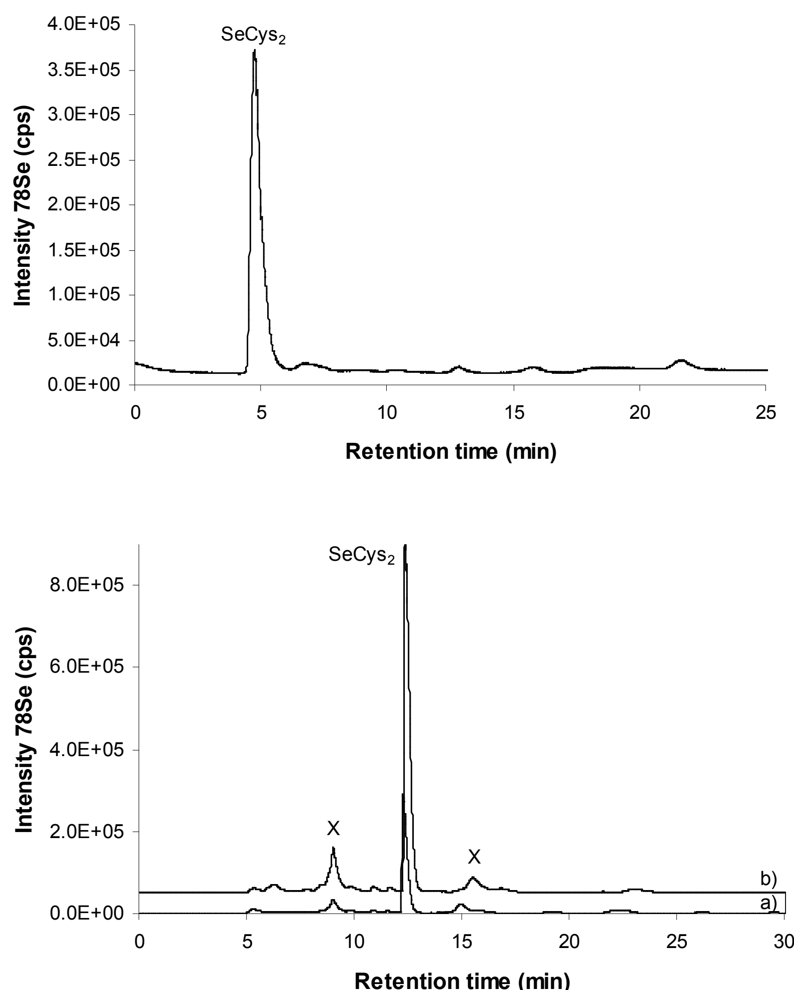


Figure 43: Chromatograms of enzymatic food supplement extract C obtained after separation on an anion exchange column (above) and on a cation exchange column (below). To form a better diagram the chromatogram obtained on a cation exchange column for sample C with $SeCys_2$ addition was transposed by $0.5x10^5$ cps upwards (below).

Suppliers often provide information on total Se content in products, but little or no information on the Se species (Figure 42, Figure 43). Since it is important to know the identity of the compounds the Se species present were investigated (Table 28). It is stated that Se was added to product A as medical yeast. We found about 75% of total Se in product A as SeMet. In product B, which does not have a declared Se form added, we detected 60% of total Se as inorganic selenium, as selenite. The declared Se species in product C was sodium selenite, but on doing speciation analysis it was not found in the extracts obtained. We were able to determine Se-cystine, which came with the void volume on the anion exchange column. To confirm this species separation on a cation exchange column and standard addition was made (Figure 43). About 55% of total Se corresponded to Se-cystine. Interferences between supplement ingredients were investigated (see section 4.1.6.2) but no effect on inorganic Se was observed. As a matter of fact, if reactions had taken place among the components during tablet, it would be difficult to say under which form Se is consumed.

From Figure 42 and Figure 43 it is seen that some other Se species were present in the extracts obtained. But, unfortunately we were not able to identify them, due to the fact that there is still lack of commercially available Se standards and their synthesis is complicated and time consuming. Moreover, the problem of identifying Se species is of major importance since their effects on human health still remain unknown. However, unidentified selenium species presented less than 5% of the main Se species determined. The major Se species identified in all products represented the quantitative amount of soluble Se. It is worrying that Se species determined and their concentrations were different in each of the three selected antioxidant food supplements. Regarding these results, the overall production of food supplements urgently needs control of Se and its species content (quality and quantity control) to assure the safety and quality of these products for the consumer's health. B'Hymer et al. (2000) indicated that labelling of yeast-based food supplements containing Se-methionine may often be inaccurate, since of the six brands of common yeast-

based Se food supplements, only two showed significant levels of selenium in the form of Se-methionine. Further, Gosetti et al. (2007) analysed commercial diet supplements and two out of six products did not have Se species present (selenite) in the supplement as declared.

