

DETERMINATION OF SELENIUM COMPOUNDS
IN SELECTED CULTIVATED AND AQUATIC
PLANTS

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Doctoral Dissertation
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DOLOČANJE SELENOVIH SPOJIN V IZBRANIH
KMETIJSKIH IN VODNIH RASTLINAH

Doktorska disertacija

Supervisor: Prof. Dr. Vekoslava Stibilj

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*Education is the most powerful weapon which you
can use to change the world.*
Nelson Mandela

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Abstract

Selenium is known as an essential element for humans and animals. It is mainly taken with food, where content is usually low. Content in plants varies from less than 0.1 µg Se/g to 0.8 µg Se/g, while in other food products, such as fish, selenium content is normally higher. To overcome low selenium content in food and low selenium status in humans, biofortification of plants is promising approach. Moreover, simultaneous biofortification with selenium and iodine is next logical step, due to low iodine intake and close relationship between selenium and iodine in thyroid gland functioning, thyroid hormone biosynthesis and metabolism. Before introduction of selenium fortified food into food chain, selenium content and distribution of selenium compounds need to be evaluated. Determination of selenium and its compounds is challenging task due to normally low content in samples, its volatility and lack of reference material for selenium compounds.

In this doctoral dissertation, plants from different families were fortified with selenium in forms of Se(IV) and Se(VI) with different fortification methods – seeds soaking and foliar application. In selected plants, selenium content and presence of selenium compounds were determined. Iodine, known as essential element for humans and animals, was simultaneously added in forms of I(-I) and I(V) to fortified solution to find out the effect of its presence on content of selenium and its compounds.

First, relevant analytical parameters for microwave oven digestion of plant samples for the determination of selenium content and method for separation and determination of selenium compounds with anion exchange column were optimized. Due to changed analytical parameters, stability tests of selenium compounds were performed. For accuracy and precision of optimized method, certified reference materials were used. In addition, other chromatographic techniques were used to verify determined selenium compounds.

With optimized methods, selenium content and compounds were measured in pea (*Pisum sativum* L.), pumpkin (*Cucurbita pepo* L.) and buckwheat (*Fagopyrum esculentum* Moench) sprouts, grown from seeds, soaked in the 10 mg Se(IV)/L and 10 mg Se(VI)/L solution. In all fortified sprouts, selenium content was enhanced according to control plants. Almost in all treatments, SeMeSeCys, SeMet, Se(IV) and Se(VI) were present. Presence of iodine (1000 mg I(-I)/L and 1000 mg I(V)/L) in soaking solution had different effect on obtained content of selenium and its compounds with no general rule.

Selenium content and its compounds were determined also in edible parts of plants – pea seeds, pumpkin seeds, buckwheat achenes and chicory leaves (*Cichorium intybus* L.) after foliar application with selenium solution (10 mg Se(IV)/L and 10 mg Se(VI)/L). In addition, selenium content was determined in other parts of pea plant. Selenium content was increased in all edible parts of fortified plants in different levels. The main selenium compound in all treated plants was SeMet, regardless of solution and plant species. The effect of iodine (1000 mg I(-I)/L and 1000

mg I(V)/L) presence on selenium and its compounds depend on the forms of iodine and plant species. Selenium compounds in pumpkin seeds and buckwheat achenes were not effect by iodine.

The effect of selenium on content of iodine and its compounds in sprouts and adult plants are described in the doctoral dissertation by Jerše (2017).

In the last part of doctoral dissertation, selenium and its compounds were determined in aquatic plant water speedwell (*Veronica anagallis-aquatica*) and sediment sampled in the three Slovenian watercourses where different activities were present: daily farming, stock raising and extensive agriculture. Before determination of selenium content in sediment samples, analytical method was optimized. Results showed no effect of agriculture on selenium content in sediments. On the other hand, *V. anagallis-aquatica* accumulated great amount of selenium without showing symptoms of toxicity. Selenium compounds Se(VI) and Se(IV) were found. Moreover, selenium content and compounds were compared with cultivated plant from present study. Despite high selenium content in *V. anagallis-aquatica*, selenium was not present in form of SeMet like we expected.

Povzetek

Selen je poznan kot esencialen element za ljudi in živali. Največji delež selena vnesemo v telo s hrano, kjer je selen prisoten v nizkih koncentracijah. Selen se v rastlinah nahaja v koncentracijskem območju manj kot 0.1 µg Se/g do 0.8 µg Se/g, medtem ko je koncentracija v ribah višja. Da bi s hrano zagotovili zadostni vnos selena, se v zadnjih letih vedno pogosteje uporabljajo metode obogatitve rastlin s selenom. Zaradi tesne povezave selena in joda pri delovanju ščitnice, sinteze ščitničnih hormonov in metabolizma ter pomanjkanja joda v prehrani, je hkratno dodajanja selena in joda smiselni korak k izboljšanju statusa selena in joda pri ljudeh. Pred uvedbo živil obogatenih s selenom, je potrebno določiti koncentracije selena in njegovih spojin. Določitev selena in njegovih spojin je težavna naloga, zaradi njegovih nizkih koncentracij, hlapnosti ter pomanjkanja ustreznih referenčnih materialov.

V doktorski disertaciji smo uporabili dva načina obogatitve rastlin s 10 mg Se/L v obliki Se(IV) in Se(VI): namakanje semen v raztopinah in foliarno škropljenje. V obogatenih rastlinah smo določili koncentracijo selena ter njegovih spojin. Ker je jod poznan kot esencialen element za ljudi, smo raztopinam v ločenih eksperimentih dodali tudi jod v dveh različnih oblikah (I(-I) in I(V)) s koncentracijo 1000 mg/L, da bi ugotovili njegov vpliv na privzem selena in metabolizem selenovih spojin.

Za določitev selena v rastlinskih vzorcih smo optimizirali metodo za razkroj z mikrovalovno pečico, medtem ko smo za določitve selenovih spojin optimizirali metodo za ločbo selenovih spojin z anionsko kolono. Zaradi spremenjenih pogojev ločbe, smo izvedli tudi test stabilnosti selenovih spojin v daljšem časovnem obdobju. Pravilnost in zanesljivost uporabljenih metode smo preverili z certificiranimi referenčnimi materiali ter z drugimi neodvisnimi metodami.

Selen in njegove spojine smo določili v obogatenih kalicah graha (*Pisum sativum* L.), buč (*Cucurbita pepo* L.) in ajde (*Fagopyrum esculentum* Moench). Koncentracije selena so bile povišane v kalicah vseh vrst v primerjavi s kontrolnimi kalicami. V kalicah smo našli sledeče selenove spojine: SeMeSeCys, SeMet, Se(IV) in Se(VI). Jod, prisoten v raztopini, je imel na privzem in metabolizem selena različen vpliv.

Foliarno škropljenje graha, ajde, buč in radiča s selenom je obogatilo seme graha, buč in ajde ter listov radiča (*Cichorium intybus* L.) v primerjavi s kontrolnimi rastlinami. Glavna identificirana selenova spojina v obogatenih semenih in listih je bila SeMet, ne glede na vrsto rastline ter obliko selena prisotnega v foliarni raztopini. Vpliv joda na koncentracijo selena in njegovih spojin je bil odvisen ob oblike joda in vrste rastline. Prisotnost joda v foliarni raztopini ni imela vpliva na vsebnost selenovih spojin v semenih buč in zrnju ajde.

Vpliv selena na vsebnost joda in jodovih spojin je opisan v doktorski disertaciji Jerše, (2017).

V zadnjem delu doktorske dizertacije smo določili selen in njegove spojine v vodni rastlini vodnem jetičniku (*Veronica*

anagallis-aquatica) in sedimentu, ki smo ju vzorčili v treh slovenskih vodotokih z različnimi vplivi v zaledju. Pred določitvijo selena v sedimentu smo optimizirali metodo razkroja z mikrovalovno pečico. Ugotovili smo, da ekstenzivno kmetijstvo nima vpliva na koncentracije selena v sedimentih. Kljub povišanim koncentracijam selena v *V. anagallis-aquatica*, rastlina ni imela vidnih znakov zastrupitve. Selen je bil v njej prisoten v obliki Se(IV) in Se(VI). Pri primerjavi vodne rastline *V. anagallis-aquatica* ter kmetijskih rastlin iz naše raziskave smo ugotovili, da kljub visokim koncentracijam selena v vodni rastlini *V. anagallis-aquatica* selen ni bil prisoten v obliki SeMet kot smo pričakovali.

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Abbreviations

BAF	...	Bioaccumulation factor
GPx	...	Glutathione peroxidase
HG-AFS	...	Hydride generation atomic fluorescence spectrometry
HPLC	...	High performance liquid chromatography
ICP-MS	...	Inductively coupled plasma mass spectrometry
DL	...	Limit of detection
DW	...	Dry weight
Se	...	Selenium
SeCys	...	Selenocysteine
SeCys ₂	...	Selenocystine
Se(IV)	...	Selenite
Se(VI)	...	Selenate
SeMeSeCys	...	Selenomethylselenocysteine
SeMet	...	Selenomethionine
SePP	...	Selenoprotein P
As	...	Arsenic
Cr	...	Chromium
Cu	...	Copper
Pb	...	Lead
Zn	...	Zinc
ICP-QQQ	...	Inductively coupled plasma tandem mass spectrometry
SEC	...	Size exclusion chromatography
AC	...	Affinity chromatography
AEC	...	Anion exchange chromatography
CEC	...	Cation exchange chromatography
RT	...	Retention time
CE	...	Capillary electrophoresis
HPLC-ICP-MS	...	High performance liquid chromatography coupled with inductively coupled plasma mass spectrometry
LC	...	Liquid chromatography
RPC	...	Reverse phase chromatography
HG	...	Hydride generation
HCL	...	Hallow cathode lamp
SPME	...	Solid phase micro extraction
ESI-MS/MS	...	Tandem mass spectrometry with electrospray ionization
FAAS	...	Flame atomic absorbance spectrometry
IEC	...	Ion exchange chromatography
TFA	...	Triflouroacetic acid
HG-AAS	...	Hydride generation atomic absorbance spectrometry
AFS	...	Atomic fluorescence spectrometry
ICP-AES	...	Inductively coupled plasma atomic emission spectrometry
GC	...	Gas chromatography
XANES	...	X-ray absorption near edge structure
EXAFS	...	Extended x-ray absorption fine structure
XRD	...	X-ray diffraction

NMR	. . .	Nuclear magnetic resonance
HPLC-HG-AFS	. . .	High performance liquid chromatography coupled to hydride generation atomic fluorescence spectroscopy
NAA	. . .	Nuclear magnetic resonance
Eh	. . .	Redox potential

Chapter 1

Introduction

Selenium, the element of the Moon, was discovered in 1817, during production of sulphuric acid (Combs & Combs, 1986). It is a chemical element with the symbol Se and atomic number 34. It is a metalloid, located in the group XVI. of the periodic table, together with the oxygen, sulphur and tellurium. Selenium belongs to the group of the rarest elements, in 70th in terms of abundance from 88 naturally occurring elements in the earth's crust. In nature it is present in four oxidation states: -II (Na_2Se , sodium selenide), 0 (Se, elemental selenium), +IV (Na_2SeO_3 , sodium selenite) and +VI (Na_2SeO_4 , sodium selenate). In addition to inorganic forms, selenium is present in the nature in organic forms: from simple organic compounds (methylselenol, dimethylselenide, diethylselenide), amino acids and low molecular compounds (selenomethionine SeMet, selenocysteine SeCys, selenocystine SeCys₂, selenomethylselenocysteine SeMeSeCys, selenoglutatione) to other compounds such as selenoproteins, selenoenzymes or selenosugars. Six stable ⁷⁴Se (0.87%), ⁷⁶Se (9.02%), ⁷⁷Se (7.58%), ⁷⁸Se (23.52%), ⁸⁰Se (49.82%) and ⁸²Se (9.19%) and three unstable (⁷²Se, ⁷⁵Se and ⁷⁹Se) isotopes are known. Unstable isotope ⁸¹Se is induced in a nuclear reactor and is not present naturally in the environment (Fernández-Martínez & Charlet, 2009). Until 1957, when Schwarz & Foltz (1957) published that additions of selenium prevented liver necrosis in rats (*Rattuss* pp.), selenium was considered a dangerous substance because of its toxicity. Its role in human health was reported in 1973, when selenium in glutathione peroxidase (GSHx), an enzyme that protects against oxidative cell damage, was discovered (Flohe, Günzler, & Schock, 1973). In the last three decades, there has been high interest in selenium research because of its known physiological importance for humans and animals (Combs & Combs, 1986; Flohé, Andreesen, Brigelius-Flohé, Maiorino, & Ursini, 2000).

1.1 Selenium Cycling

The global selenium distribution is mainly determined by a various sources that affect its presence in the environment (Figure 1.1). In the atmosphere, natural (crustal erosion, biological production of volatile organoselenium compounds, volcanoes, aerosol particles associated with sea spray) and anthropogenic (metal refining and coal combustion) sources are present. Natural sources contribute only 50 – 65% of total emissions. Several physical and chemical transformations occur before selenium is deposited in the ground (Sharma et al., 2014; Wen & Carignan, 2007). It can be removed from the ecosystem and bound in soil through natural processes of chemical and microbial reduction of selenate Se(VI) to selenite Se(IV) . The reduction is followed by adsorption to clay, reaction with iron species, and co-precipitation or settling. After it has settled into the sediment, chemical and microbial reduction may occur, causing insoluble organic, elemental or adsorbed selenium species. Some organic forms may be released into the atmosphere through volatilization by chemical or microbial activity in the water and sediment or by direct release from plants. Immobilization processes effectively remove selenium from the ecosystem, especially in slow-moving or still-water areas (Lemly, 1999).

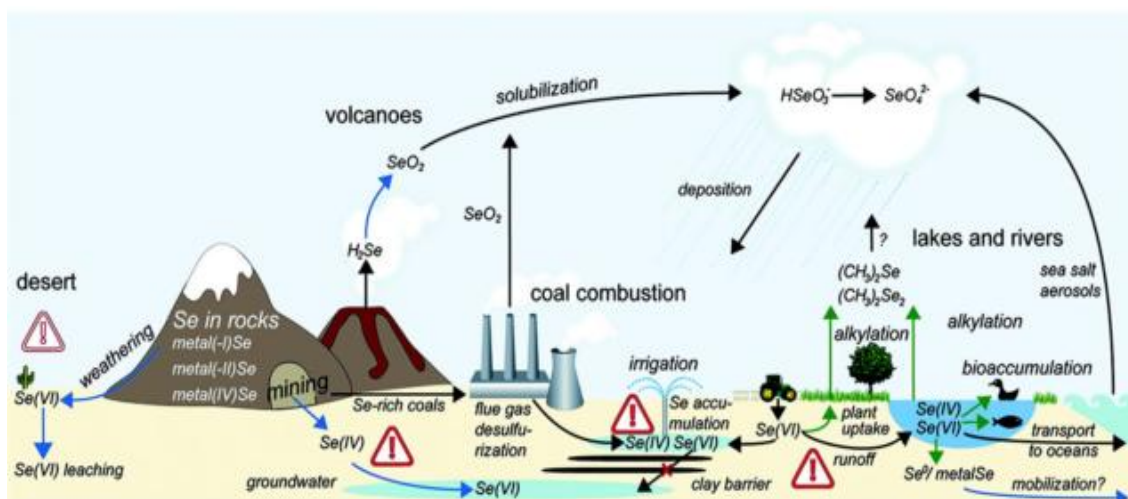


Figure 1.1: Schematic global cycle of selenium. Blue arrows indicate processes that involved oxidation, green arrows indicate processes that involve reduction. Warning symbols indicate specific environmental settings that are at risk of either developing selenium deficiency (open warning symbol) or selenium excess (shaded warning symbol) (Winkel et al., 2012).

The most significant transport processes for selenium are hydrological processes. About 14,000 tons of selenium per year are transferred along aquatic pathways from the continents to the oceans, in two possible ways: via selenium-bearing sediments, responsible for 85% of this transport, or via soluble compounds (Fernández-Martínez & Charlet, 2009).

In order to achieve selenium monitoring successfully, it is important to pay attention to its cycling in waters and, even more, bioaccumulation in water organisms. When dissolved selenium enters the aquatic ecosystem three things can happen:

- It can be absorbed or ingested by organisms,
- It can bind or complex with particulate matter or surface sediments, or

- It can remain free in solution.

Over a period of time, most of the selenium is either taken up by organisms or bound to particulate matter. Because of deposition and settling of particulate matter (sedimentation), most of the biologically incorporated selenium usually accumulates in the top layer of sediment. However, biological, chemical and physical processes move selenium out of, and into the sediments, so the sediments are only a temporary reservoir for selenium. Aquatic systems are dynamic, and selenium can be moved back into the biota and remain at elevated levels for years after waterborne inputs of selenium are stopped (Lemly, 1999, 2004).

1.1.1 Selenium in the Slovenian environment

Slovenia is known to be a country with low selenium concentrations in soil. In research by Pirc & Šajn (1997), less than 0.1 to 0.7 µg Se/g were found in Slovenian soils. The addition of up to 0.5 µg Se/g in the forms of Na₂SeO₃ (sodium selenite) or Na₂SeO₄ (sodium selenate) in feedstuff is allowed according to legislation in European Union (Council Directive 2004/C 50/01, 2004) and Slovenian legislation (RS, 2002). Selenium addition in feedstuff, in the form of Na₂SeO₃ or Na₂SeO₄, started in Slovenia in 1989 (SFRJ, 1989). While animals do not absorb whole selenium from feedstuff, part of it is directly extracted (Nahapetian, Janghorbani, & Young, 1983) and can potentially be released into the environment by the use of liquid and slurry manure.

The selenium content in the Slovenian environment is not well studied. There have been only a few studies in which the selenium content was determined (Byrne, Ravnik, & Kosta, 1976; Gnamuš, Byrne, & Horvat, 2000). In recent years, only one study by Mechora, Germ & Stibilj (2014) was published, in which different aquatic plants in Slovenian watercourses with different land use in their catchment area were investigated. Selenium content was highest in aquatic moss *Fontinalis antipyretica* (3.038 µg Se/g DW) and *V. anagallis-aquatica* (1.507 µg Se/g DW).

1.2 Selenium Essentiality and Dietary Intake

Until 1957, when Schwarz and Foltz discovered a connection between selenium deficiency and liver, muscle and heart diseases, it was thought that selenium is only toxic. Nowadays we know, that the range between deficiency, essentiality and toxicity of selenium is narrow and that the difference between intake deficiency and toxicity is also small: less than 0.1 µg Se/g of body weight for dietary deficiency and more than 1 µg Se/g for toxic effect (Levander, 1987). An adequate selenium intake has an important role in human and animals health (B'Hymer & Caruso, 2006; D'Ulivo, 1997; Fernández-Martínez & Charlet, 2009; Pyrzynska, 2002b). Fernández-Martínez & Charlet (2009) published that less than 40 µg per day is deficiency and more than 400 µg Se per day is toxic. Selenium is constitutional for various enzymes from the glutathione peroxidases family (which has the oxidative role of peroxides and hydroperoxides). It has been found that selenium can play an important role in anti-carcinogenic activity and prevention of heavy metal toxic effects (Pyrzynska, 2002b). It has been considered to be an exhibitor of anti-diabetic and insulin-mimetic properties for a long time. In 2010, a study in which participants with long term high selenium intake (200 µg/day for a period of 12 years) were more likely to develop type 2 diabetes mellitus than those in the group with placebo effect (Steinbrenner, Speckmann, Pinto, & Sies, 2010).