Table 28: Agreement between declared and determined Se values and the species present (µg Se/tablet for product A, B, C and µg Se/g sample for CRM).

	Se content		Speciation analysis					
product	declared value	determined value	extraction with diethylether		enzymatic extraction		declared Se species added	determined Se species
			soluble	insoluble	soluble	insoluble		
A	15	14.4 ± 0.9	0.3 ± 0.1	13.8 ± 0.2	12.2 ± 0.9	1.4 ± 0.2	Se as medical yeast	75 % of total Se/ tablet (87 % of soluble Se/ tablet) as Se-methionine
B	10	9.4 ± 0.5	-	-	5.8 ± 0.1	3.7 ± 0.1	Not stated	60 % of total Se/ tablet (97 % of soluble Se/ tablet) as selenite
C	10	69.1 ± 5.0	-	-	40.4 ± 2.0	27.8 ± 1.9	Se as sodium selenite	55 % of total Se/ tablet (93 % of soluble Se/ tablet) as Se-cystine

Results reported are given as the average of two determinations along with the absolute error.

5 Conclusions

Although Se has not been confirmed to be an essential micronutrient in higher plants, it is known that some are capable of accumulating appreciable amounts of the element, though the physiological response to selenium is plant-dependent. Some plant species are Se tolerant and accumulate very high concentrations of Se (Se accumulators), but most plants are Se non-accumulators and are Se-sensitive. A knowledge of total Se content is important, but information on the elemental species present in plants is essential since the bioavailability of Se and hence the element accumulation in the organism depends on the species.

This study provides information on Se and its species distribution in Se-enriched non-accumulating plants, namely potato tubers (*Solanum tuberosum* L.) cv. Desiree, common buckwheat (*Fagopyrum esculentum*) sprouts, cv. Darja and the leafy vegetables: chicory (*Cichorium intybus* L. cv. 'Anivip' and 'Monivip'), dandelion (*Taraxacum officinale Waggner*), garden rocket (*Eruca sativa* Mill.) and wild rocket (*Diplotaxis tenuifolia* DC.). These cultivated plants were selected due to their high worldwide consumption and great nutritional value, especially for vegetarians. Plants were grown under various environmental conditions, and exposed to inorganic Se. According to the plant species, optimal growing conditions were selected. We focused particularly on Se uptake by plants, incorporation into proteins and volatilisation of the element. Moreover, the food industry is a very important sector and contributes to ensuring that products with benefits to population health and welfare are produced. It is essential to sustain and improve the Se content in food, with special stress on the Se species present. Se-enriched plants could be a key factor in achieving this and could represent an alternative to food supplements. Since presently there is no verification of Se and its species content in food supplements, they were examined, with regard to the agreement between Se and its species contents determined and the values advertised. To acquire this information, the development and optimisation of analytical methodologies for Se species characterization in these matrices were crucial. Analytical methods for separation and detection of soluble (HPLC-ICP-MS) and volatile Se species (SPME-GC-MS) were developed.

In plants, Se is mainly incorporated in proteins. Before a chromatographic separation, the analytes have to be enzymatically extracted from the solid plant sample in which identification and determination of selenium species is intended. For this purpose, several commercially available enzymes (protease, lipase, cellulase, amylase) can be used. However, we first checked if they are pure enough to be used for selenium speciation analysis. Most of the enzymes studied contained detectable amounts of selenium. In some cases we found large variations in Se content between different lots of the same enzyme. The presence of selenium species could be due to the preparation procedure used for the enzyme, as a degradation product. Therefore, correction was and should be always made for Se species from the enzyme used, otherwise results for such samples will be too high.