The daily intake of selenium for humans and animals depends on its concentration in food and the amount of food consumed. The Recommended Daily Intake (RDI) for selenium is set at 70 μg per day for men and women in the European Union to maximize synthesis of the selenoprotein glutathione peroxidase (GPx) ("Dietary Reference Values for selenium," 2014; Stoffaneller & Morse, 2015). Pograjc, Stibilj, & Falnoga (2012) reported that daily intake in Slovenian recruits was $48 \pm 10 \mu\text{g/day}$, which is below the recommended value for the European Union. In cereals and grains, the selenium content varied from less than 0.1 to more than 0.8 $\mu\text{g Se/g}$, while in fruits and vegetables, the content is less than 0.1 $\mu\text{g Se/g}$. Concentrations of selenium are greater in animals and animal products: in seafood from 0.4 – 1.5 $\mu\text{g Se/g}$ and in muscle meat 0.1 – 0.4 $\mu\text{g Se/g}$ (DRI, 2000).

For humans, in addition to dietary intake the bioavailability of ingested selenium is also important (Navarro-Alarcon & Cabrera-Vique, 2008). Bioavailability is expressed as the proportion of ingested nutrient that is used for normal physiological functions or storage (Tapiero, Townsend, & Tew, 2003). Selenium bioavailability depends on its chemical forms in ingested food. Identified selenocompounds in plants are selenate, selenite, SeCys, SeMet, selenohomocysteine, selenomethyl, selenocysteine, γ -glutamyl-selenocystathionine etc. On the other hand, SeCys, selenotrisulfides of cystine, selenate and selenite have been found in animal tissues. Organic compounds of selenium are more bioavailable than inorganic forms. Around 80% of ingested selenium is absorbed (depend on the types of food consumed). The availability of nutrient is influenced by the total protein, fat and presence of heavy metals (Navarro-Alarcon & Cabrera-Vique, 2008).

Most common selenium sources in food are organoselenium compounds (Table 1.1). Inorganic compounds (selenite, selenate) are present only as supplements or contaminants. SeCys is present in selenoproteins forms (selenoenzymes, transcription factors) in plants and animals, while SeMet is present as SeMet randomly substituting Met in general proteins and also as free SeMet in seleno accumulator plants. Ingested selenium is metabolized to different forms (Figure 1.2). There are many different input selenium compounds and their metabolic pathways for selenocompounds, but almost all led to intermediate hydrogen selenide (H_2Se) (B'Hymer & Caruso, 2006; Suzuki, 2005). Selenide H_2Se is used for the synthesis of selenoproteins, which are important for normal cell functioning and human health. SeCys are directly synthesized on t-RNA and they are designated t-RNA^{[Ser]Sec} complex. That complex reads the UGA codon and is used for the integration of SeCys into the amino acidic sequence to form selenoproteins. SeCys is admitted as the 21st amino acid, because of synthesis, which is genetically encoded in the ribosome-mediated system (Roman, Jitaru, & Barbante, 2014).

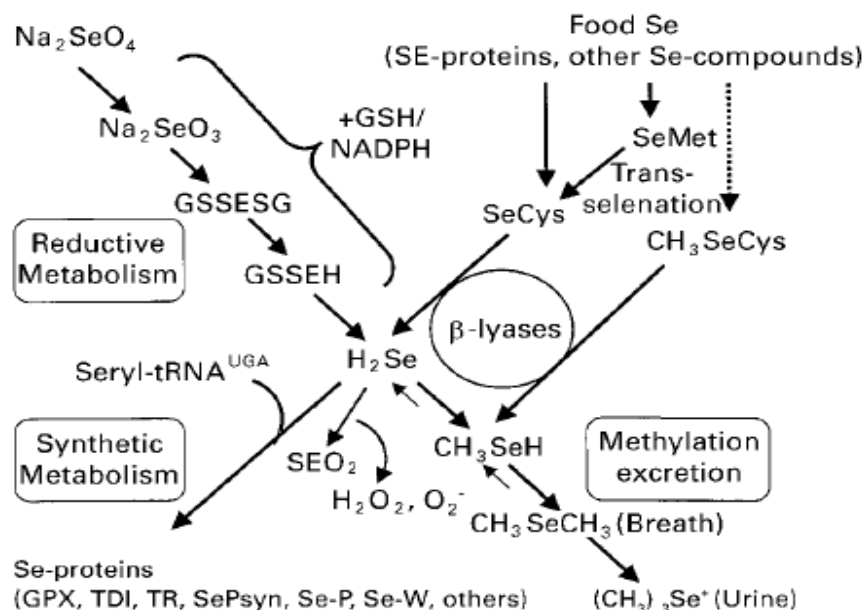


Figure 1.2: Pathways of selenium metabolism in humans (Combs, 2001).

Table 1.1: Selenium compounds in environmental in biological systems (summarized from Uden, Boakye, Kahakachchi, & Tyson, (2004)).

Group	Selenium compound	Chemical form
Inorganic forms	Elemental selenium	Se
	Selenide	Se ²⁻
	Selenate	SeO ₄ ²⁻
	Selenite	SeO ₃ ²⁻
Organic forms	Methyl selenole	MeSeH
	Dimethylselenide	Me ₂ Se
	Dimethyldiselenide	Me ₂ Se ₂
	Trimethylselenonium ion	Me ₃ Se ⁺
	Dimethylselenone	Me ₂ SeO ₂
	Dimethylselenoxide	Me ₂ SeO
	Dimethylselenosulfide	MeSSeMe
	Selenocyanate	SeCN ⁻
	Selenourea	Se=C(NH ₂) ₂
	Methyl selenonyl ion	MeSe(O)O ⁻
Amino acids and simple molecular forms	Selenomethionine (SeMet)	H ₃ CSe-CH ₂ CH ₂ CH(NH ₂)COOH
	Selenocysteine (SeCys)	HSe-CH ₂ CH(NH ₂)-COOH
	Selenocystine (SeCys ₂)	COOH-CH(NH ₂)CH ₂ -Se-Se-CH ₂ CH(NH ₂)COOH
	Selenomethyl selenocysteine (SeMeSeCys)	H ₃ C-Se-CH ₂ CH(NH ₂)-COOH
	Selenocysteine acid	
	Selenomethyl selenomethionine (SeMeSeMet)	[Me ₂ Se ⁺ -CH ₂ CH ₂ CH(NH ₂)-COOH]X ⁻
	S-(seleno methyl)cysteine	H ₃ CSe-S-CH ₂ CH(NH ₂)-COOH

	Selenomethionine selenoxide	$\text{Me-SeO-CH}_2\text{CH}_2\text{CH (NH}_2\text{) -COOH}$
	Seleno homocystine	$\text{HOOC-CH (NH}_2\text{) CH}_2\text{CH}_2\text{-Se-Se-CH}_2\text{CH}_2\text{CH (NH}_2\text{) COOH}$
	γ -glutamyl-Se-methylselenocysteine	$\text{H}_3\text{N}^+\text{-CH}_2\text{CH}_2\text{CO-NH-CH (COO-) CH}_2\text{-Se-CH}_3$
	Se-adenosyl seleno homocysteine	$\text{H}_2\text{N-CH (COOH) CH}_2\text{CH}_2\text{SeCH}_2\text{C}_4\text{H}_5\text{O}_3\text{C}_5\text{N}_4$
	Selenocystatione	$\text{HOOC-CH (NH}_2\text{) CH}_2\text{-Se-CH}_2\text{CH}_2\text{CH (NH}_2\text{) COOH}$
	Selenocystamine	$\text{H}_2\text{NCH}_2\text{CH}_2\text{SeSeCH}_2\text{CH}_2\text{NH}_2$
	Seleno holine	$[\text{Me}_2\text{Se}^+\text{-CH}_2\text{CH}_2]\text{X}^-$
	Selenobetaine	$[\text{Me}_2\text{Se}^+\text{CH}_2\text{COOH}]\text{X}^-$
	Seleno peptide	
Other forms	Selenoproteins	
	Selenoenzymes	
	Selenosugars	
	Se-metal metallothioneine	

1.3 Selenium Compounds in Higher Plants

Selenium has never been shown to be essential for higher plants, although it is taken up and assimilated readily because of its chemical similarity to sulphur. In plants, selenium comes mainly from the soil and it is distributed in all parts of plants as selenate and selenite (Figure 1.3). The rate of uptake depends on plant-specific ability, and soil parameters such as: pH, Eh, water regime, clay content, organic matter, cation exchange capacity, nutrient balance, and the concentration of other trace elements (Fernández-Martínez & Charlet, 2009). Selenate is taken up via high-affinity sulphate transporters and metabolism via the sulphate assimilation pathways (Terry, Zayed, de Souza, & Tarun, 2000). It was thought that selenite is absorbed passively (Sors, Ellis, & Salt, 2005; Terry et al., 2000), but Winkel et al. (2015) reported that selenite uptake is an active process mediated via phosphate transporters. Elemental selenium and most selenides, however, are insoluble and unavailable for plant uptake. Uptake from soil is affected by irrigation, aeration, liming and selenium fertilization (Carvalho & Martin, 2001; Combs, 2001; Terry et al., 2000). The transport and distribution of selenium in various plant parts differ according to the selenium compounds, plant phase of development and its physiological condition. Distribution also depends on the form of selenium and its concentration supplied to the roots, as well as the nature and concentration of other substances, especially sulphates (El-Ramady, Abdalla, Alshaal, et al., 2015; Terry et al., 2000). When selenite and selenate are taken up from soil, they are reduced to selenide by glutathione. With the help of the enzyme, selenide is metabolized to SeCys. Selenium metabolic pathways are different for seleno accumulator and non-accumulator plants. Seleno accumulating plants are plants that can accumulate significant concentrations of selenium without showing symptoms of toxicity (Lemly, 2004; Ximénez-Embún, Alonso, Madrid-Albarrán, & Cámara, 2004). In selenium accumulator plants (*Astragalus*, *Stanleya*, *Morinda*, *Neptunia*, *Oenopsis* and *Xylorhiza*) concentrations are up to 4000 $\mu\text{g Se/g DW}$ (Terry et al., 2000). In non-accumulating plants concentrations of selenium in plants are under 25 $\mu\text{g Se/g DW}$ (Wang, Xie, & Peng, 1996). SeCys can be incorporated into proteins or transformed into SeMet in non-

accumulating plants. SeMet can be incorporated into proteins, methylated, converted into DMSe and volatilized. In selenium accumulating plants, SeCys can be transformed into numerous selenium amino acids, in which SeMeSeCys is the predominant organic selenium constituent (El-Ramady, Abdalla, Alshaal, et al., 2015; Terry et al., 2000).

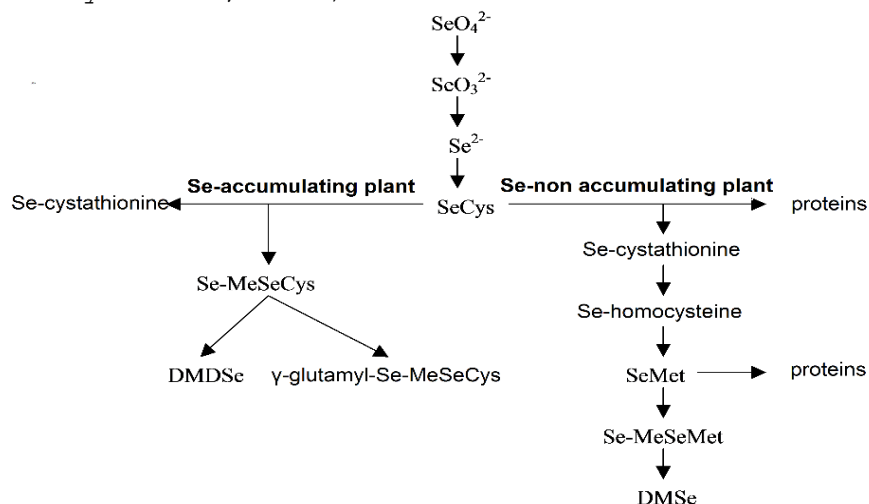


Figure 1.3: Pathways of selenium metabolism in plant (modified from Terry, Zayed, de Souza & Tarun (2000)).

El-Ramady et al. (2015) reviewed the physiological role of selenium in higher plants. It enhances the production of stress hormones, protects plants against UV stress, low and high temperature, heavy metal toxicity and delays senescence by activation of an antioxidant defence system and through some other physiological processes. A highly increased selenium content in plant can cause symptoms of toxicity, including growth inhibition, withering and drying of leaves, decreased protein synthesis, and premature death of the plant. Major forms of selenium available for plants are selenite and selenate. They can be either beneficial or toxic, depending on exposure and absorption levels. Which of those two, selenate or selenite, excerpts higher toxicity is not clear. Terry et al. (2000) wrote that a higher toxicity of selenite might be a consequence of faster conversion of Se(IV) to selenoamino acids, which are probably incorporated into plant proteins where they replace sulphur.

On the other hand, aquatic plants are able to absorb nutrients not only via the roots, but also via the shoots (Melzer and Kaiser, 1986). Some studies indicate that roots are an important site of nutrient uptake for aquatic plants in flowing waters (Chambers, Prepas, Bothwell, & Hamilton, 1989), but in many plants the leaves are certainly very significant in mineral nutrition (Hutchinson, 1975). Aquatic plants from natural freshwater ecosystems contained less than 0.6 µg Se/g DW (Maier, Nelson, Bailey, Klaine, & Knight, 1998), while according to United States Department of the Interior, (1998) selenium in aquatic plants normally ranges between 0.1 and 2.0 µg Se/g DW.

1.3.1 Selenium fortified plants

Different ways of selenium biofortification have been investigated over the years in order to overcome the naturally low selenium content in soils and crops in several areas including most parts of Europe. What is biofortification, though? It is an economical, safe agricultural technique with the aim of enriching the content

of a particular nutrient in diet, and increase the content of a micronutrient in edible portions of plants (El-Ramady, Abdalla, Taha, et al., 2015). There are several selenium biofortification methods, such as soil application, foliar application and seeds soaking with selenium or seeds priming as well as *in vitro* experiments (Table 1.2).

Soil application was first introduced in the 1960s with spraying selenite and selenate onto the soil surface. The first studies, conducted in USA and New Zealand, showed a promising approach for increasing the selenium content in plants. In the past, there have been many studies, in which soil application increased selenium content in wheat, barley, rye grass etc. On the one hand, the selenium content was increased, but the selenium toxic effect needs to be taken into account. In soil application, losses caused by soil adsorption, chemical or microbiologically mediated conversions or losses can occur. Soil application is a recommended technique for crops prone to late-season moisture or heat stress (El-Ramady, Abdalla, Alshaal, et al., 2015; El-Ramady, Abdalla, Taha, et al., 2015).

In the literature data, foliar application is the most effective method of biofortification with selenium. Foliar spraying has a high degree of uptake and assimilation by the plant. The efficiency of foliar spraying depends on ion absorption through aboveground plant organs and its mobility. The selenium ion diffuses from the leaf surface to epidermal cells, where high concentrations can damage the cells. Selenium ions are distributed from there among other plant organs (El-Ramady, Abdalla, Alshaal, et al., 2015; Ježek et al., 2012). In the literature, different plant species have been enriched by foliar application with Se(IV) or Se(VI) in different concentrations: carrot, cabbage, red cabbage, pumpkin, broccoli, buckwheat, bean etc. (Table 1.2).

The other method of selenium biofortification is seed treatment. Seeds are soaked in solution for an appropriate time and placed on plates to start germination. There have been only a few studies in which this approach has been applied to enhance the selenium content in edible parts of plant (Table 1.2).

1.3.1.1 Selenium compounds in selenium fortified plants

Selenium compounds have been determined in plants species after biofortification with selenium. Different methods, selenium forms in solutions, extraction procedures, separation methods and detection systems were used (Table 1.2). Anion exchange chromatography and detection by ICP-MS was the most used method for determination of selenium compounds in enriched plant samples. The determined selenium compounds depended on the plant species, biofortification method and form of selenium in the fortified solution (SeMet, SeMeSeCys, Se(IV), Se(VI), SeCys₂ and others).

Table 1.2: Overview of different selenium fertilization and determination of selenium compounds in selenium enriched plants.

Plant	Treatment	Plant part	Solution	Extraction	Separation	Detection	Identified Se compounds	Reference
Pumpkin <i>Cucurbita pepo</i> L.	foliar	seeds	Se (IV)	Protease XIV	HPLC <i>anion exchange</i> : Hamilton PRP X-100 and <i>cation exchange</i> : Hamilton PRP X-200	HG-AFS	SeMet	Smrkolj, Stibilj, Kreft, & Kapolna, 2005
Bean <i>Phaseolus vulgaris</i> L.	foliar	seeds	Se (VI)	Protease XIV	HPLC <i>anion exchange</i> : Hamilton PRPX-100 and <i>cation exchange</i> : Hamilton PRPX-200	HG-AFS	SeMet, SeMeSeCys	Smrkolj, Osvald, Osvald, & Stibilj, 2007
Carrot <i>Daucus carota</i>	foliar	leaves roots leaves roots	Se (IV) Se (IV)	proteolytic	HPLC <i>anion exchange</i> : ION-120 (Transgenomic)	ICP-MS	Se (IV), Se (VI), SeMet Se (IV), SeMet, γ -glu-MeSeCys Se (IV), Se (VI), SeMet Se (VI), SeMet, γ -glu-MeSeCys	Kápolna, Hillestrøm, Laursen, Husted, & Larsen, 2009
Buckwheat <i>Fagopyrum esculentum</i>	foliar	seeds leaves	Se (VI)	Protease XIV	HPLC <i>anion exchange</i> : Hamilton PRP X-100 and <i>cation exchange</i> : Zorbax 300-SCX	HF-AFS / ICP-MS	SeMet Se (VI)	Vogrinčič, Cuderman, Kreft, & Stibilj, 2009
Cabbage <i>Brassica oleracea</i> cv. Pandion Red cabbage <i>Brassica oleracea</i> cv. Efrurtsko rano	foliar foliar	roots leaves stems roots leaves stems	Se (VI) Se (VI)	Protease XIV	HPLC <i>anion exchange</i> : Hamilton PRP X-100 and <i>cation exchange</i> : Zorbax 300-SCX	ICP-MS	SeMet, SeMeSeCys Se (IV), Se (VI), SeMet Se (IV), SeMet SeMet Se (IV), Se (VI), SeMet SeMet	Mechora, Germ, & Stibilj, 2012b
Broccoli <i>Brassica oleracea</i>	foliar	flower heads	Se (VI)	Protease XIV	HPLC <i>reverse phase</i> : Puroshper STAR-C8e	ICP-MS	Se (VI), SeMet, SeMeSeCys, SeCys ₂	Šindelářová et al., 2015
Wheat sprouts <i>Triticum aestivum</i> Alfalfa sprouts	seed soaking	whole plant	Se (VI)	water	HPLC <i>anion exchange</i> : Hamilton PRP X-100	ICP-MS	Se (IV), Se (VI) Se (IV), Se (VI)	Lintschinger, Fuchs, Moser, Kuehnelt, &

Plant	Treatment	Plant part	Solution	Extraction	Separation	Detection	Identified Se compounds	Reference
<i>Medicago sativa</i>								Goessler, 2000
Buckwheat sprouts <i>Fagopyrum esculentum</i>	seed soaking	whole plant	Se (VI) Se (IV)	0,3 M HCl Protease XIV 0,3 M HCl Protease XIV	HPLC <i>anion exchange</i> : Hamilton PRP X-100 and <i>cation exchange</i> : Zorbax 300-SCX	ICP-MS	Se (IV), Se (VI) Se (VI), SeMet SeMeSeCys SeMet, SeMeSeCys	Cuderman et al., 2010
Alfalfa sprouts		shoot					Se (IV), Se (VI), SeMet, SeCys ₂	Funes-Collado,
Lentil sprouts	seed soaking	shoot	Se (IV) in	Protease XIV	HPLC <i>anion exchange</i> : Hamilton PRP X-100	ICP-MS	Se (IV), Se (VI), SeMet, SeCys ₂	Morell-Garcia,
Soy sprouts		shoot	Se (VI)				Se (IV), Se (VI), SeMet, SeCys ₂	Rubio, &
		root					Se (IV), Se (VI), SeMet, SeCys ₂	López-Sánchez, 2013
Buckwheat sprouts <i>Fagopyrum esculentum</i> Broccoli sprouts <i>Brassica oleracea</i> Kaiware daikon sprouts <i>Raphanus sativus</i> Soybean sprouts <i>Glycine max</i>	hydroponic	whole plant	Se (IV)	0,2 M HCl	HPLC <i>reverse phase</i> : Devolosil RP-Aqueous (Nomura Chemical, Seto, Japan)	ICP-MS	SeMeSeCys Se (IV), SeMeSeCys SeMet, SeMeSeCys SeMet, SeMeSeCys	Sugihara et al., 2004
Chicory <i>Cichorium intybus</i>	aeroponic	/	Se (VI)	Protease XIV	HPLC <i>anion exchange</i> : Hamilton PRPX-100 and <i>cation exchange</i> : Hamilton PRPX-200	HG-AFS	Se (IV), Se (VI), SeMet, SeMeSeCys, SeCys ₂	Mazej, Osvald, & Stibilj, 2008
Chickpea <i>Cicer arietinum</i>	semi-hydroponic	roots	Se (IV)	Protease XIV	HPLC <i>anion exchange</i> : AS 11 (Thermo), <i>size exclusion</i> : 300x10mm, filled with TSK 55 S HW	ICP-MS	Se (IV), Se (VI), SeMet, SeCys, GPx	Lyubenova, Sabodash, Schröder, &

Plant	Treatment	Plant part	Solution	Extraction	Separation	Detection	Identified Se compounds	Reference
		shoots			and 250x10mm, filled with TSK 40 S SW (Tosoh Hass, Stuttgart) and <i>affinity</i> : Amersham (GE Healthcare Europe GmbH)		Se(IV), Se(VI), SeMet, SeCys, GPx	Michalke, 2015
Mushroom <i>P. eryngii</i> , <i>P. nameko</i>	hydroponic	/	Se(IV) and Se(VI)	phosphoric acid	HPLC <i>anion exchange</i> : Supelco LC-SAX1 and <i>cation exchange</i> : Zorbax SAX	ICP-MS	Se(IV), Se(VI), organic Se	Niedzielski et al., 2015

1.4 Selenium and Iodine Fortified Plants

Like selenium, iodine is also known to be an essential element for humans. It is a component of thyroid hormones, thyroxine T₄ (3,5,3',5'-tetraiodothyronine) and T₃ (3,5,3'-triiodothyronine). These hormones are necessary for controlling metabolic rate, growth, development of body structures as well as neuronal function and development. Selenium is known to be a cofactor of the enzyme iodothyronine deiodinase, responsible for conversion of the hormone T₄ to the active form T₃ (Risher & Keith, 2009). Because of the close relationship between selenium and iodine in thyroid gland functioning, thyroid hormone biosynthesis and metabolism, biofortification with both elements at the same time is the next logical step in further biofortification research. In general, iodized salt is the most important source of iodine, but alternative sources are needed, particularly for patients with cardiovascular diseases and persons with alternative dietary habits who do not consume iodized salt. The most promising approaches are soil biofortification and foliar application (Lawson, Daum, Czauderna, Meuser, & Hartling, 2015).