Further, the enzymes mentioned were evaluated in order to obtain the highest selenium extraction efficiency from plant samples. A one-step extraction, using the non-specific enzyme protease from *Streptomyces griseus*, using a ratio between the enzyme and sample of 1:10, was found to be the most appropriate, regardless of the sample used. 24 h incubation with constant stirring (200 rpm) and temperature (37 °C) was preferred to exposure to ultrasound, due to the higher extraction efficiency achieved. For the separation of soluble Se species (Se(VI), Se(IV), SeMet, $SeCys_2$, SeMeSeCys), anion (Hamilton PRP-X 100) and cation (Zorbax 300-SCX) exchange chromatography was selected. Gradient elution, using 3 mM and 10 mM citrate buffer (pH 4.8) in 2% MeOH was chosen for anion exchange chromatography and isocratic elution with 3 mM pyridine solution (pH 2.1) in 2% MeOH was chosen for cation exchange chromatography. ICP-MS with an octopole reaction system was used as the detection system. Hydrogen, with a flow rate between 4 and 4.5 mL/min was used as the reaction/collision gas. The most appropriate isotope for detection was found to be ^{78}Se. Since there are no certified reference materials available for the contents of Se species in materials of plant origin, the accuracy of Se species determination using the developed method was checked by analysing the certified reference material Selenium Enriched Yeast, SELM-1, which is certified for total as well as for SeMet content, and the standard reference material Durum Wheat Flour, NIST RM 8436, that is certified for total Se content and for SeMet literature data exist. All the results obtained were in agreement with the certified and literature

data.Since the stability of Se species in the matrix examined is rarely reported in the literature, we considered reactions that can take place between Se species and the matrix components (common phenolic compounds, such as the tannins, flavonoids and several other antioxidants, like coenzyme Q10, beta-carotene, vitamin E) during the extraction and during extract storage. The results showed a strong decrease in Se(IV) (100 ng/g solution) response when phenolics were added together in the ratio 1:100 (rutin (0.2 mg/g solution) : tannin (20 mg/g solution)), higher in water (60%) and lower in enzyme (20%) solution. The response was comparable to the one obtained for buckwheat seeds, where the natural ratio between flavonoids corresponds to the tests performed *in-vitro*. It is most likely that Se(IV) was reduced to Se(0) or that an insoluble complex was formed, which was no longer detectable by the separation and detection system used. Signals for other Se species and for other parts of buckwheat, as well as for potato tubers and leafy vegetables, were stable. Se species were stable in the presence of other antioxidants; also the extracts after hydrolysis were stable for 30 days with proper storage at minus 20 °C, regardless of the plant material used.

The next important information is the distribution of selenium between insoluble, soluble and volatile fractions (mass balance calculations), which are often overlooked. A mass balance calculation is the key factor in determining species volatility during sample preparation. Since not all of the mass balances were quantitative, a method for the determination of volatile organo seleno compounds (DMSe and DMDSe) was developed, using SPME-GC-MS. Headspace sampling was needed, and therefore the incubation time was shortened to 30 min, using ultrasound treatment instead. Due to its better sensitivity (approximately 100-times for DMSe and 2-times for DMDSe), a 75 μm Carboxen-PDMS fiber coating was chosen over 100 μm PDMS, taking 15 min as the optimal adsorption time. For the separation of Se species a non-polar DB-5 MS column was applied.

The methods developed were used to determine Se species transformation in selected plants, enriched with Se. Potato was enriched in Se by foliar spraying with an aqueous solution containing 10 mg Se per L in the form of Na_2SeO_4. Four combinations of treatments were utilised: well watered plants with and without Se foliar spraying, and drought exposed plants with and without Se foliar spraying. Well-watered potatoes foliarly sprayed with Se(VI) solution took up more Se (1101 ng/g DM) than drought exposed ones (347 ng/g DM). Enzymatic extraction gave almost quantitative extraction efficiency. Approximately 80% of Se was present in soluble form, of which SeMet and Se(VI) shared equal parts, 30-40% each. The Se species present in Se-enriched potato were not treatment dependent, and the ability of the plant to transform inorganic Se into organic forms was not reduced by water deficiency. Apparently, potato tubers were able to transform a high amount of added Se to SeMet, indicating that the added Se was accessible to plant metabolism.

Seeds of common buckwheat, cv. Darja, were soaked (4 h) in various concentrations of Na_2SeO_4 (5, 10, 20 mg/L), Na_2SeO_3 (5, 10, 20 mg/L) and SeMet (10 mg/L). When the buckwheat sprouts developed two extendend cotyledon leafs sprout sampling was done. The results showed that the uptake of selenium by seeds was dependent on the form and concentrations of selenium in the solution used for soaking, and the order was as follows: Se(VI) > SeMet > Se(IV). Soluble Se represented 36-42% for Se(VI), 19-22% for Se(IV) and around 17% for SeMet sprouts, regardless of the extraction procedure used. Low extraction efficiency could correspond to the plant developing a protective mechanism, despite the short growing period of buckwheat sprouts (< 22 days). One possibility is the formation of the insoluble selenobis(S-glutathionyl)-arsinium ion (Gailer et al., 2000) which could prevent Se transformation. Sprouts had the ability to transform Se to SeMet, but its content was low (< 8 %), irrespective of the soaking solution used. This shows that buckwheat sprouts were not able to take up and/or transform Se(VI), Se(IV) or SeMet present in the soaking solution to SeMet, indicating that added Se was not accessible to plant metabolism. Other soluble Se species found were Se(IV) and Se(VI), as well as an unknown species in Se(IV) treated sprouts. Further, by performing mass balance calculation, about 20-35% losses in Se(VI) and SeMet dosed sprouts were observed. On analysing volatile Se species, DMSe was found at trace level, while the DMDSe content was below the detection limit of the method. From the low extraction efficiency and mass balance calculation we assumed that an insoluble Se component was formed, which was difficult to decompose. Therefore, higher temperatures were needed and microwave digestion with ICP-MS detection was used, instead of mineralization in a closed system and HG-AFS detection. Then a quantitative mass balance was achieved. Additionally, Se(VI) addition had no influence on the content of several other elements, important for plant metabolism (S, Mn, Fe, Cu, Zn, Mo and Cd), probably due to a relatively low plant Se content (below 15 μg/g DM).

Dandelion, salad rocket, wild rocket and two cultivars of chicory were foliarly sprayed twice (at 5 day intervals) with a nutrient solution of various concentrations (1+1; 2+2; 5+5; 10+0; 10+10 and 10+50 mg

Se/L) in the form of Na_2SeO_4. Interestingly, no toxic effects on plants were observed despite the high Se concentrations used. The Se content in plants varied from less than 1 μg/g DM (those sprayed with 1+1 mg Se/L) to as high as 100 μg/g DM (those sprayed with 10+50 mg Se/L). About 50% of the total Se was present in soluble form, regardless of the concentration range and vegetable analysed, indicating that 50% of total Se remained in insoluble form. Beside inorganic Se (Se(VI), i.e. the form with which the plants were sprayed), SeMet was identified in the extracts after enzymatic hydrolysis. Apparently, the ability of plants to convert inorganic Se into SeMet decreased with increasing concentration, regardless of plant species. Vegetables were able to convert 7-40% of total Se to SeMet, when sprayed with 2+2 and 5+5 mg Se/L. On increasing the Se content in the spraying solution to 10+50 mg Se/L, the Se content decreased to 2-4%. There were probably some protective mechanisms operating, similar to those of Se accumulating plants. One possibility is storage of Se(VI) in vacuoles, as this is the main form found in extracts. Since SeMeSeCys was present in the 10+50 extracts, the other possibility is the formation of the dipeptide γ-glutamyl-SeMeSeCys (Terry et al., 2000). Additionally, unknown Se species were found in plants sprayed with 2+2 and 10+50 mg Se/L, but they were present only in trace amounts. By indicating the effect of high Se(VI) addition on the content of several other elements important for plant metabolism, the S content was found to decrease with increasing Se content in Se-enriched chicory and dandelion, showing an antagonistic relationship between Se and S. In contrast, for the S content in rocket an inverse relation was observed. Some correlations could also be drawn for other elements (Mn, Fe, Cu, Zn, Mo and Cd). These differences could be attributed to the high Se content in plants in comparison with potato and buckwheat sprouts, where these effects were not observed. The high Se content lead to the activation of plant detoxification mechanisms, explaining the response of other essential elements and the lower SeMet content.

Among the Se species, SeMet is one of the most interesting for human health because it is considered to have the highest rate of absorption and retention in the tissues which serve as a safe and stable storage mode for Se. From the results obtained, it can be concluded that despite the high total Se concentration found in Se-enriched plants, the amount of Se as SeMet could be significantly lower, and depends considerably on the plant type. Additionally, part of soluble Se unstayed identified for all the investigated plants, amounting to approximately 10-20%. This could be ascribed to losses of unknown Se species on the column (formation of high-molecular weight species), which we were unable to determine with the system used. Another problem is the high content of insoluble Se, especially in the case of buckwheat sprouts, where more than 60% of total Se stayed in the residue. To obtain further insight into this, other techniques should be applied.