There are only three studies in which the two elements were applied at the same time (Table 1.3) (Smoleń et al., 2016; Smoleń, Kowalska, & Sady, 2014; Zhu, Huang, Hu, Liu, & Christie, 2004).

The first research of simultaneously fortified plants with selenium and iodine was published in 2004 (Zhu et al., 2004). Spinach (*Spinacia oleracea* L.) was hydroponically grown for 21 days in a growth room. I(V) and Se(VI) were used in the experiment in three concentrations: 0, 10 and 50 µM (0, 0.8 and 4 mg Se/L and 0, 1.3 and 6.3 mg I/L). Plants were divided into leaves and roots, and determined for selenium and iodine content. They found that the selenium concentration in shoots and root tissue was increased by increasing the concentration of the selenium solution. The selenium content was not affected by iodine presence in the solution. The results of this study indicated that there was no inhibitory effect on plant uptake of Se(VI) (Zhu et al., 2004).

Smoleń et al., (2014) introduced double biofortification of selenium (Se(VI)) and iodine (I(V)) on lettuce cv. 'Melodion' and observed the interactions between them. Selenium and iodine were introduced with two steps: i) the nutrient medium and ii) with foliar application. In the nutrient medium, five sub-experiments were conducted: i) control, ii) 0.5 mg Se/L, iii) 1 mg I/L, iv) 0.5 mg Se/L + 1 mg I/L and v) 1.5 mg Se/L + 1 mg I/L. In each sub-experiment, the following solutions were added: i) distilled water (control), ii) 0.005% Se (~ 50 mg Se/L), iii) 0.05% I (~ 500 mg I/L) and iv) 0.005% Se + 0.05% I (~ 50 mg Se/L + ~ 500 mg I/L). Foliar spraying was conducted five times. The lettuce was harvested and divided into leaves and roots, in which selenium and iodine content were determined. As in the study by Zhu et al. (2004) increasing the selenium concentration in the solution medium increased the selenium content in roots and leaves, with a higher amount in the roots. Simultaneous addition of selenium and iodine in the nutrition medium did not effect the selenium content in leaves and roots tissue. On the other hand, a significantly higher selenium content in leaves was observed when iodine was added to the foliar solution. The results indicated a synergistic effect of selenium and iodine absorption in leaves (Smoleń et al., 2014).

In 2016, the first study was published, in which selenium and iodine in different forms had been used for the simultaneous biofortification. Carrot (*Daucus carota* L.) was fortified with

two approaches: soil fortification and as a top-dressing (foliar application). Carrot was cultivated in the field in 2012-2014. Selenium was applied before sowing (before ridge formation) in the following combinations: control, KI, KIO₃, Na₂SeO₄, Na₂SeO₃, KI + Na₂SeO₄, KIO₃ + Na₂SeO₄, KI + Na₂SeO₃ and KIO₃ + Na₂SeO₃. After sowing, the carrot was fortified (top-dressing) with 0.5 kg Se/ha and 2.5 kg I/ha in each treatment. The total amount of applied selenium and iodine was 1 kg Se/ha and 5 kg I/ha. The carrot was harvest as the whole plant, and later divided into roots and leaves. In addition to selenium and iodine determination the content of SeMet was also ascertained. Higher uptake of selenium and content of SeMet were found, when Se(VI) was applied. In each year, a varied effect of the relation between application of Se(VI) alone or in combination with I(-I) or I(V) was observed. Iodine in both forms had a negative impact on selenium content when it was present with Se(IV) in the fortified solutions. In the same solutions, no interactions were observed in terms of SeMet content (Smoleń et al., 2016).

Table 1.3: Literature data for selenium and iodine enriched plants.

Plant	Treatment	Solution	Concentration of solution	Plant part	I effect on Se uptake	Reference
Spinach <i>Spinacia oleracea</i> L.	hydroponic	Se(VI), I(V)	0 - 4 mg Se/L, 0 - 6.3 mg I/L	roots, leaves	no effect	(Zhu et al., 2004)
Letucce 'Melodion' cv.	hydroponic sysetm of NFT and foliar application	Se(VI), I(V)	0.5 mg Se/L - 1.5 mg Se/L, 1 mg I/L and 0.005% Se, 0.05% I	roots, leaves	increase	(Smoleń et al., 2014)
Carrot <i>Daucus carota</i> L.	soil fertilization and top-dressing	Se(IV). Se(VI), I(- I), I(V)	1 kg Se/ha, 5 kg I/ha	roots, leaves	diverse	(Smoleń et al., 2016)

1.5 Methods for Determination of Selenium and Its Compounds

1.5.1 Determination of selenium content

Even today, with all the advantages of modern analytical equipment available, the determination of selenium in different samples is a particular challenge because of its often very low concentrations and the ease by which it can be lost during sample preparation (Reilly, 1996). There are many different detection techniques for selenium determination. The most frequently used analytical techniques are hydride generation atomic absorption spectrometry (HG-AAS), hydride generation atomic fluorescence spectrometry (HG-AFS), inductively coupled plasma atomic emission spectrometry (ICP-AES), inductively coupled plasma mass spectrometry (ICP-MS) and neutron activation analysis (NAA). Chosen detection system will depend on several factors such as the nature of samples for analysis, the level of required sensitivity, the number of samples, which other elements need to be determined and the required limit of detection (Table 1.4) (Caruso & Montes-Bayon, 2003; Reilly, 1996).

Table 1.4: Comparison of limits of detection for selenium determination in different samples (summarized from Kristan, Arribére, & Stibilj, 2013; Pinho, Canário, Cesário, & Vale, 2005; Smrkolj & Stibilj, 2004).

Analytical technique	Limits of detection (ng/g)
HG-AAS	33 – 100
NAA	50
HG-AFS	0,14
ICP-AES	850
ICP-MS	30
ETV-ICP-MS	400

1.5.1.1 HG-AFS

Hydride generation atomic fluorescence spectrometry (HG-AFS) is a method with great sensitivity, few matrix interferences and relatively inexpensive equipment. Hydride generation is a separation technique, whereby selenium is separated from the digested solution by two processes: hydride formation from sample solution and transport of hydrides with inert gas to an atomizer. Since only Se(IV) forms the hydride (H_2Se), pre-treatment consisting of sample decomposition and reduction of Se(VI) is needed. For formation of H_2Se , the reducing agent mostly used is sodium tetrahydroborate ($NaBH_4$). When H_2Se is formed, it flows with inert gas argon through drying gas nitrogen to an atomizer. In principle, the AFS technique is light absorption, when free atoms absorb light from linear and continuous sources. When atoms absorb energy, electrons are excited to higher energy levels. The excitation is normally short lived - release of the excess energy occurs. Fluorescence light is measured with a detector positioned at a 90° angle to the light beam. The detectors are based on photomultiplier tubes (Dedina, 1995; Vandecastelle & Block, 1993). HG-AFS systems have been used successfully to obtain selenium contents in samples of vegetables, fish, muscle, eggs, milk etc. (Kristan et al., 2013; Smrkolj, Pograjc, Hlastan-Ribič, & Stibilj, 2005; Smrkolj & Stibilj, 2004).

1.5.1.2. ICP-MS

Inductively coupled plasma mass spectrometry (ICP-MS) is a technique that can be used for reliable and sensitive detection of trace elements in biological and environmental samples. It has many advantages, such as a wide range of sample types, high sensitivity, minimal sample preparation and sample size needed, simultaneous multi-elemental analysis and high species throughput. It has been suggested that determination of selenium with ICP-MS is not possible because of the high ionization potential causing a smaller proportion of atoms in the plasma to be converted into ions thus lowering instrument sensitivity (Miksa, Buckley, Carpenter, & Poppenga, 2005). The major disadvantages for selenium determination by ICP-MS are spectral and non-spectral interference (Table 1.5). The use of the most abundant isotopes (^{76}Se , ^{78}Se and ^{80}Se) is not appropriate, due to the interferences of the argon dimmers. Less abundant isotopes (^{77}Se and ^{82}Se) are frequently used. Non-spectral interferences are usually found during ICP-MS measurements, mainly from matrix and instrument space charge effects occurring in the interface region between the skimmer and the ions lens, causing an overall analyte sensitivity drop. Interferences can also cause a change in the analytical signal (drift), which can be corrected if signals are monitored by internal standardization (Pinho et al., 2005). Several reports have confirmed that detection of selenium by ICP-MS in various samples, such as hair, brain, liver, serum, plasma, sediment, soil etc., is possible (Dias et al., 2005; Inagaki et al., 2006; Miksa et al., 2005; Pinho et al., 2005). To avoid multiple spectral interference in real world samples, triple quadrupole ICP-MS or ICP-QQQ was introduced for selenium determination in complex matrix samples. ICP-QQQ features an additional quadrupole mass filter (Q1), situated in front of a conventional collision/reaction cell and quadrupole mass filter (Q2). Q1 act as an additional mass filter, allowing only the target analyte mass to enter the cell. For selenium measurement, O_2 is used in a reaction cell. Q2 is set to measure the analyte reaction product ($m/z+16$) to avoid double charges and dimer ions (Sugiyama, 2012).

Table 1.5: Spectral interferences on major selenium isotopes.

Se isotopes m/z	Abundance %	Interference						
		Isobaric	Argide	Oxide	Hydride	Chloride	Doubly-charged	Dimer
77	7.63		$^{39}\text{K}^{38}\text{Ar}^+$	$^{61}\text{Ni}^{16}\text{O}^+$, $^{59}\text{Co}^{18}\text{O}^+$	$^{76}\text{GeH}^+$, $^{76}\text{SeH}^+$	$^{40}\text{Ar}^{37}\text{Cl}^+$, $^{40}\text{Ca}^{37}\text{Cl}^+$	$^{154}\text{Sm}^{++}$, $^{154}\text{Gd}^{++}$	
78	23.77	$^{78}\text{Kr}^+$	$^{40}\text{Ca}^{38}\text{Ar}^+$	$^{62}\text{Ni}^{16}\text{O}^+$	$^{77}\text{SeH}^+$	$^{41}\text{K}^{37}\text{Cl}^+$	$^{156}\text{Gd}^{++}$, $^{156}\text{Dy}^{++}$	$^{38}\text{Ar}^{40}\text{Ar}^+$, $^{39}\text{K}^{39}\text{K}^+$
80	49.61	$^{80}\text{Kr}^+$	$^{40}\text{Ca}^{40}\text{Ar}^+$	$^{64}\text{Ni}^{16}\text{O}^+$, $^{64}\text{Zn}^{16}\text{O}^+$, $^{32}\text{S}_2^{16}\text{O}^+$, $^{32}\text{S}^{16}\text{O}_3^+$	$^{79}\text{BrH}^+$	$^{45}\text{Sc}^{35}\text{Cl}^+$	$^{160}\text{Gd}^{++}$	$^{40}\text{Ar}^{40}\text{Ar}^+$, $^{40}\text{Ca}^{40}\text{Ca}^+$
82	8.73	$^{82}\text{Kr}^+$	$^{42}\text{Ca}^{40}\text{Ar}^+$	$^{66}\text{Zn}^{16}\text{O}^+$, $^{32}\text{S}^{34}\text{S}^{16}\text{S}^+$, $^{34}\text{S}^{16}\text{O}_3^+$	$^{81}\text{BrH}^+$	$^{45}\text{Sc}^{37}\text{Cl}^+$	$^{162}\text{Dy}^{++}$, $^{162}\text{Er}^{++}$	

The most important part of determination of trace elements is the preparation of samples for measurement. Some methods, such as INAA, can be performed without destroying the sample but digestion has to be performed in most other techniques. These processes usually involve some forms of oxidation, such as dry or wet digestion with acid. Acid digestion as a far more common procedure. In this type of digestion, different mixtures of acid ($\text{HNO}_3/\text{HClO}_4/\text{HF}/\text{HCl}/\text{H}_2\text{SO}_4$) and H_2O_2 can be used, depending on the samples and methods (Reilly, 1996). Acid digestion can be done in open or closed vessel systems. The closed system is better than

the open one, because of the higher than atmospheric pressure inside the vessel (increased temperature of the solution), avoidance of atmospheric contamination and, most important, volatile elements such as selenium will remain in the solution. All these advantages also reduce the digestion time. In recent years, the most convenient technique has been microwave digestion. This is closed digestion with controlled temperature and pressure inside the vessels (Technologies, 2000).

1.5.2 Determination of selenium compounds

The best known selenium compounds are oxyanions (selenite - Se(IV) and selenate - Se(VI)) and some organic compounds (SeMet, SeCys and SeMeSeCys) but other selenium compounds can also be determined in different samples. Determination of selenium compounds will lead to legislation based on maximum selenium compounds content rather than total selenium content.

For studies of compounds determination, the combination and coupling of different technologies and element-selective or molecule-selective detection systems are needed. Many methodological developments are necessary, mostly for coupling and quality control strategies. Researchers have shown that changes in original compounds information can easily occur during sampling, sample preparation, storage, speciation and detection. One of the important tasks in methods development is quality controlled analytical procedure (Michalke, 2003).

1.5.2.1 Sampling and sample preparation

Sampling is one of the most critical step in analysis of compounds. There are interferences such as sampling per se, representativeness, wall effects (compounds can be adsorbed to the sampler zone), contaminations from sampler, alteration of biological or chemical equilibria, oxidation by oxygen, exceeded range of solubility (precipitation) etc. In biological samples, contamination occurs from syringes, metal scalpels and other metal tools, which can change a compound's pattern. These can cause changes to a secondarily altered pattern of another element (Michalke, 2003).

In plant samples, the cleaning process (soil, dust and sediment) is normally the first step in preparation for measurement. Solid samples required mechanical processing, such as grinding and drying. Grinding is important for homogenizing the sample, because the highest ratio between surface and volume is reached. This is important for later extraction. Instead of drying, it is sometimes better to use lyophilisation, since some compounds can evaporate or decompose in the drying process (Emons, 2003).

The sample can only be stored for a short time, unless longer storage time is unavoidable in the case of replicate measurements of a single sample and in general for most applications. Clean room conditions, clean vials and other equipment must be provided. Short-term storage at 4 °C or long-term freeze-drying or shock-freezing at - 80 °C is advisable (Michalke, 2003). Storage materials also played an important role (polytetrafluoroethylene PTFE, polyethylene or polycarbonate), because they can be a source of an unwanted chemical reaction between samples and storage materials (Emons, 2003).

1.5.2.2 Extraction

Determination of selenium compounds often requires an extraction process, whereby selenium compounds from the matrix are obtained. It is necessary for the chemical forms of the analyte not to be

modified and that the equilibrium between various compounds present is not disturbed (Pyrzynska, 2002a). A problem in that kind of determination is a lack of references material, which could be added during the extraction procedure for quality control during measurement (Thiry, Ruttens, De Temmerman, Schneider, & Pussemier, 2012).

It is known that selenium is usually bound to proteins (Zachara, 1992), so for determination of selenium compounds, enzymatic extraction is a frequently used method, with soft conditions (37 °C, pH 7.5) and minimized risk of compounds transformation, while allowing high extraction efficiencies. Extractions are often combined with ultra-sound or micro-wave energies (Thiry et al., 2012). The predominant extraction procedure in the literature is extraction with enzyme Protease XIV (Kristan et al., 2013; Mechora, Cuderman, Stibilj, & Germ, 2011). Cuderman & Stibilj (2009) reported on the critical use of commercially available enzymes for determination of selenium compounds. They found SeMet, Se(IV), Se(VI) and some unknown compounds in several commercially available enzymes. Hot water extraction is one of the options for compounds determination, because selenoamino acids are water-soluble (Pyrzynska, 2002a). If selenium compounds are incorporated into proteins, this method cannot be used (Cuderman et al., 2010). Extraction of selenium compounds is also possible with sodium hydroxide instead of water when separation and identification is made with SEC-ICP-MS (high molecular weight) (Wróbel et al., 2004).

1.5.2.3 Methods for determination of selenium compounds

1.5.2.3.1 Direct selenium compounds determination

Two rear compounds determination methods without a previous separation procedure are nuclear magnetic resonance (NMR) or voltammetry. These two techniques are rare in practice in real life, because of high concentration ranges in NMR and nearly impossible quantification with voltammetry in samples with a high organic load (Michalke, 2003). The use of complementary techniques (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 structure (XANES), x-ray diffraction (XRD), microprobe analysis etc.) that give access to structural information of a solid sample are important. These techniques have become increasingly popular due to the minimum sample preparation needed and the resulting decreased risk of species transformation (Caruso & Montes-Bayon, 2003).

1.5.2.3.2 Hyphenated techniques for selenium compounds determination

After extraction, soluble compounds have to be separated, identified and correctly quantified. The choice of separation techniques depends on various physicochemical properties, such as volatility, charge and polarity (Caruso & Montes-Bayon, 2003). Separation is usually made by liquid chromatography (HPLC-high performance liquid chromatography), gas chromatography (GC), capillary electrophoresis (EC) etc. (Caruso & Montes-Bayon, 2003; Thiry et al., 2012). These techniques are coupled with detection systems such as UV spectrometry, hydride generation atomic fluorescence spectrometry (HG-AFS), flame atomic absorption spectrometry (FAAS), electrospray ionization tandem mass spectrometry (ESI-MS/MS), inductively coupled plasma atomic emission spectrometry (ICP-AES) and inductively coupled plasma mass spectrometry (ICP-MS) (Gong, Xu, Lee, & Zhang, 2012; Pedersen & Larsen, 1997).

Gas chromatography (GC) separates volatile selenium compounds (methylselenol MeSeH , dimethylselenide Me_2Se , dimethyldiselenide Me_2Se_2) or compounds previously derivatized (sediments, water). Capillary columns with bounded phase are mostly used (Michalke, 2003). The major advantage of this technique is that there is no need for transformation of the compounds into an aerosol or gaseous phase. Nowadays, the combination of GC and detection of compounds with ICP-MS has become a good method for the determination of volatile selenium compounds. It requires the high resolving power of GC and the sensitivity and specificity of ICP-MS. Input of the sample is 100%, which improves the limits of detection. There are some of limitations. The first is maintenance of the analyte in the gas phase during their transport from GC to ICP-MS, to avoid condensation effects. The next limitation is of the need for an additional carrier gas to achieve a sufficient flow in the central channel of the plasma by effluent from the gas chromatograph (Michalke, 2003). Volatile dimethyl selenium compounds often require utilized purge and cryogenic trapping systems or headspace sampling using solid phase micro extraction SPME. On the other hand, some non-volatile selenium species (SeMet and SeCys) can be derivative for analysis by GC, which is a valuable alternative for HPLC, because of its higher sensitivity (Michalke, 2003).

Capillary electrophoresis (CE) has the capacity to separate a wide range of analyte, from large biomolecules to small inorganic ions. It can also separate positive, neutral and negative ions in a single run with high separation efficiencies. The advantages of CE are the possibility of analyzing relatively labile compounds because of the absence of chromatographic packing, nanoliter sample volumes, small amounts of reagents used and relatively low costs of capillary columns (Caruso & Montes-Bayon, 2003). The total amount of a sample which can be applied is often below 1 ng, so absolute detection limits are at pg levels. By coupling CE to ICP-MS the injected volume can be minimized, which is good from the environmental point of view. On the other hand, small amount of injected volume is unsuitable for a mass-responsive detector such as the ICP-MS, where actual concentrations detectable may be in the range of mg/kg. Nowadays, a commercial interface is used, which optimizes electrophoretic and nebulizer flows independently, so that dilution and sample consumption is minimal (Caruso & Montes-Bayon, 2003; Michalke, 2005). Michalke et al. (2005) used CE-ICP-MS for identification and determination of selenogluthathione in human milk and differentiation of methionine, selenomethionine, cystamine, and selenocystamine. An application of CE-ICP-MS has also been shown for the analysis of selenoamino acids in selenized yeast (Uden, 2002).

1.5.2.3.2.1 Liquid chromatography

The type of liquid chromatography (LC or HPLC) is chosen on the basis of the relative polarity, solubility and molecular weight of the species of interest (Caruso & Montes-Bayon, 2003). One of the most important advantages of LC is the extensive flexibility of separation mechanisms, with the use of different mobile and stationary phases. It is important to select the right mechanism and reagents to obtained original compounds information. Many stationary phases, buffers and organic modifiers can denature native compounds. Chelating eluents or ion exchangers may cause re-complexation of free or labile bound metal species (Michalke, 2003). A general disadvantage of using LC for separation analysis is the existence of a stationary phase, which provides a large surface on which adsorption effects, contamination, or miscellaneous alterations of the compound can occur (Harms & Schwedt, 1994).

LC has been used for the separation of thermally unstable and non-volatile organic selenium compounds, which are commonly found in biological samples (Pedersen & Larsen, 1997). It is by far the most widely used technique in the literature because of its inherent advantage of performing separations of analyte which only need to be soluble (B'Hymer & Caruso, 2006). LC has also been widely applied to separate selenium compounds because of good reproducibility and simple online interfaces for the detector (Gong et al., 2012). HPLC coupled with ICP-MS can provide low detection limits for selenium and can be improved by the use of the collision/reaction cell. Various types of chromatography have been used for determination of selenium compounds in plant samples (Table 1.2).

REVERSED PHASE CHROMATOGRAPHY (RPC): Reversed phase HPLC-ICP-MS is the most popular mode nowadays for organic selenium compounds analysis, because of its ultra-sensitivity (Gong et al., 2012). In RCP, compounds are separated according to their polarity and their corresponding affinity with non-polar stationary phase (C_8 and C_{18}) usually coated with an ion-pairing agent (trifluoroacetic acid TFA) added to the relatively polar mobile phase. This method is appropriate for many organic compounds (charged or not) (Thiry et. al., 2012).

ION-EXCHANGE CHROMATOGRAPHY (IEC): Selenium compounds determination can also be performed by using IEC. Anion-exchange and cation-exchange chromatography are complementary techniques, which are able to assure satisfactory resolution of all selenium compounds in extracts of biological samples. In this approach, the analyte anions and the ions of the mobile phase compete for the oppositely charged functional group ions in the stationary phase. Although anion exchange provides poor separation, it allows the separation of selenite and selenate, which cannot be accomplished with reversed-phase ion-pairing HPLC. Another advantage of IEC is use of an aqueous buffer, which makes this technique compatible with element selective detectors such as an ICP-MS. IEC is also used in separation analyses as a pre-concentration step and cleanup step prior to analysis (Bird et al., 1997; Caruso & Montes-Bayon, 2003). In the polar phase, better systems for IEC separation of polar compounds of selenium include phosphate, citrate and formate (B'Hymer & Caruso, 2006). IEC techniques have been used in many studies devoted to determination of selenium compounds in plant samples (Table 1.2). Coupling of HPLC with UV-HG-AFS also occurs in the literature. Mazej et. al. (2006) developed a method for determination of selenium compounds in plant leaves by HPLC-UV-HG-AFS. They used water extraction for water-soluble selenium compounds and enzymatic hydrolysis for selenium compounds bound to proteins. Fractionation was done by ion exchange chromatography (anion and cation column) and detection limits were 2-10 ng Se/g of supernatant (Mazej, Falnoga, Veber, & Stibilj, 2006). Mazej et al. (2008) also published a study of selenium compounds in leaves of chicory, dandelion, lamb's lettuce and parsley. Extractions and speciation were performed by the same procedure as in previously described research.

SIZE EXCLUSION CHROMATOGRAPHY (SEC): SEC is a method based on the molecular weights of the analyte. Secondary absorption and ion-exchange effects can play an important role and separation of small compounds with similar molecular masses can be achieved in addition to the possibility of the detection of selenoproteins (Uden, 2002). The basic principle of SEC is the difference in molecular weights of analytes. Certain molecular sizes may diffuse into a uniform network of pores. Molecules that are larger than the pore size are not retained by the column, whereas those of smaller size can diffuse into the pores, thus eluting slower than

larger molecules. SEC is applied for biomolecules with a molecular weight between 1000 and 100 000 Da. If SEC is coupled with ICP-MS, this technique is by far the most popular for the first screening of an unknown sample, especially when elemental macromolecular compounds may be present. In contrast to other LC methods, the solvent peak elutes last here, because it is usually also the smallest. Size exclusion is widely used as a purification step followed by complementary LC in multidimensional schemes. For example, selenocompounds are first fractionated by SEC and the collected results are further processed by other methods of LC, such as reverse phase or ion exchange. All these multidimensional schemes are necessary not only for improving resolution in separation but also to remove the matrix component (Caruso & Montes-Bayon, 2003). SEC-HPLC-ICP-MS has enabled the detection of several metals bound to a variety of macromolecular ligands, but success for selenoproteins has been limited. Selenoprotein-P was first measured by SEC chromatography, but lack of resolution enables separation of the major seleno proteins (Uden, 2002). Moreno et al., (2004) published the results of a study of selenium compounds in biological tissues (fish, shellfish), vegetal tissues and selenized yeast. High molecular weight selenoproteins were obtained by SEC coupled to ICP-MS or UV spectrophotometers, after water extraction.

Chapter 2

Aims and Hypothesis

2.1 Goals of the Thesis

The goals of the thesis were:

- To optimize a method for determination of selenium content and content of selenium compounds in plant samples,
- To determine selenium content and compounds in selenium enriched plant samples (pea, buckwheat, pumpkin, chicory) after two biofortification methods (seed soaking and foliar application),
- To evaluate differences between selenium content and compounds in sprouts and adult plants,
- To study the effect of simultaneous soaking or foliar spraying with selenium and iodine (both in different forms) on selenium content and compounds in sprouts and adult plants,
- To study seasonal variations of selenium in three Slovenian watercourses in sediment and aquatic plant *Veronica anagallis-aquatica* to discover whether extensive agriculture has any influence on selenium content,
- To discover the differences between selenium compounds in selected cultivated and aquatic plant *Veronica anagallis-aquatica*.

2.2 Hypothesis

- Different treatments will increase selenium content in cultivated plants regarding the selenium forms in soaking or in foliar solution,
- After enzymatic extraction, the main selenium compound will be SeMet regardless of plant species and biofortification method,
- The content in sprouts grown from seeds soaked in the solution will be higher in comparison with foliar treated adult plants,
- Simultaneous treatment with iodine (different forms) will affect the content of selenium and its compounds in enriched plant samples,

- Extensive agriculture has an influence on selenium content in aquatic plant *Veronica anagallis-aquatica* and associated sediment samples,
- Selenium content in aquatic plant *V. anagallis-aquatica* will be higher than in cultivated plants,
- Selenium compounds in aquatic plant *V. anagallis-aquatica* will be the same as selenium compounds in cultivated plants.

2.3 Selection of Cultivated and Aquatic Plants

Plants in this thesis were chosen from different groups due to differences in agricultural and nutritional properties: green pea (*Pisum sativum* L., cultivar 'Petit Provençal') from the leguminous group, pumpkin (*Cucurbita pepo* L., cultivar 'Slovenska Golica') from the oil plant group, buckwheat (*Fagopyrum esculentum* Moench, cultivar 'Darja') from the pseudocereals group and chicory (*Cichorium intybus* L. var. *foliosum*, cultivar 'Leonardo') from the leafy vegetables group.

The importance of the family of leguminous plants is obvious, due the use of plants for food and feedstuffs. There are many leguminous plants that are very important in human and animal nutrition: bean, pea, chickpea, lupine and lentil sprouts (Marton, Mandoki, Caspo, Caspo-Kiss, 2010) especially in the Asian market. Peas are normally consumed as dry seeds. They are cultivated around the world for animal and human consumption. The major producers are Canada, the Russian Federation, China, the USA and India (Dahl, Foster, & Tyler, 2012). Peas are an inexpensive, readily available source of protein, complex carbohydrates, vitamins and minerals. The most important are pea protein, including lysine and other essential amino acids (Garousi, Kovács, Domokos-Szabolcsy, & Veres, 2017). Many health benefits are connected with pea consumption and are derived from the concentration and properties of starch, protein, fiber, vitamins, minerals and phytochemicals: cardiovascular health, weight management, gastrointestinal function and homeostasis, antioxidant activity etc. (Dahl et al., 2012).

Pumpkin is classified as an oil plant. Pumpkin crops are traditional in Central Europe, because of their high nutritional value and particular culinary attraction. Seeds are noted for their high content of sterols, vitamin B and E, minerals Ca, K, P, Mg, Fe and Zn (El-Adawy & Taha, 2001). Oil content in pumpkin seeds varies and is genetically dependent. Seeds may contain up to 50% of oil and have been used for centuries in traditional medicine: problems with kidney or urinary tract, and against tapeworms (Kreft, Stibilj, & Trkov, 2002; Stibilj, Kreft, Smrkolj, & Osvald, 2004). Pumpkin is a traditional crop plant in the eastern part of Slovenia.

Buckwheat, from the group of pseudocereals, is a traditional crop with high nutritional value. It is rich in vitamin B, minerals Zn, Cu and Mg, phenolic compounds with antioxidant activity (rutin and quercetin), lysine and it has balanced amino acid composition. On the other hand, allergic reactions can be caused by ingesting buckwheat and its products or by exposure to buckwheat dust: asthma, asthmatic attacks, urticaria and gastrointestinal disorders. It does not contain gluten and is appropriate for patients with coeliac disease. Buckwheat is used in many different food products around the world (Asia, Europe, South Africa and the USA). The most popular buckwheat dish is buckwheat noodles, consumed mainly in Japan, China and Italy (Holasova et al., 2002; Kreft, Fabjan, & Yasumoto, 2006). Buckwheat sprouts were introduced as a new source of vegetable. They have an attractive

fragrance and soft and crispy texture, and high amounts of amino acids, minerals and crude fiber. Rutin and lysine contents are much higher than in buckwheat seeds. They are widely available and consumed in Asia (Lintschinger et al., 2000). Buckwheat contains a high level of rutin in nearly all parts, including seeds. The role of rutin in buckwheat plants is efficiently scavenging free radicals in vitro. It also has antifungal activity (Suzuki et al., 2015).

Chicory is a bushy perennial herb. The leaves are used as salad vegetables, consumed as a raw salad in winter time. It is a typical Mediterranean plant, indigenous to Europe, Western Asia and North America, resistant to low temperatures (Rossetto et al., 2005). Red chicory is a vegetable with high antioxidant activity. The presence of water-soluble flavonoids has many beneficial health effects: protection against cancer, cardiovascular diseases, aging etc. (Rossetto et al., 2005).

All selected plants are cultivated in Slovenia (Table 2.1). Chicory was in the 9th place among all vegetables and in 3rd among leafy vegetables in terms of production (tons) in Slovenia in 2012 ("Statistični letopis republike Slovenije," 2013).

Table 2.1: Production of selected plants in Slovenia in 2012 ("Statistični letopis republike Slovenije," 2013).

Plants	Production (ha)	Production (t)
Pea	57	186
Pumpkin (for oil)	4512	2542
Buckwheat	1676	1340
Chicory	202	2562

Research has been done on sprouts and adult plants. Sprouts are an important source of protein, vitamins and minerals and other important health-maintaining nutrients. Because sprouts are consumed at the beginning of the growing phase, their nutrient content remains very high (vitamins, minerals, enzymes and amino acids). The most popular are the sprouts of adzuki bean, Alfalfa, broccoli, buckwheat, clover, mungo bean etc. Fat content, fatty acid composition, protein content and amino acid composition, amount of free amino acids, vitamin content, the possibility of production of selenium enriched sprouts and contribution to better status of the population made sprouts interesting for investigation (Marton, Mandoki, Caspo, Caspo-Kiss, 2010).

The second part of the study was performed on aquatic plants from an agricultural area. Aquatic plants can also be a source of food for herbivores and detritivores. This can lead to bioaccumulation of toxic levels of trace elements in the food chain (Cardwell, Hawker, & Greenway, 2002; Samecka-Cymerman & Kempers, 2004). Aquatic plants can be used for biomonitoring low levels of trace element in the environment (Samecka-Cymerman & Kempers, 2004). *V. anagallis-aquatica* was chosen for our research because it is an amphibious vascular plant, widely distributed in Slovenia, Europe (especially in the Mediterranean area) and Asia (Harput, Varel, Nagatsu, & Saracoglu, 2004; Martinčič et al., 1999; Shahzad, Parveen, & Fatema, 2011). It is a perennial herb with a root-like subterranean stem, commonly horizontal in position. It occurs in moist, wet and semi-aquatic habitats (Hadi, Ahmad, & Ali, 2014). The roots and leaves are used for the treatment of various diseases: scurvy, Alzheimer's disease, jaundice, blood impurity and many more (Shahzad et al., 2011).

Chapter 3

Materials and Methods

3.1 Chemicals and Standards

For preparation of samples, ultra-pure water (18 MΩ/cm) obtained from a Milli-Q purification device (Millipore Corporation, Bedford, MA, USA) was used. The following chemicals were used: 65% HNO₃ (s.p., Merck), 40% HF (s.p., Merck), 36% HCl (s.p., Merck), 99.9% H₃BO₃ (s.p., Merck), 32% H₂O₂ (s.p., Merck), NaBH₄ (> 99%, p.a., Sigma Aldrich), NaOH (p.a., Carlo Erba), V₂O₅ (p.a., Merck), TRIS (≥ 99.9%, p.a., Carl Roth), ammonium acetate (Fractopur, Sigma Aldrich), acetic acid (p.a., Sigma Aldrich), diammoniumhydrogen citrate (p.a., Fluka), citric acid (p.a., Fluka), methanol (HPLC-PLUS-gradient, Carlo Erba), pyridine (> 99%, for spectroscopy, Acros Organics), diethyl ether (ISO, Merck) and enzyme Protease from *Streptomyces griseus* (type XIV, bacterial, 4.4 units/mg solid, Sigma-Aldrich, P-5147).

Selenium solutions were prepared from: Na₂SeO₃ (Se(IV), > 99%, Sigma-Aldrich), Na₂SeO₄ (Se(VI), Sigma-Aldrich, SigmaUltra), selenomethionine (SeMet, > 99%, Fluka Chemie), selenocystine (SeCys₂, > 98%, Fluka Chemie), and selenomethylselenocystine (SeMeSeCys, > 98%, Fluka Chemie). Stock solutions of selenium compounds (about 1 mg of Se/g) were prepared in water and stored at 4 °C. For determination of selenium compounds, 100 ng Se/g was prepared daily in solution of enzyme. For determination of selenium with HG-AFS, 100 ng Se/g of daily solution of Se(IV) was prepared and further diluted to appropriate concentrations and amounts of acids to obtain the same acidic media as in the solutions after digestion of samples.

For selenium determination a series of composite calibration standard solutions were prepared from commercially available multi-elemental XVI standard solution (Merck, USA). A 1 mg/g standard was used to prepare standards ranging from 0.05 to 100 ng/g and measured on ICP-MS or ICP-QQQ. Standards were prepared in the same acidic media as samples.

For accuracy and precision of all the methods and results obtained, several reference materials were used: NIST 1570a (Trace elements in spinach leaves), NIST 8436 (Durum wheat flour), NIST 8418 (Wheat gluten), NIST 1567a (Wheat flour), BCR 320R (Channel sediments), SELM-1 (Selenium enriched yeast). Reference materials were used in each series of samples.

3.2 Biofortification of Cultivated Plants

3.2.1 Sprouts

3.2.1.1 Pea sprouts

Experiments with pea sprouts (*Pisum sativum* L., cultivar 'Petit Provençal') were performed in June and July 2013 at the Biotechnical Faculty. Seeds (161.0 ± 0.2 g) were soaked for 8 h in 200 mL of Milli-Q water, a solution of selenium (10 mg Se/L), a solution of iodine (1000 mg I/L) and a combination of two elements in the same concentrations in different forms. Selenium was present in the forms of selenite Se(IV) or selenate Se(VI) and iodine in the forms of iodide I(-I) or iodate I(V). After soaking, pea seeds were distributed on plastic plates, covered with filter paper and put in a bright air-conditioned room. Each treatment was performed in duplicate in each experiment. During germination, seeds were treated with tap water as necessary. The experiment was performed in two parts. Pea sprouts grown from seeds soaked in a solution of Se(VI) and its combinations with iodine were grown at an average daily 21 °C and nightly 15 °C temperature for 14 days. Pea sprouts grown from seeds soaked in a solution of Se(IV) and its combinations with iodine were grown at an average daily 22 °C and nightly 18 °C temperature for 12 days. Control plants were present in both experiments. Sampling of sprouts was done when the sprouts had developed two true leaves. Procedure of breeding is shown in Figure 3.1. Seed germination was $(96 \pm 2)\%$ in the first and $(95 \pm 2)\%$ in the second experiment. Sprouts samples were separated into stems with leaves (in this study designated sprouts), roots and seeds residues. All plant parts were lyophilized at -50 °C (1-16 LSC, Christ Gamma), milled and homogenized with a planetary micro mill (MM 200, Retsch, Haan, Germany). Samples were stored at -20 °C until analysis. The detailed growing conditions are published in study by Jerše et al. (2017).