Since there is no verification of the selenium and its species content in marketed food supplements, which are widely used in recent years, the method developed was used for the analysis of such antioxidant food supplements, with special stress on the Se species present. Se values obtained for products A and B were in agreement with the advertised values, while on the other hand, the value of Se obtained for product C far exceeded the declared value: instead of 10 μg Se per tablet, 69 μg of Se per tablet was found and therefore supplement C represents a potential risk of Se poisoning. The Se species added was declared in one out of the three products, namely product C (Se as sodium selenite). In product A Se was added as medical yeast. About 75% of total Se in product A was present as SeMet. In product B which did not have a declared form of Se added, we detected 60% of total Se as inorganic selenium, as selenite. The declared Se species in the product C was sodium selenite, but on performing speciation analysis it was not found. About 55% of the total Se corresponded to Se-cystine. However, the main ingredients in product C (coenzyme Q10, beta-carotene, vitamin E), as well as temperature and/or incubation, did not affect the presence and transformation of inorganic Se species. Therefore, the overall production and sale of food supplements urgently needs to be controlled for Se and its species content (quality and quantity control) to assure the safety and quality of these products.

Further work in Se speciation should include development of efforts for the identification of all soluble Se-compounds present in various samples. The complexity of these matrices together with the unavailability of Se-compounds as standards makes the determination of the unidentified Se species difficult. These efforts will require the synthesis of new Se standards, and the development of a more appropriate enzyme in order to improve the efficiency of hydrolysis of Se-proteins. In recent years, molecule-specific methods, such as ES and/or MALDI mass spectrometry, which provide structural information on the compounds analysed, have been applied to the identification of Se species.

For insoluble Se compounds, techniques that enable analysis of solid sample, such as XANES and/or XPS need to be introduced to Se speciation analysis. Applying these methods to these matrices will constitute an important tool to determine and identify additional Se species than those usually determined.

6 Acknowledgements / Zahvala

Doktorsko disertacijo sem opravljala na Odseku za znanosti o okolju Instituta Jožef Stefan. Mentorici prof. dr. Vekoslavi Stibilj se iskreno zahvaljujem za zanimivo temo doktorske disertacije, strokovno vodenje, usmerjanje, predloge, življenjske nasvete in vsestransko oporo, ki mi jo je nudila med nastajanjem dela.

Hvala predsednici komisije za zagovor prof. dr. Mileni Horvat ter članoma komisije za zagovor akademiku prof. dr. Ivanu Kreftu in doc. dr. Radmili Milačič za pregled naloge. Akademiku prof. dr. Ivanu Kreftu gre posebna zahvala za oskrbo poskusov na Biotehniški fakulteti.

Hvala vsem sodelavcem z Odseka za znanosti o okolju za pomoč pri delu, prijaznost, spodbude in ustvarjanje prijetnega delovnega vzdušja.

Posebna zahvala gre fantu Matjažu, vsem domačim in prijateljem, ker ste mi vedno stali ob strani, me spodbujali in verjeli vame.

Hvala Ministrstvu za visoko šolstvo, znanost in tehnologijo Republike Slovenije za financiranje.

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Appendix: Personal bibliography for the period 2005-2010

PETRA CUDERMAN [26453]

ARTICLES AND OTHER COMPONENT PARTS

1.01 Original scientific article

1. CUDERMAN, Petra, HEATH, Ester. Determination of UV filters and antimicrobial agents in environmental water samples. *Anal. bioanal. chem.*, 2007, vol. 387, no. 4, str. 1343-1350. [COBISS.SI-ID 20651559]

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CUDERMAN, Petra, STIBILJ, Vekoslava. How safe are antioxidant food supplements containing selenium? *Acta Chimica Slovenica*

CUDERMAN, Petra, ŽNIDARČIČ, Dragan, OSVALD, Jože, STIBILJ, Vekoslava. Selenium species in selected leafy vegetables sprayed with Se(VI) in the growing period. *Journal of the Science of Food and Agriculture*

1.08 Published scientific conference contribution

7. CUDERMAN, Petra, HEATH, Ester, ZUPANČIČ-KRALJ, Lucija. Določanje nekaterih ostankov kozmetičnih izdelkov v vodah = Determination of some personal care products residues in water. V: GLAVIČ, Peter (ur.), BRODNJAK-VONČINA, Darinka (ur.). *Slovenski kemijski dnevi 2005, Maribor, 22. in 23. september 2005*. Maribor: FKKT, 2005, str. [1-10], graf. prikazi. [COBISS.SI-ID 27021573]

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9. CUDERMAN, Petra, HEATH, Ester, ZUPANČIČ-KRALJ, Lucija. Determination of personal care products in water = Določitev ostankov sredstev za osebno nego v vodnih vzorcih. V: 12th Young Investigators` Seminar on Analytical Chemistry, Sarajevo, July 5-10, 2005. *Book of abstracts*. Sarajevo: University of Sarajevo, Faculty of Natural Sciences and Mathematics, Department of Chemistry, 2005, str. 83. [COBISS.SI-ID 19656231]

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