Figure 3.1: Soaking of pea seeds, distribution on plated with moist paper and sprouts before sampling. Photo: Nina Kacjan Maršič.

3.2.1.2 Pumpkin sprouts

Pumpkin sprouts (*Cucurbita pepo* L., cultivar 'Slovenska Golica') were grown in December 2013 in a growing chamber at the Biotechnical Faculty. Pumpkin seeds (0.2 g) were soaked in various solutions of selenium, iodine and their combinations. Selenium was present in the soaking solutions in the forms of Se(IV) and Se(VI), in two concentrations: 2.5 mg Se/L and 10 mg Se/L. Seeds were also soaked in a solution of selenium (10 mg Se(IV)/L or 10 mg Se(VI)/L) in combination with iodine (1000 mg I(-I)/L or 1000

mg I(V)/L). The experiment was performed in two parts: i) only soaking in solutions of selenium and ii) combinations of the two elements. In both parts of the experiment, a control plant grown in Milli-Q water was present. Each treatment was performed in 1-3 replicates. Seeds were soaked for 4 h and scrolled into filter paper in a growing chamber (average temperature 22 °C and humidity 70 - 80%). Sampling of sprouts was done when the sprouts had developed two true leaves (the first after 10 days and the second after 12 days) (Figure 3.2). Sprouts were sampled as whole plant, lyophilized at - 50 °C (1-16 LSC, Christ Gamma), milled and homogenized with a planetary micro mill (MM 200, Retsch, Haan, Germany). Samples were stored at - 20 °C until analysis.



Figure 3.2: Pumpkin sprouts before sampling. Photo: Nina Kacjan Maršić.

3.2.1.3 Buckwheat sprouts

In June and July 2015, achenes (0.024 ± 0.002 g) of common buckwheat (*Fagopyrum esculentum* Moench, cultivar 'Darja') were soaked in different solutions of selenium and iodine to grown common buckwheat sprouts at the Biotechnical Faculty. In June 2015, a solution of selenium (Se(IV) and Se(VI)) at concentration 10 mg Se/L and its combination with 1000 mg I/L (I(-I) and I(V)) was used. In the second experiment, achenes were soaked in a combination of 10 mg Se/L and 1500 mg I/L in all forms. In both experiments, a control group (Milli-Q water) of sprouts was present. After 4h soaking, achenes were arranged on plates, each treatment in three replicates. In the first experiment, sprouts were sampled after 13 days, while in the second one only after 10 days, when two true leaves had developed (Figure 3.3). The temperature during the experiment varied from (22 - 24) °C. In both experiments, (71 - 89)% of seeds germinated, with the exception of Se(IV) (49%). The detailed growing conditions are described in a study by (Germ et al., 2015). Further sample preparation was the same as for pea sprouts.



Figure 3.3: Buckwheat sprouts before sampling. Photo: Nina Kacjan Maršič.

3.2.2 Adult plants

3.2.2.1 Pea

Pea seeds (*Pisum sativum* L., cultivar 'Petit Provençal') were sown in March 2015 in the experimental field of the Biotechnical Faculty. Foliar application with selenium and iodine was performed 8 weeks after sowing, at the beginning of blooming. Different solutions, similar as for seed soaking, were used: 10 mg Se(IV)/L, 10 mg Se(VI)/L, 10 mg Se(IV)/L+1000 mg I(-I)/L, 10 mg Se(IV)/L+1000 mg I(V)/L, 10 mg Se(VI)/L+1000 mg I(-I)/L, 10 mg Se(VI)/L+1000 mg I(V)/L and a control solution (Milli-Q water). Each experiment was performed in three replicates. The arrangement of the blocks on the field is shown in Figure 3.4. Pea plant was sampled twice in the mature phase. At the first sampling (19 days after foliar application), pods and leaves, while 29 days after spraying, pods, leaves, stems and roots were sampled. Pods were afterwards divided into seeds and peel. For further analysis, pods from the first sampling and leaves from the second sampling were used. The yield of pea seeds in the first sampling was lower than in second one, but with no statistical differences. Seeds, pod shells and leaves from the first sampling were lyophilized at -50 °C (1-16 LSC, Christ Gamma), milled and homogenized with a planetary micro mill (MM 200, Retsch, Haan, Germany). Leaves, stems and roots from second sampling were lyophilized (1-16 LSC Plus, Christ), homogenized and only leaves and stems were milled with a planetary micro mill (Fritisch, Pulverisette 7 or 14, Idar-Oberstein, Germany). Roots were cut into small parts with scissors. All samples were stored at -20 °C until analysis. Detailed growing conditions are published by Jerše et al. (2018).



Figure 3.4: Pea plants – arrangement of the blocks on the field.
Photo: Lara Škof.

3.2.2.2 Pumpkin

Pumpkin seeds (*Cucurbita pepo* L., cultivar 'Slovenska Golica') were sown in April 2014 and transplanted onto the field of the Biotechnical Faculty one month later. Plants were foliar sprayed in three replicates, when the plants had developed three to four leaves (3 weeks after transplanting, at the beginning of blooming) (Figure 3.5). For foliar application, selenium (10 mg Se(IV)/L, 10 mg Se(VI)/L), iodine (1000 mg I(-I)/L, 1000 mg I(V)/L) and a combination of the two solutions were used. Seeds were sampled approximately 3.5 months after sowing, lyophilized, milled in a knife mill (Grindmix GM 200, Retsch) after freezing in liquid nitrogen and stored at -20°C for further analysis.



Figure 3.5: Pumpkin fruits approximately 2 weeks before sampling. Photo: Marjetka Pirc.

3.2.2.3 Buckwheat

Buckwheat plants were grown in the field in the Biotechnical Faculty from achenes (*Fagopyrum esculentum* Moench, cultivar 'Darja') that were sown in July 2014. Plants were foliar sprayed in the blooming period (4 weeks after sowing) with selenium and iodine solutions in different forms and combinations in four replicates (Figure 3.6). Selenium was present in solution in a concentration of 10 mg Se/L in the forms of Se(IV) and Se(VI) and iodine in a concentration of 1000 mg Se/L in forms of I(-I) and I(V). Achenes (also called seeds or grains) were sampled in the mature phase (7 weeks after foliar spraying), lyophilized, milled (M20, IKA® Werke, Staufen, Germany) and stored at -20°C until

analysis. The detailed growing conditions were published by Germ et al. (submitted).



Figure 3.6: Foliar sprayed buckwheat plants. Photo: Janja Turk.

3.2.2.4 Chicory

In July 2015, seedlings (approximately one month old) of chicory (*Cichorium intybus* L. var. *foliosum*, cultivar 'Leonardo') were transplanted onto the experimental field at the Biotechnical faculty. Plants were foliar sprayed (two weeks later, in the rosette phase) with a solution of Milli-Q water (control), 10 mg Se/L (Se(IV) and Se(VI)), 1000 mg I/L (I(-I) and I(V)) and a combination of the two in three replicates. Chicory leaves were sampled in two parts: i) outer leaves during the growing period (5 weeks after foliar application, at the beginning of the head formatting) and ii) inner leaves in the phase of technological maturity (11 weeks after foliar application) (Figure 3.7). Samples were lyophilized, homogenized, milled (Pulverisette 7, Fritsch, Idar-Oberstein, Germany) and stored at - 20 °C until analysis (Kroflič et al., 2017).



Figure 3.7: Chicory heads before second sampling. Photo: Nina Kacjan Maršič.

3.2.3 Marking of samples

In order to facilitate following treatments through the doctoral thesis, treatments are marked as follows: plant name and treatment. For example: pea sprouts grown from the pea seeds soaked in a solution of 10 mg/L Se(IV) and 1000 mg/L I(V) will be written as pea sprouts Se(IV)+I(V).

3.3 Aquatic Plants and River Sediments

The aquatic plant water speedwell (*Veronica anagallis-aquatica* L.) (Figure 3.8) was sampled in three watercourses in two Slovenian regions: Central and Notranjska in the years 2009 – 2011 and in 2013. Different sampling sites were chosen based on the presence of different activities in these regions: dairy farming, stock raising and agriculture (Figure 3.9). Plants were collected from one sampling site on the Lipsenjščica watercourse (minor human impact, mowed meadows), at two sites on the Pšata watercourse (near a cattle farm) and at five sampling sites on the Žerovniščica watercourse (urban selenium source – in the village, nearby fields and dairy farms with cattle). All sampling sites in Žerovniščica watercourse were located within around 1 km. The Lipsenjščica watercourse was chosen as a non-point selenium source. *V. anagallis-aquatica* was not always found at all sampling sites. In addition to aquatic plants, associated sediments were sampled. In 2009, three samplings were performed (April, July and October) due to rain in February and in 2010 and 2011 four samplings took place (February, April, July and October). The detailed sampling procedure in the years 2009 – 2011 is given elsewhere (Mechora et al., 2014). In 2013, sampling was done only twice (July and September), due to a very long and cold winter (average temperatures in March were 3 °C lower in 2013 than in 2011) and, on the other hand, a very hot and dry summer (days with temperatures above 30 °C in 2013 were 31, while in 2011 only 20; rainfall from June to August was on average 72 mm per month in 2013, while in 2011 it was 103 mm per month).

The collected plant samples were rinsed in water from the watercourses to remove adhered material, placed in plastic bags and stored in a freezer at – 20 °C until processing. In the years 2009 – 2011, whole plants were sampled. Samples were freeze-dried (APLHA 1-4, Osterod and Herz, Germany) and milled in agate bowls at 2600 rpm for 6 minutes. In larger plants (above 40 cm), stems were hard to mill. We decided to separate the plants in 2013 into parts (leaves, stems, roots), with the same further procedure. All samples were stored at – 20 °C until analysis (Kroflič, Germ, Mechora, & Stibilj, 2016).



Figure 3.8: Aquatic plant *V. anagallis-aquatica* before sampling.
Photo: Urška Remic.

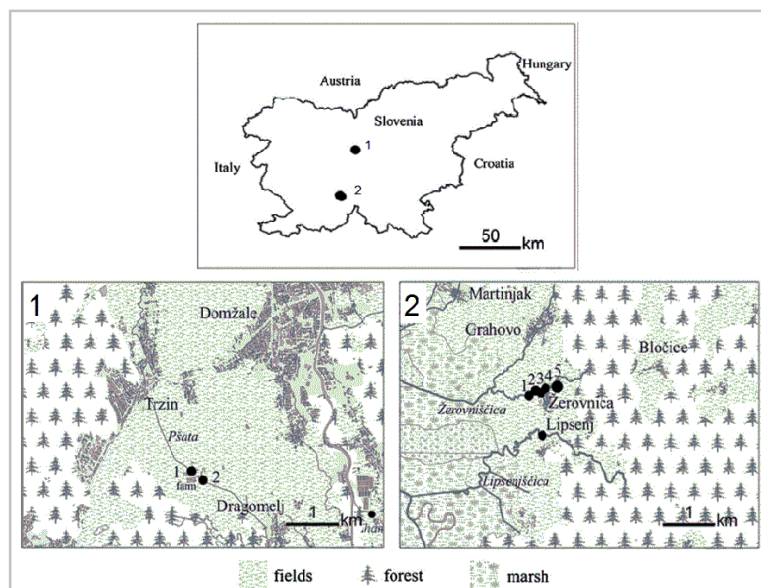


Figure 3.9: Map of sampling sites with locations 1 (Central region) and 2 (Notranjska region). The names of the watercourses are written in italics. Sampling sites are marked with Arabic numbers and symbol •.

Around 2 kg of sediments were collected and dried in air to a constant mass (Figure 3.10). Samples were then sieved through a 1 mm sieve and particles smaller than 1 mm were ground into fine powder by milling in agate bowls (Pulverisette 7, Fritsch, Idar-Oberstein, Germany) at 2500 rpm for 7 minutes.



Figure 3.10: Drying of sediment samples. Photo: Urška Remic.

3.4 Methods

3.4.1 Selenium content in plant and sediment samples

3.4.1.1 Determination of selenium content in plant samples by HG-AFS

For selenium determination, 0.20 g of lyophilized and milled plant sample was digested in 50 mL closed PTFE tubes with 0.5 mL H_2SO_4 and 1.5 mL HNO_3 for 4h at 80 °C and 1 h at 130 °C on an aluminum block. The solution was cooled to the room temperature and 2 mL of H_2O_2 was added. It was then heated for 10 min at 115 °C. 0.1 mL of HF was added to leaves, stems, roots and heated for 10 min at 115 °C. At the end, 2 mL of H_2O_2 was added and it was heated for 10 min at 115 °C. To the cooled solutions, 0.1 mL of V_2O_5 in H_2SO_4 was added and they were heated at 115 °C until the solution became blue. At the end of the digestion, 2.5 mL of HCl was added and

the solution was heated for 10 min at 90 °C. Samples were diluted with Milli-Q water in polypropylene tubes (Sarstedt, Nümbrecht, Germany) to appropriate concentrations. Selenium content was determined by hydride generation atomic fluorescence spectrometry HG-AFS (Excalibur, PS Analytical, Orpington, UK). The exact procedure is described in detail elsewhere (Smrkolj & Stibilj, 2004). Accuracy and precision were checked with standard reference material Trace elements in spinach leaves (NIST 1570a).

For determination of selenium in the soluble part of a plant sample after enzymatic extraction, 0.5 mL HNO₃ was added to approximately 1 g of extract and heated for 10 min to 80 °C and 30 min at 160 °C in closed PTFE vessels. The solution was cooled to room temperature and 0.5 mL H₂O₂ was added three times and evaporated to half volume at 120 °C. For reduction, 0.5 mL HCl was added and it was heated for 15 min at 90 °C. The further procedure was the same as for plant samples.

3.4.1.2 Determination of selenium content in plant samples by ICP-MS/ICP-QQQ

Plants were digested in two microwave systems: Ethos One (Milestone, Italy) and Ultrawave single reaction digestion system (Milestone, Italy). Approximately 0.3 g of homogenized and lyophilized plant sample, 6 mL HNO₃, 2 mL H₂O₂ and 0.1 mL HF were placed in the microwave system Ethos One in a closed Teflon tube. The temperature program for digestion was 40 min long, with a 20 min ramp to 200 °C and then 20 min at a constant temperature of 200 °C (1200 W). A lower amount of HNO₃ (4 mL), no H₂O₂ addition and 0.1 mL HF (where needed) was used for the Ultrawave digestion system. Digestion was performed in glass and Teflon (presence of HF) tubes. The temperature program was shortened: 20 minute ramp to 220 °C and 15 minute hold at 220 °C (1500W). After both digestions, samples were cooled to room temperature, quantitatively transferred to polypropylene tubes (Sarstedt, Nümbrecht, Germany) and diluted with Milli-q water to appropriate volume (30 mL). Samples were stored at 4 °C until analysis.

An external calibration technique was applied for the quantitative analysis of the samples. Seven standard solutions were prepared by diluting a standard stock solution with the same percentage of acids present in the sample. The range of content was from 0.05 to 100 ng/g, including the calibration blank (0 ng/g).

Two systems were used for detection: ICP-MS (7500ce, Agilent Technologies, Tokyo, Japan) and ICP-QQQ (8800, Agilent Technologies, Tokyo, Japan). Operating conditions of ICP-MS and ICP-QQQ are described in Table 3.1.

Table 3.1: Optimal conditions for ICP-MS/ICP-QQQ.

	ICP-MS	ICP-QQQ
<i>Nebulizer</i> ^a	Babington	Babington/MicroMist
<i>Plasma conditions</i>		
RF power (W)	1500	1550
Plasma gas flow rate (L/min)	15	15
Carrier gas flow rate (L/min) ^b	0.9	0.9

Makeup gas flow rate (L/min) ^b	0.1	0.1
<i>Octopole reaction cell</i>		
Hydrogen gas flow rate (mL/min)	4	/
Oxygen gas flow rate (%)	/	30
<i>Measuring parameters</i>		
m/z monitored	⁷⁷ Se, ⁷⁸ Se	⁷⁷ Se, ⁷⁸ Se, ^{77→93} Se, ^{78→94} Se
integration time (s)	0.1	3.0

^a Babington nebulizer was used when HF was present in the digested samples

^b parameters were daily changed to obtain optimal sensitivity

Verification of the optimized method was made with certified reference material Trace elements in spinach leaves (NIST 1570a). When selenium was determined by ICP-MS, good agreement between certified (117 ± 9) ng/g and obtained (124 ± 34) ng/g values was found. Detection by ICP-QQQ showed a good match between certified (117 ± 9) ng/g and obtained (121 ± 3) ng/g values. In addition, certified reference material Durum wheat flour (NIST 8436) and Wholemeal flour (BCR 189) were used. Approximately equal values for obtained and certified values were found in both certified reference materials. Both methods for determination of selenium in plant and seed samples were compared with a previously described independent method HG-AFS with digestion on an aluminum block.

3.4.1.3 Determination of selenium content in sediment by ICP-MS

Around 0.25 g of milled sediments, 4 mL HNO₃, 2 mL HF and 1 mL HCl were placed in a microwave system (Ethos One, Millstone, Italy) in a closed Teflon tube. The following temperature program was used: 30 min ramp to 210 °C, 60 min hold with power of 1200 W and then cooling. When the vessels were cooled to room temperature, 15 mL of 4% aqueous solution H₃BO₃ was added to each vessel and the following program was used: 15 min ramp to 220 °C, 30 min hold with power of 1200 W and then cooling to room temperature. The solution was transferred to polyethylene tubes (Sarstedt, Nümbrecht, Germany) and diluted with Milli-Q water to appropriate volume. Selenium was determined by ICP-MS. Optimal conditions are described in Table 3.1.

The certified reference material Trace elements in river sediments (BCR 320R) was also analysed, whereby good agreement for selenium content was found between obtained (0.97 ± 0.20) µg/g and indicative values (0.96 ± 0.18) µg/g.

3.4.2 Selenium compounds in plant samples

3.4.2.1 Lipid extraction of pumpkin seeds

Due to the high amount of lipids in pumpkin seeds, extraction with diethyl ether is needed before determination of soluble selenium compounds. Five g of homogenized and lyophilized pumpkin seeds was weighted in Duran bottles with a cover and 75 mL of diethyl ether was added. Extraction was performed for 8 h at the room temperature. The organic phase was removed and samples were extracted twice with 50 mL of diethyl ether for 2 h. All organic phases were collected and left overnight, until the residue of diethyl ether completely evaporated. Seed residue was lyophilized and selenium compounds were determined (Smrkolj, Stibilj, Kreft, & Kapolna, 2005).

3.4.2.2 Enzymatic extraction of soluble selenium compounds

For determination of selenium compounds in plant samples, 0.6 g was extracted with 60 mg of enzyme Protease XIV in 8 mL Milli-Q water by shaking in a water bath (SW22, Julaba) for 24 h at 37 °C and 200 rpm, followed by centrifugation (Centrifuge 5804 R, Eppendorf) at 11000 rpm at 4 °C for 60 min. After centrifugation, the supernatant was filtered through 0.45 µm Millex PVDF filters (Millipore Corporation) and stored at - 20 °C until analysis.

The general scheme of determination of selenium and its compounds in selected samples is shown below in Figure 3.11.

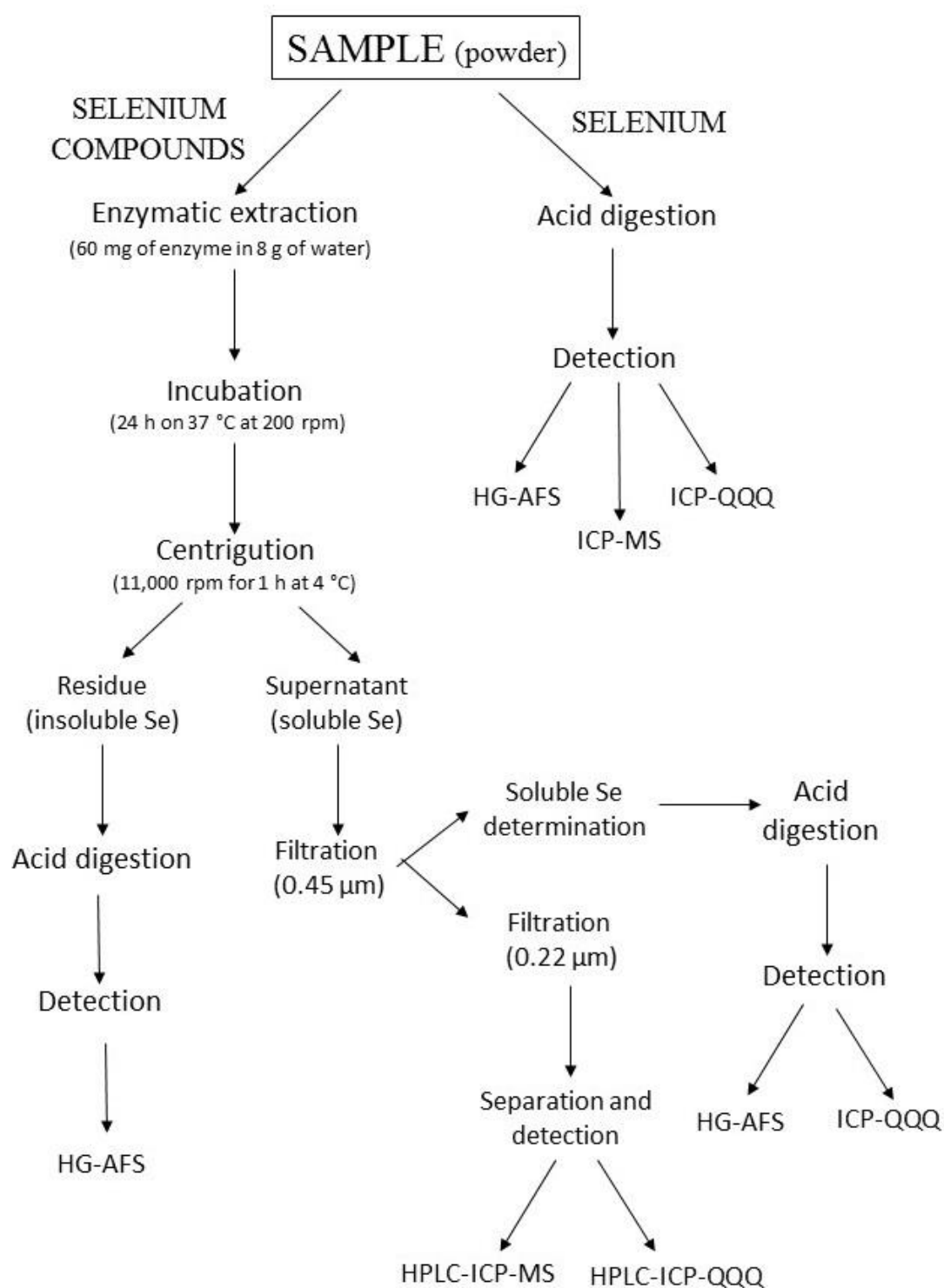


Figure 3.11: General scheme of sample pre-treatment and determination of selenium and its compounds in selected plant samples.

3.4.2.3 Separation and detection of soluble selenium compounds

For determination of soluble selenium compounds, the chromatographic system consisted of a high performed liquid chromatography pump (Series 1100, Agilent, Germany), equipped with a manual injector Rheodyne (Model 7725i, Cotati, CA) with a 50 μ L loop. The outlet of the column was directly connected to the concentric nebulizer of the ICP-MS. Treatment of the data obtained was performed with MassHunter and Agilent ChemStation software. Data processing was based on the peak area. The other hyphenated chromatographic system was composed of high performed liquid chromatography pump (Series 1260 Infinity, Agilent Technologies, Germany) and auto sampler, connected to inductively coupled plasma tandem mass spectrometry ICP-QQQ (8800, Agilent Technologies, Tokyo, Japan). A 50 μ L loop was used for determination. Data obtained from MassHunter were furthermore processed in MassHunter software, based on the peak area of identified compounds.

3.4.2.3.1 Anion exchange chromatography

3.4.2.3.1.1 Hamilton PRP-X100

For separation of selenium compounds (SeMet, SeMeSeCys, SeCys₂, Se(IV) and Se(VI)) in enzymatic extracts, a Hamilton PRP-X100 (4.1 mm x 250 mm x 10 μ m) anion exchange column with gradient elution of 1 and 10 mM citrate buffer (pH 4.8) in 2% methanol (to increase the sensitivity of the selenium signal) was used. Optimal conditions for selenium compounds separation are described in Table 3.3 and Table 3.3. For detection of selenium compounds separated on a Hamilton PRP-X100 column two different detection systems were used: ICP-MS or ICP-QQQ. LOD and LOQ were measured for both systems (Table 3.4). Calculation for the sample was based on 0.6 g of sample and 8 mL volume of extract.

Selenium compounds were measured on ICP-MS mainly as ⁷⁸Se isotope, using an octopole reaction cell, with hydrogen as the reaction gas (flow 4 mL/min) while on ICP-QQQ as mass shift ⁷⁷→⁹³Se and ⁷⁸→⁹⁴Se using an octopole reaction cell with oxygen as the reaction gas (30% of total flow). Selenium standards (SeMet, SeMeSeCys, SeCys₂, Se(IV) and Se(VI)) in enzyme at an approximate concentration of 100 ng Se/g were used for identification of selenium compounds in plant samples. Standards were measured on a daily basis to ensure the correct peak area for each selenium compound. Retention times for selenium standards in water were: 5.5 min for SeCys₂, 6.7 min for SeMeSeCys, 11.1 min for SeMet, 12.7 min for Se(IV) and 28.3 min for Se(VI). In addition to selenium standards, mobile phases and enzyme solutions were injected on a daily basis. Soluble selenium compounds in samples were confirmed by the standard addition method.

Table 3.2: Operating conditions for HPLC-ICP-MS.

Parameter	Value
HPLC	
<i>instrument:</i> Agilent 1100	
<u><i>Anion exchange chromatography:</i></u>	
HAMILTON PRP-X100 column	4.1 mm × 250 mm × 10 µm
mobile phase A	1 mM citrate buffer in 2% MeOH (pH 4.8)
mobile phase B	10 mM citrate buffer in 2% MeOH (pH 4.8)
gradient	35 min: 0–14 min 0% B to 50% B, 14.1–15 min 50% B to 100% B, 15.1–25 min 100% B, 25.1–27 min 100% B to 0% B, 27.1–35 min 0% B
flow rate (mL/min)	0.5
injected volume (µL)	50
<u><i>Cation exchange chromatography</i></u>	
ZORBAX 300-SCX column	4.6 mm × 250 mm × 5 µm
mobile phase	3 mM pyridine solution in 2% MeOH (pH 2.1)
isocratic	32 min
flow rate (mL/min)	0.5
injected volume (µL)	50
ICP-MS	
<i>instrument:</i> Agilent 7500ce	
<i>nebuliser:</i>	MicroMist
<i>plasma:</i>	
RF power (W)	1500
outer gas flow rate (L/min)	15.0
carrier gas flow rate (L/min)	0.90
make up gas flow rate (L/min)	0.20
<i>octopole reaction cell:</i>	
H ₂ gas flow rate (mL/min)	4.0
<i>measuring parameters:</i>	
m/z monitored	⁷⁷ Se, ⁷⁸ Se (mostly used),
Integration time (s)	0.3

Table 3.3: Operating conditions for HPLC-ICP-QQQ.

Parameter	Value
HPLC	
instrument: Agilent 1200	
Anion exchange chromatography:	
HAMILTON PRP-X 100 column	4.1 mm × 250 mm × 10 µm
mobile phase A	1 mM citrate buffer in 2% MeOH (pH 4.8)
mobile phase B	10 mM citrate buffer in 2% MeOH (pH 4.8)
gradient	35 min: 0–14 min 0% B to 50% B, 14.1–15 min 50% B to 100% B, 15.1–25 min 100% B, 25.1–27 min 100% B to 0% B, 27.1–35 min 0% B
flow rate (mL/min)	0.5
injected volume (µL)	50
ICP-QQQ	
instrument: Agilent 8800	
nebuliser:	MicroMist
plasma:	
RF power (W)	1550
outer gas flow rate (L/min)	15.0
carrier gas flow rate (L/min)	1.05
octopole reaction cell:	
O ₂ gas flow rate (%)	30
measuring parameters:	
m/z monitored	⁷⁷ Se, ⁷⁸ Se (mostly used)
Integration time (s)	0.1

Table 3.4: LODs and LOQs of separated selenium compounds on Hamilton PRP-X100.

Se compound	ICP-MS			ICP-QQQ		
	LOD (ng/g) solution	LOQ (ng/g) sample ^a	LOQ (ng/g) solution	LOD (ng/g) solution	LOQ (ng/g) sample ^a	LOQ (ng/g) solution
SeCys ₂	0.1	1	0.3	0.1	1	0.3
SeMeSeCys	0.3	4	1.0	0.3	4	1.0
SeMet	0.2	3	0.7	0.4	5	1.3
Se(IV)	0.6	8	2.0	0.8	11	2.7
Se(VI)	0.2	3	0.7	1.3	17	4.3

^a 0.6g of sample diluted in 8 mL of enzymatic solution

3.4.2.3.1.1 Thermo IonPac AS11

Soluble selenium compounds were measured on a Thermo IonPac AS11 anion exchange column to confirm the selenium compounds obtained with an Hamilton PRP-X100 column. For the chromatographic system, high performance liquid chromatography HPLC Knauer 1100 Smartline directly connected by an ICP-MS NexIon 300D (PerkinElmer, USA) was applied. Peaks were identified with the PeakFit 4.12 program. Operation conditions are described in Table 3.6.

An anion exchange column Thermo IonPac AS11 and mobile phase acetate buffer were used for separation of selenium compounds in a concentration of 100 ng Se/g (Figure 3.12). Selenium compounds were separated in methane mode, but because of problems with a lack of methane, some samples were measured in helium mode. Methane mode is much more sensitive and low contents of selenium

compounds can be detected. Commercially available standards were: SeMet, SeMeSeCys, SeCys₂, Se(IV), Se(VI) and GPx. SePP was prepared in the laboratory. Retention times of separated selenium compounds on Thermo IonPac AS11 are noted in Table 3.6. SeMet and SeMeSeCys were eluted at very similar retention times, less than 0.6 min apart. This was also seen for separation of GPx and SeCys₂. Injection of GPx also caused the appearance of a peak at the retention time 13.1 min, specific for Se(IV). When SeCys₂ was injected, in addition to the peak at retention time 9.9 min, one peak appeared at retention time 11.2 min, with unknown identity. When SeCys₂ and GPx were injected simultaneously, peaks were not separated. The presence of GPx needed to be confirmed with another chromatographic technique - affinity chromatography. Identification of SeMet, Se(VI) and Se(IV) was possible with an anion exchange column Thermo IonPac11.

Table 3.5: Operating conditions for separation on anion column Thermo IonPac11.

Parameter	Value
HPLC	
instrument: Knauer 1100 Smartline	
<u>Anion exchange chromatography:</u>	
Thermo IonPac AS11 column	250 x 4 mm ID
mobile phase A	3.3 mM Tris-acetate buffer (pH=8.0)
mobile phase B	10 mM Tris-HAc + 500 mM NH ₄ Ac (pH=8.0)
gradient	60 min:0-3 min 0% B, 3-110 min 100% B to 40% B, 10-23 min 40% B to 55% B, 23-26 min 55% B to 57% B, 26-28 min 57% B to 100% B, 28-52 min 100% B, 52-60 min 100% B to 0% B
flow rate (mL/min)	0.8
injected volume (μL)	20
ICP-MS	
instrument: NexIon 300D	
nebuliser:	Glass
plasma:	
RF power (W)	1300
carrier gas flow rate (L/min)	0.92
octopole reaction cell:	
CH ₄ gas flow rate (mL/min)	0.58
He gas flow rate (mL/min)	2.8
measuring parameters:	
m/z monitored	⁷⁷ Se, ⁷⁸ Se, ⁸⁰ Se (mostly used)

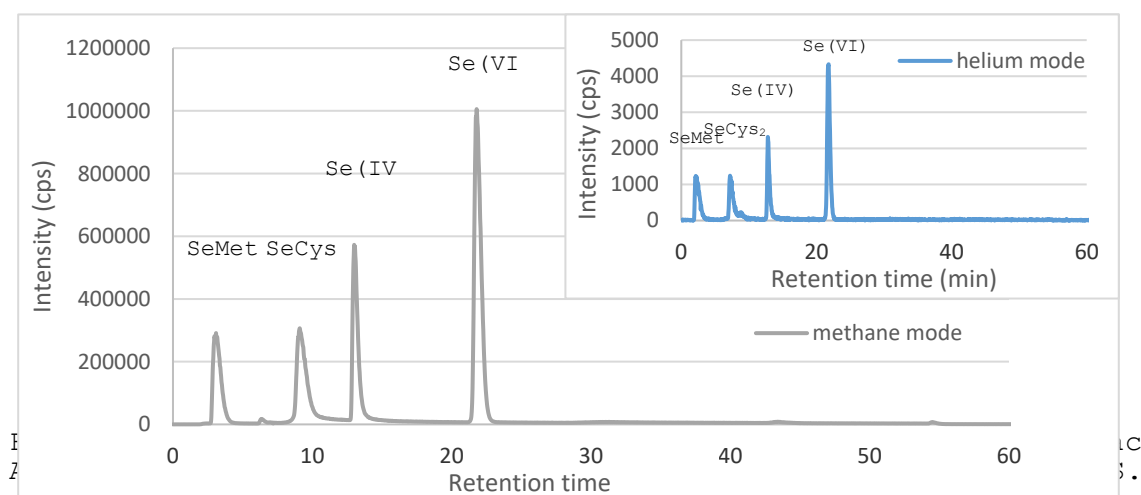


Table 3.6: Retention times of selenium standards on a Thermo IonPac AS11 column.

Se compound	Retention time
SePP	2.2 min
SeMet	3.1 min
SeMeSeCys	3.7 min
SeCys ₂	9.9 min and 11.2 min
GPx	9.9 min and 13.1 min
Se(IV)	13.1 min
Se(VI)	21.7 min

3.4.2.3.2 Cation exchange chromatography

Because of elution of SeCys₂ in the void volume of the Hamilton PRP-X100, cation exchange column Zorbax 300-SCX (4.6 mm x 250 mm x 5 µm) was used in previous studied studies for determination in plant samples (Cuderman, Kreft, Germ, Kovačević, & Stibilj, 2008). A 3 mM pyridine solution (pH 2.1) in 2% methanol was used for separation. The method and operating conditions are given in Table 3.2.

Five selenium standards were prepared for separation on a Zorbax 300-SCX: SeCys₂, SeMeSeCys, SeMet, Se(IV) and Se(VI). Retention times of Se(IV) and Se(VI) were not separated. This column is mainly used for determination of SeCys₂ in enzymatic extracts (Figure 3.13).

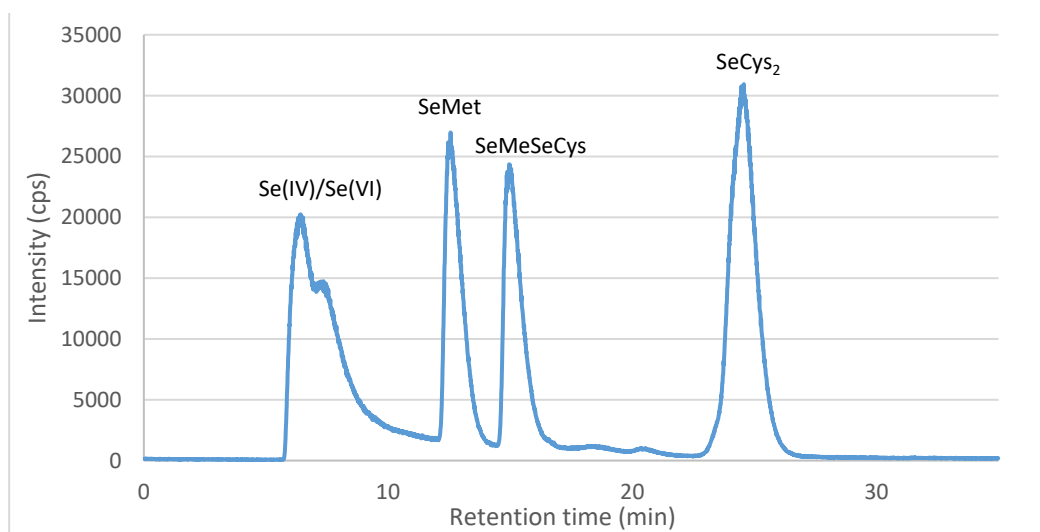


Figure 3.13: Separation of five selenium standards on cation exchange column Zorbax 300-SCX by HPLC-ICP-MS.

3.4.2.3.3 Affinity chromatography

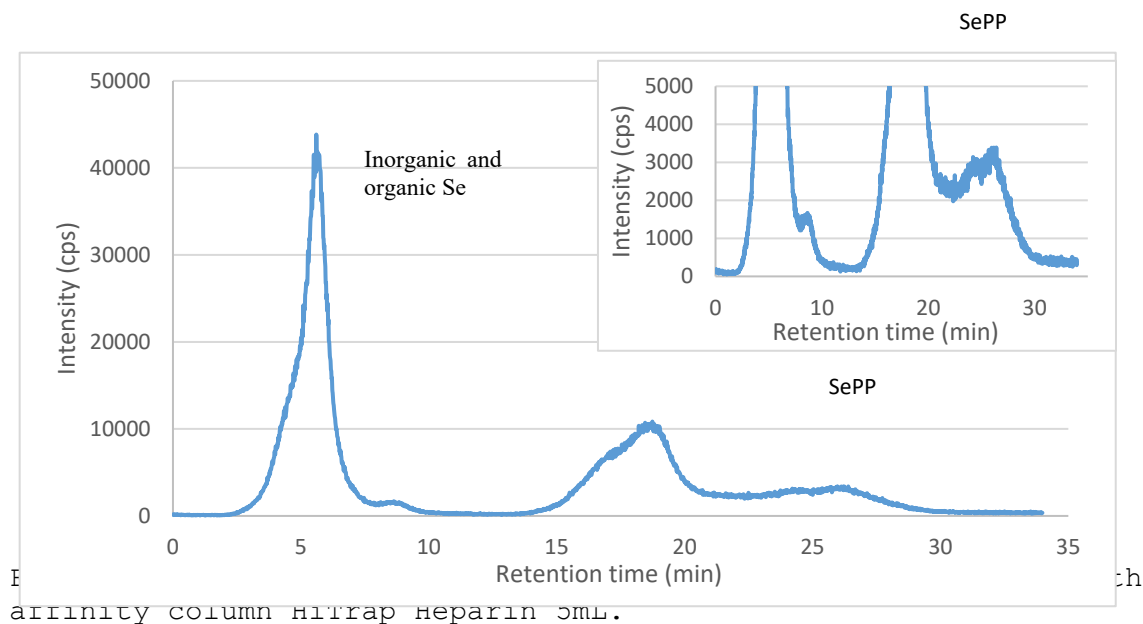
Affinity chromatography is important for confirmation of SePP and GPx in samples. An affinity column HiTrap Heparin 5 mL was applied for separation. In addition to five known standards, two additional standards (GPx and SePP) were used. Selenium was measured as ⁷⁸Se in a reaction cell, using methane (0.58 mL/min) or helium gas (2.8 mL/min) (Table 3.7). SePP was eluted at around 27 min, while GPx at around 23 min. Other selenium compounds (Se(IV), Se(VI), SeMet) were eluted at retention times from 6.3 to 8.0 min and were not well separated.

3.4.2.3.3.1 Purification of selenoprotein P from human serum

For identification of SePP on anion exchange column, SePP needs to be previously purified. A column filled with Heparin was used for purification of SePP from human serum. First, serum was injected into an HPLC system, coupled with ICP-MS to obtain the presence of SePP (eluted at around 27 minutes (Figure 3.14)). Second, HPLC was disconnected from the ICP-MS and four fractions were collected. Times of fractions: 3.33 – 6.66 min, 6.66 – 13.33 min, 13.33 – 21.66 min and 21.66 – 28.3 min. In the first fraction mainly inorganic selenium and in the fourth fraction SePP were present (low content).

Table 3.7: Operation conditions on affinity column HiTrap Heparin 5 mL.

Parameter	Value
HPLC	
instrument: Knauer 1100 Smartline	
<u>Affinity chromatography:</u>	
HiTrap Heparin 5 mL column	
mobile phase A	50 mM NH ₄ Ac (pH=5.8)
mobile phase B	10 mM Tris-HAc + 500 mM NH ₄ Ac (pH=8.0)
gradient	30 min:0–8 min 0% B, 8–20 min 0% B to 100% B, 20–30 min 100% B to 0% B
flow rate (mL/min)	1.0
injected volume (μL)	20
ICP-MS	
instrument: NexIon 300D	
nebuliser:	Glass
plasma:	
RF power (W)	1300
carrier gas flow rate (L/min)	0.92
octopole reaction cell:	
CH ₄ gas flow rate (mL/min)	0.58
He gas flow rate (mL/min)	2.8
measuring parameters:	
m/z monitored	⁷⁷ Se, ⁷⁸ Se (mostly used), ⁸⁰ Se



The last fraction was separated on an anion exchange column IonPac AS11 to confirm SePP presence. SePP was eluted at 2.2 min but its concentration was very low (Figure 3.15). SePP was stored at - 20 °C until injection.

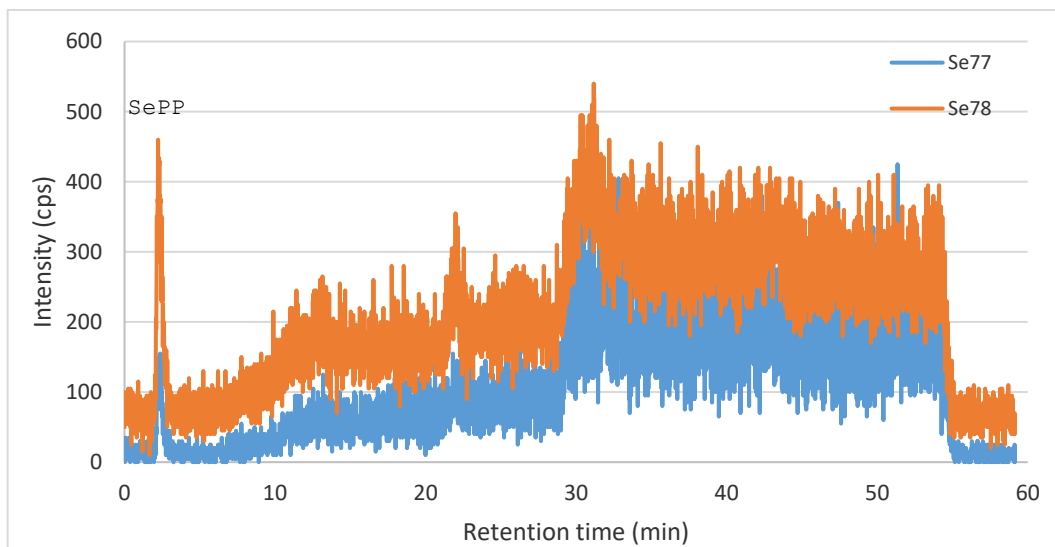


Figure 3.15: Chromatogram of fraction 4 (SePP) on anion exchange column Thermo IonPac AS11.

3.4.2.3.4 Size exclusion chromatography

In addition, selenium compounds in selected samples were separated on two connected size exclusion columns SEC: SK 55 S HW (600 x 10 mm) and TSK 40 S SW (250 x 8 mm). With this configuration, the separation range is from 200 to 0.1 molecular weight (MW). The method and operating conditions are given in Table 3.8.

3.4.2.3.4.1 Mass calibration

We first need to perform mass calibration, to obtain a linear function for calculation of unknown masses. For mass calibration ^{55}Mn , ^{63}Cu , ^{57}Fe , ^{66}Zn , ^{77}Se , ^{78}Se , ^{82}Se need to be measured. We also used UW light at 280 nm to confirm the presence of proteins.

Proteins with known masses were separated on the SEC. Calibration curves are obtained from the graph of $\log(\text{kDa})$ vs. RT (min): one for high kDa mass and one for low kDa mass. We separated albumin, SeMet and Se(VI). Due to previous results from anion exchange chromatography, the focus was only on SeMet and Se(VI) compounds.

$$\text{High molecular weight: } y = -0.0359x + 6.6726, R^2 = 0.9975$$

$$\text{Low molecular weight: } y = -0.0213x + 3.7818, R^2 = 0.9304$$

Se(VI) is a smaller molecule than SeMet, but was eluted earlier. This may be because of Se(VI) to proteins with molecular weight around 60 kDa (like albumin). Peaks of SeMet and Se(VI) were covered with retention times of albumin (Table 3.9).

Table 3.8: Operation conditions on size exclusion column.

Parameter	Value
HPLC	
instrument: Knauer 1100 Smartline	
<u>Size exclusion chromatography:</u>	
SK 55 S HW, TSK 40 S SW column	600 x 10 mm, 250 x 8 mm
mobile phase A	50 mM NH ₄ Ac (pH=5.8)
mobile phase B	10 mM Tris-HAc + 500 mM NH ₄ Ac (pH=8.0)
gradient	120 min: 80% A and 20% B
flow rate (mL/min)	1.0
injected volume (μL)	20
ICP-MS	
instrument: NexIon 300D	
nebuliser:	Glass
plasma:	
RF power (W)	1300
carrier gas flow rate (L/min)	0.92
octopole reaction cell:	
CH ₄ gas flow rate (mL/min)	0.58
He gas flow rate (mL/min)	2.8
measuring parameters:	
m/z monitored	⁷⁷ Se, ⁷⁸ Se, ⁸⁰ Se (mostly used)

Table 3.9: Retention times for selenium compounds separation with SEC.

Standards	MW (Da)	Detection	RT (min)
Albumin	66400	UV	46.1, 51.9 (highest) and 56.8 min; 63.3, 70.9 and 77.7 min (highest)
SeMet	197	ICP-MS	63.5, 67.5 and 70.9 min (highest)
Se(VI)	143	ICP-MS	52.4 and 56.0 min (highest)

3.5 Statistical Data

Statistical analysis was performed with the Statgraphics Centurion XVI program (Statgraphics, Herndon, VA, USA). Analysis of variance (ANOVA) was used to assess the effect of treatment with selenium, iodine and their combinations on selenium and other measured parameters (agricultural data). Differences between treatments were estimated using Tukey's (HSD) post-hoc test at a significance level of $p = 0.05$.

Chapter 4

Results and Discussion

4.1 Method Optimization for Some Analytical Parameters

4.1.1 Optimization of digestion procedures

4.1.1.1 Optimization of digestion in the Ultrawave digestion system for selenium determination in plant samples

The digestion procedure for selenium determination in plant samples was optimized in the Ultrawave single reaction digestion system (Ultrawave), which can reach higher temperature and pressure, rather than the previously used digestion system Ethos One (Cuderman, 2010). Different certified reference materials were used for optimization: Trace elements in spinach leaves (NIST 1570a) and Durum wheat flour (NIST 8436). Various parameters were changed during optimization procedure: temperature of digestion (200 °C or 220 °C), volume of HNO₃ (4 – 6 mL) and presence of 1 mL H₂O₂. The amount of HF (0.1 mL) and the same temperature program were used for all digestion procedures: 15 min ramp to 200 °C (or 220 °C) and 20 min hold at the temperature. Solutions were diluted with Milli-Q water to an appropriate volume (30 mL) in polypropylene tubes (Sarstedt, Nümbrecht, Germany) and the selenium content was measured by ICP-QQQ (optimal conditions in Table 3.1). An external calibration technique was applied for the quantitative analysis of the samples. Seven standard solutions were prepared by diluting a standard stock solution with the same percentage of acids present in the sample (13% HNO₃). The range of concentrations was from 0.05 to 100 ng/g, including the calibration blank (0 ng/g).

Comparison of certified and obtained values of certified reference material Trace elements in spinach leaves (NIST 1570a) showed, that overestimated selenium content were present where the excess of HNO₃ (5 – 6 mL) and the H₂O₂ were present (Table 4.1). In further work, only 4 mL of HNO₃ (with no H₂O₂) was used for digestion of different plant materials at 220 °C. No residues were present in any of the digested solutions. The method was also used to determine the selenium content in certified reference material Durum wheat flour (NIST 8436) and Wholemeal flour (BCR 189). Good agreement between measured and certified values was found with the use of 4 mL HNO₃ for digestion in the Ultrawave digestion system (Table 4.2).

Table 4.1: Comparison of certified and determined values in certified reference material Trace elements in spinach leaves (NIST 1570a) by ICP-QQQ.

Digestion Temperature	Acids	Measured value (ng/g)	Certified value (ng/g)	Recovery (%)
1	6 mL HNO ₃ , 1 mL H ₂ O ₂ , 0.1 mL HF	141 ± 3	117 ± 9	121
2	200 °C 6 mL HNO ₃ , 0.1 mL HF	151 ± 6		129
3	5 mL HNO ₃ , 0.1 mL HF	151 ± 4		129
4	220 °C 4 mL HNO ₃ , 0.1 mL HF	121 ± 3		103

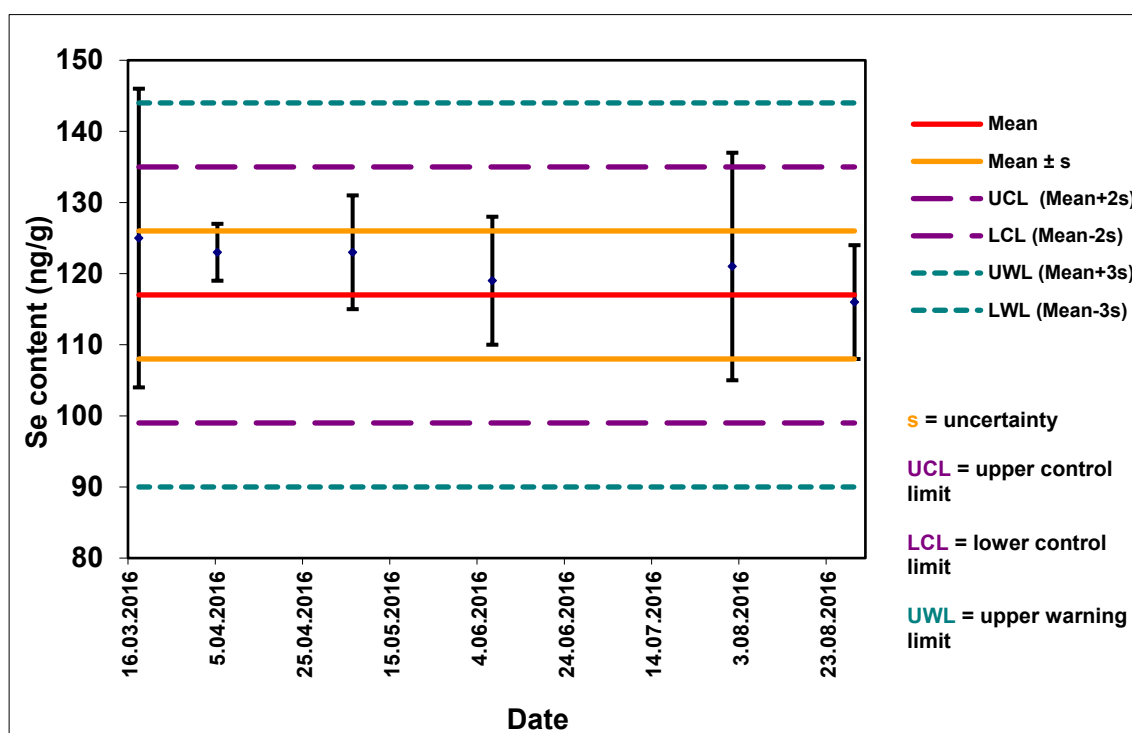


Figure 4.1: Control chart for selenium content in certified reference material Trace elements in spinach leaves (NIST 1570a).

Table 4.2: Comparison of certified and determined values of certified reference materials Durum wheat flour (NIST 8436) and Wholemeal flour (BCR 189) by ICP-QQQ.

Certified reference material	Temperature	Acids	Measured value (ng/g)	Certified value (ng/g)
Durum wheat flour (NIST 8436)	220 °C	4 mL HNO ₃	1167 ± 13	1140 ± 10
Wholemeal flour (BCR 189)			132 ± 11	132 ± 10

4.1.1.1.1 Selenium determination by ICP-QQQ and HG-AFS

The comparison among the selenium contents in buckwheat sprouts, buckwheat achenes, pumpkin seeds and pea seeds was determined with two independent methods (Figure 4.2). In the first method, acid digestion was performed in an Ultrawave digestion system whereby selenium was detected by ICP-QQQ, while a closed tube in hot plate was used for acid digestion in the second method, whereby selenium was detected by HG-AFS (Smrkolj & Stibilj, 2004). Good agreement between the both methods was observed.

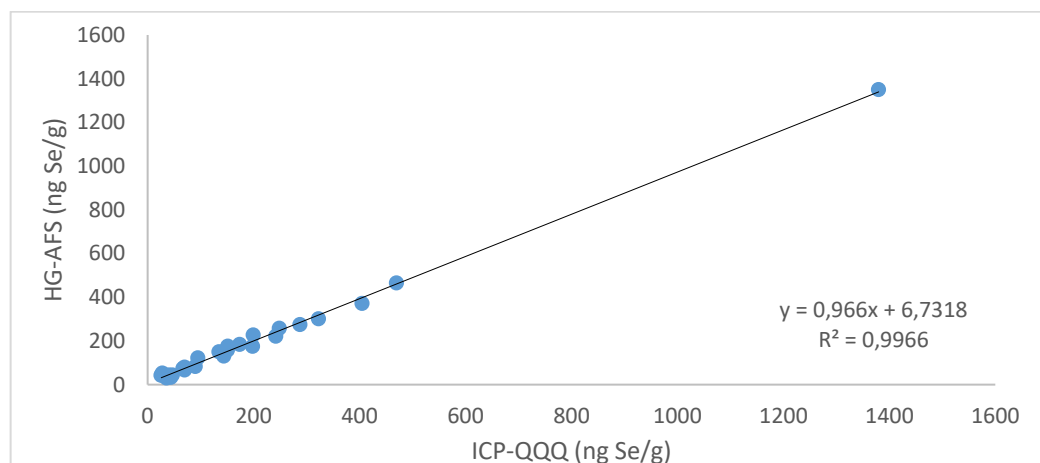


Figure 4.2: Comparison of selenium determination with two independent methods.

4.1.1.2 Optimization of microwave digestion for determination of selenium in sediment samples

An analytical procedure for digestion of marine sediment (Ščančar, Zuliani, Turk, & Milačič, 2007) was modified for determination of selenium in sediment samples from watercourses. For optimization, certified reference material Trace elements in channel sediments (BCR 320R) was used. Variable parameters were: time of digestion, temperature, volume of H_3BO_3 and temperature in the second step of digestion (Table 4.3). Digestion at 200 °C was not completed, due to residues in the digested solution. The temperature was raised to 220 °C and 230 °C, at which good agreement was found between certified and obtained values of BCR 320R, but vessels were started to melt at the set temperature and time of digestion. A raised volume of H_3BO_3 caused better results with fewer insoluble particles in the digested solution. A lower temperature in the first step of digestion (200 °C) and 220 °C at the second step were chosen for the digestion procedure.

This digestion procedure 6 (Table 4.3) was the best choice due to the obtained solution being without insoluble residue. Digested samples were diluted to an appropriate volume in polypropylene tubes (Sarstedt, Nümbrecht, Germany) and the selenium content was measured by ICP-MS. An external calibration technique was applied for the quantitative analysis of the samples. Seven standard solutions were prepared by diluting a standard stock solution with the same percentage of acids present in the sample (0.1% HNO_3). The range of concentrations was from 0.05 to 100 ng/g, including the calibration blank (0 ng/g). The correction factor for selenium was 0.9999. The good agreement between indicative (0.96 ± 0.18) µg/g and obtained values (1.04 ± 0.14) µg/g of certified reference material Trace elements in channel sediments (BCR 320R) was found. Calculation of LOD and LOQ in ICP-MS was based on the sample blanks. LOD (0.52 µg/g) and LOQ (1.72

µg/g) were calculated from 14 sample blanks measured five times, with a correction for sample weight and dilution.

Table 4.3: Temperature programs for optimization of the digestion procedure for sediment samples.

Digestion procedure	First step of digestion Chemicals	Temp. program	Second step of digestion Chemicals	Temp. program	Selenium content (µg/g DW)
1		30 min to 200 °C 45 min on 200 °C 1200 W	12.5 ml 4% H ₃ BO ₃	15 min to 200 °C 30 min on 200 °C	/
2		30 min to 200 °C 60 min on 200 °C		1200 W	0.93 ± 0.18
3	4 ml HNO ₃ 2 ml HF	30 min to 220 °C 60 min on 220 °C		15 min to 220 °C 30 min on 220 °C	0.97 ± 0.11
4	1 ml HCl	30 min to 230 °C 60 min on 230 °C	15 ml 4% H ₃ BO ₃	15 min to 230 °C 30 min on 230 °C	1.07 ± 0.32
5		30 min to 200 °C 60 min on 200 °C		15 min to 220 °C	0.82 ± 0.21
6		30 min to 210 °C 60 min on 210 °C		30 min on 220 °C	1.04 ± 0.14

4.1.2 Optimization of the analytical procedure for determination of selenium compounds

4.1.2.1 Optimized analytical parameters for separation of selenium compounds

4.1.2.1.1 Anion exchange chromatography

4.1.2.1.1.1 The effect of buffer solution on separation

An anion exchange column Hamilton PRP-X100 (4.1 mm x 250 mm x 10 µm) was used for the separation of five selenium compounds (Se(IV), Se(VI), SeMet, SeMeSeCys, SeCys₂) in plant samples (Cuderman et al., 2008; Mechora, Germ, & Stibilj, 2012a). The concentration and pH of the mobile phase (citric buffer) were previously optimized in a study by Cuderman et al. (2008). Due to better separation of the two selenium compounds (Se(IV) and SeMet) eluted at similar retention times, instead of 3 and 10 mM (pH=4.8), 1 and 10 mM citric buffer (pH=4.8) was used in the present work

(Figure 4.3). Separated selenium compounds were measured on an ICP-MS/ICP-QQQ detection system.

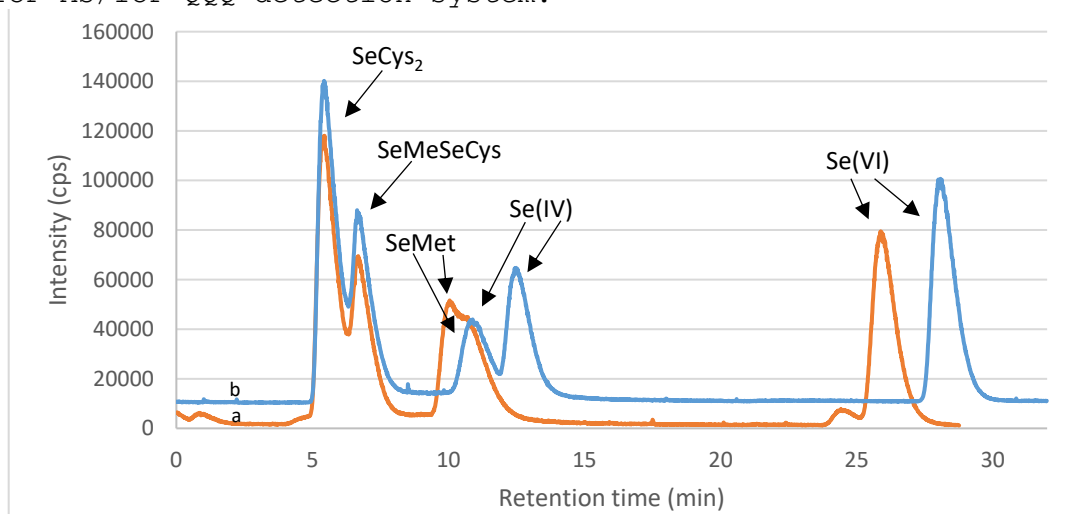


Figure 4.3: Separation of 100 ng/g selenium standards on an anion exchange column Hamilton PRP-X100 in a) 3 and 10 mM citrate elution buffer (pH=4.8) (Cuderman et al., 2008) and b) 1 and 10 mM citrate elution buffer (pH=4.8) by ICP-MS.

4.1.2.1.1.2 The effect of enzyme Protease XIV on separation

Five peaks were detected in an solution of five selenium standards (Se(IV), Se(VI), SeMet, SeMeSeCys, SeCys₂) prepared in Milli-Q water. When the selenium standard solution was prepared in enzyme Protease XIV (needed for extraction of selenium compounds from the sample) six peaks appeared (Figure 4.4). The peak for Se(IV) was divided into two peaks. Standard Se(IV) probably reacted with one of the components of the enzyme that cause elution of standard Se(IV) at a different retention time. Se(IV) was also eluted at two retention times in enzymatic extracts of aquatic plants (Figure 4.5) and other samples analyzed in this work.

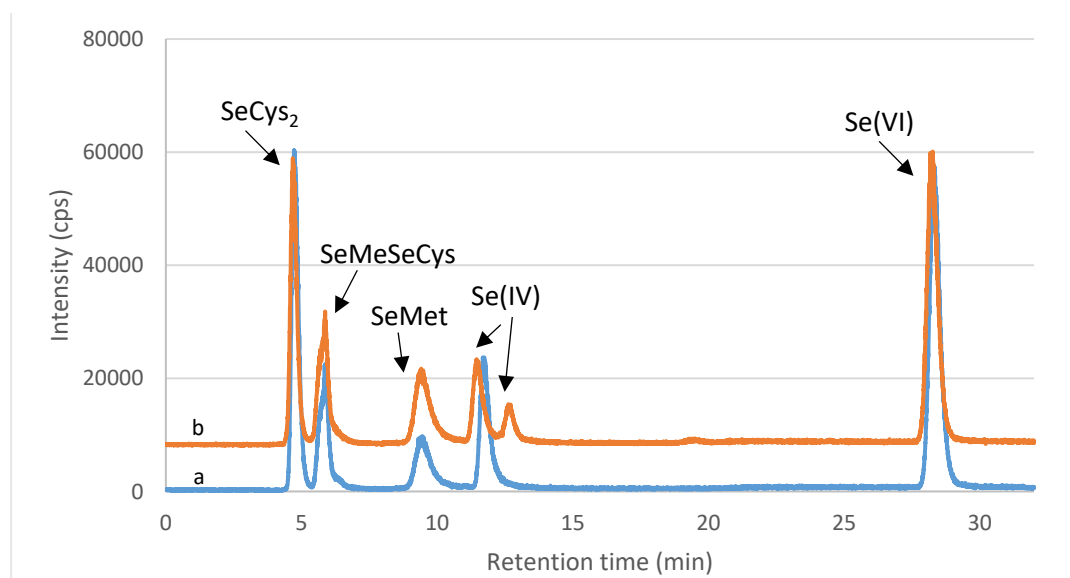


Figure 4.4: Separation of different solutions of selenium standard compounds (100 ng/g) on Hamilton PRP-X100 anion exchange column (a) in Milli-Q water and (b) in water-soluble fraction of enzyme Protease XIV by ICP-QQQ.

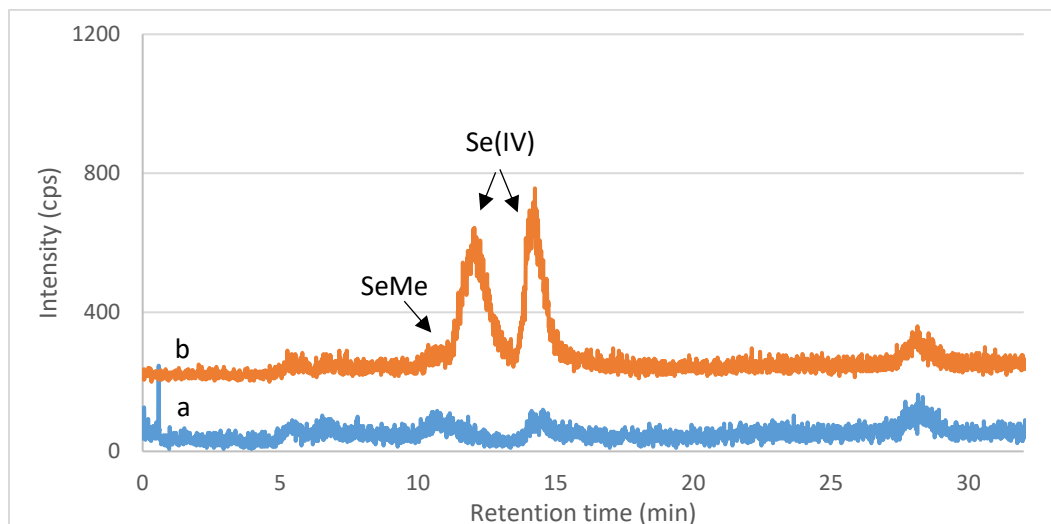


Figure 4.5: Chromatogram of enzymatic extract of aquatic plant sample (a) without standard addition, (b) with addition of 20 ng/g Se(IV) on Hamilton PRP-X100 anion exchange column by ICP-MS.

4.1.2.1.1.3 Stability of selenium compounds in enzymatic extract

Stability studies were performed in four parts. In the first part, 100 ng/g selenium standards (Se(IV), Se(VI), SeMet, SeMeSeCys, SeCys₂) prepared in enzyme Protease XIV were separated on an anion exchange column Hamilton PRP-X100: (a) immediately after preparation of standards, (b) 24 h stored at 4 °C and (c) 48 h stored at 4 °C (Figure 4.6). From the obtained results it can be concluded that the selenium compounds were stable for 48 h when stored at 4 °C.

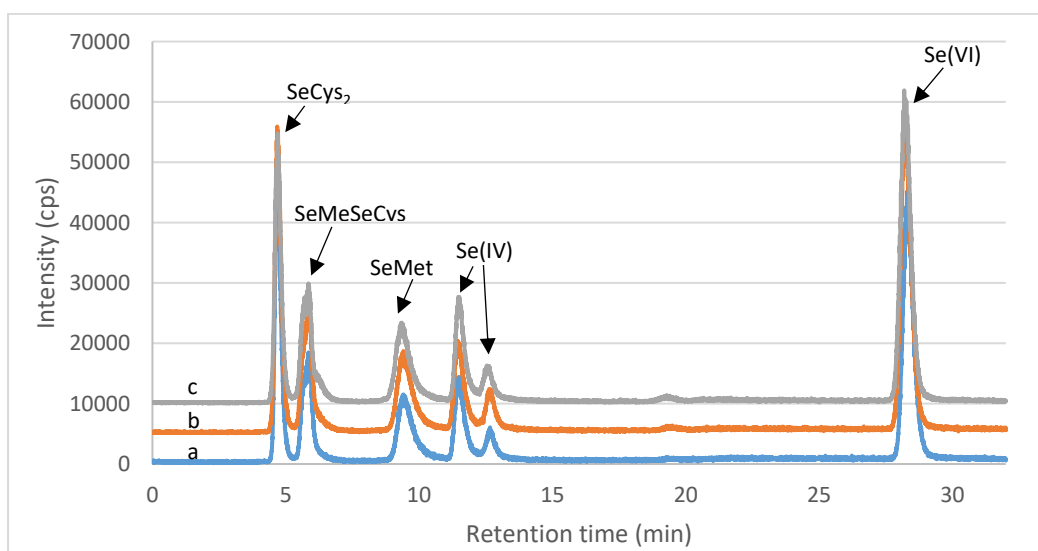


Figure 4.6: Stability of 100 ng/g selenium standards in enzyme Protease XIV solution (a) immediately after preparation, (b) 24 h stored at 4 °C and (c) 48 h stored at 4 °C, separated on Hamilton PRP-X100 by ICP-QQQ.

In the second part of the stability research, selenium standards were separated on a Hamilton PRP-X100 anion exchange column in 24 hours' storage at room temperature. Five selenium standards (Se(IV), Se(VI), SeMet, SeMeSeCys, SeCys₂) were prepared at a concentration of 20 ng/g in water (Figure 4.7) and in enzyme Protease type XIV (Figure 4.8). According to our results, selenium compounds in water are stable at room temperature for approximately 6 hours. After 18h, standards Se(IV) and Se(VI) in water had different retention times and different intensities. Selenium standards in enzyme had similar trend (after 21 h), while peaks for Se(IV) and Se(VI) were eluted at a different retention times with different intensities. The intensity of Se(VI) was probably higher due to oxidation of other selenium compounds to the highest selenium oxidation state Se(VI). All selenium compounds had higher intensity after 21 h, which is in contrast with expectations. It may be because of a higher sensitivity of ICP-QQQ after 21h of measurements. Overall, selenium compounds were stable in enzymatic extract for approximately 12 h at room temperature.

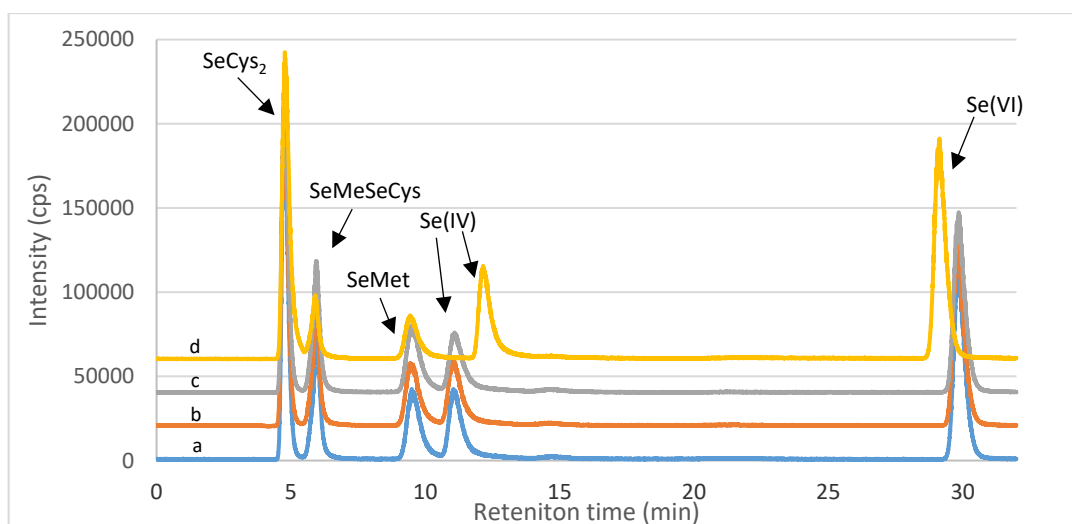


Figure 4.7: Stability of 20 ng/g selenium standards in water separated on Hamilton PRP-X100 by ICP-QQQ at room temperature: a) immediately after preparation, b) 4 h after preparation, c) 6 h after preparation and d) 18 h after preparation.

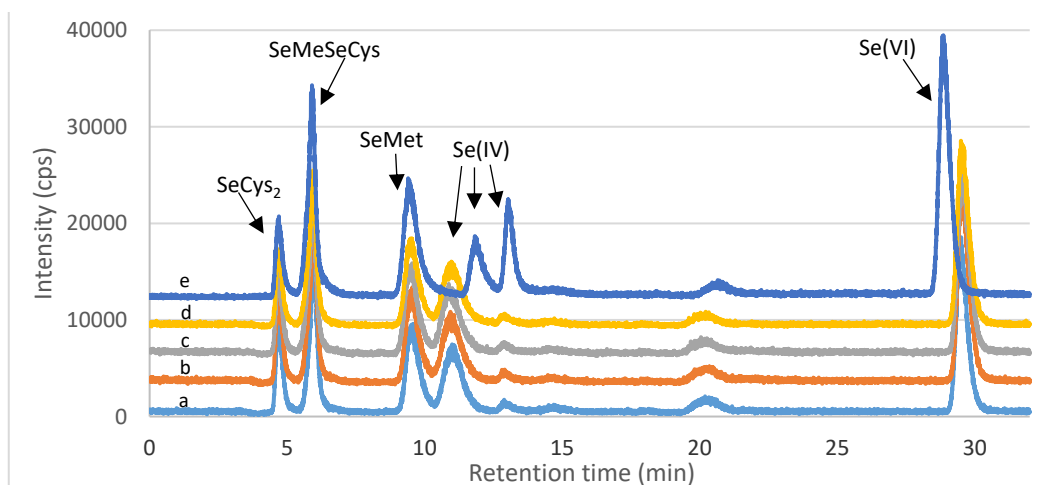


Figure 4.8: Stability of 20 ng/g selenium standards in enzyme Protease XIV separated on Hamilton PRP-X100 by ICP-QQQ at room

temperature: a) immediately after preparation, b) 5 h after preparation, c) 10 h after preparation, d) 12 h after preparation and e) 21 h after preparation.

In the third part, the stability of selenium compounds in enzymatic extracts during storage at $-20\text{ }^{\circ}\text{C}$ after a longer period of time was investigated. Some of the plant samples (pea sprouts) were extracted within 2 months' time and separated on a Hamilton PRP-X100 anion exchange column (Figure 4.9). Chromatograms of pea sprout samples extracted at various days were compared. A second extraction was performed at Helmholtz Zentrum München (HZM) with the same extraction procedure. Samples were frozen at $-20\text{ }^{\circ}\text{C}$ immediately after extraction. Regardless of the different time and location of extraction, the same selenium compounds were found in all extracts prepared at different times.

It can be concluded that selenium compounds in enzymatic extracts are stable for 2 months at a temperature $-20\text{ }^{\circ}\text{C}$.

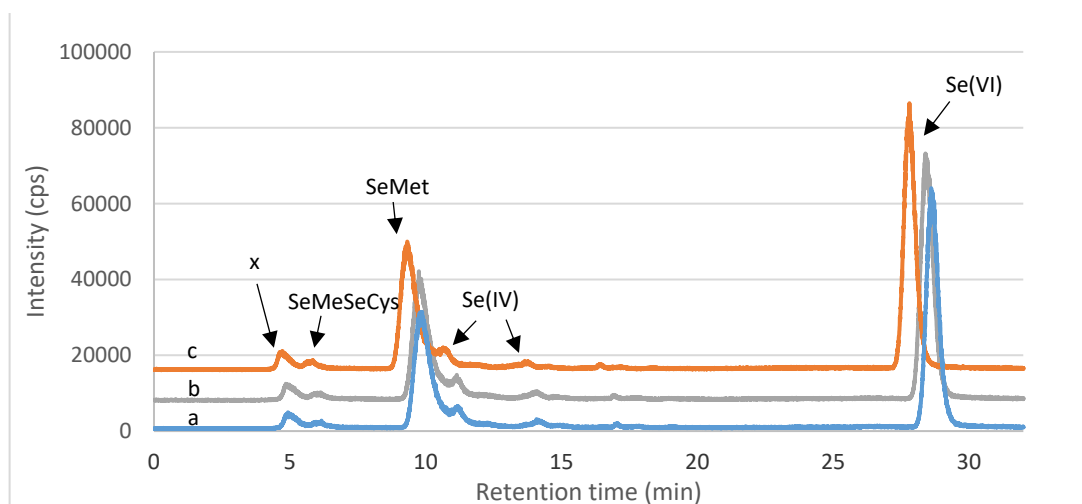


Figure 4.9: Chromatograms of pea sprouts Se(VI)+I(V), extracted within 2 months' time: (a) 0 days (5th September 2016), (b) 20 days (20th September 2016) and (c) 59 days (5th November 2016) and separated on a Hamilton PRP-X100 by ICP-QQQ.

In the last part, a stability test of real samples at room temperature was performed. Separation of selenium compounds in root samples of pea sprout Se(VI) was performed 29 hours after extraction (Figure 4.10). The same trend of Se(IV) and Se(VI) elution was present as when selenium standards stored at the room temperature were separated (Figure 4.8). Overall, selenium compounds in plant extract were stable for approximately 12h at the room temperature.

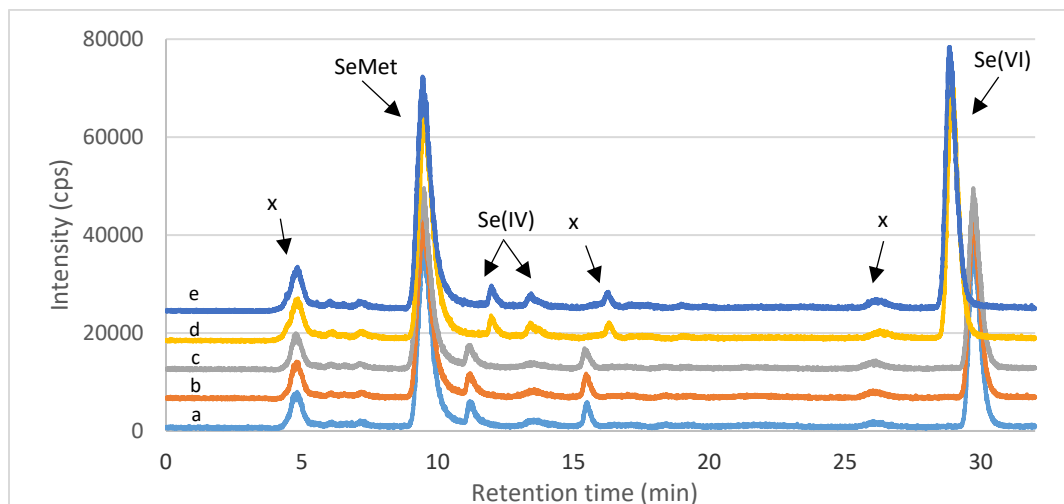


Figure 4.10: Stability of selenium compounds in roots of pea sprouts Se(VI) separated on Hamilton PRP-X100 by ICP-QQQ at room temperature: a) immediately after preparation, b) 5 h after preparation, c) 12 h after preparation, d) 24 h after preparation and e) 29 h after preparation.

4.1.2.2 Verification of the method

There is a lack of certified reference material for determination of selenium compounds in plants. Only one reference material, Selenium enriched yeast (SELM-1), has certified values for SeMet, Met and total selenium content (Mester et al., 2006). Good agreement for SeMet was found between our results and the certified value (Table 4.4). The accuracy and precision of SeMet determination was checked with literature data in certified reference materials: Durum wheat flour (NIST 8436) and Wheat gluten (NIST 8418), and good agreements were found (Table 4.4).

Table 4.4: SeMet content in reference materials.

Certified reference material	Found value (n)	SeMet ($\mu\text{g Se/g}$)	
		Certified value	Literature data
Selenium enriched yeast (SELM-1)	1233 ± 188 (8)	1380	1441 ± 100 (a)
Durum wheat flour (NIST 8436)	1.24 ± 0.08 (14)	/	1.21 ± 0.03 (b)
			1.47 ± 0.03 (c)
Wheat gluten (NIST 8418)	0.75 ± 0.15 (3)	/	0.59 ± 0.04 (b)
			0.57 ± 0.04 (c)
			0.61 ± 0.07 (d)

(n) number of measurements

(a) Cuderman & Stibilj, 2010

(b) Wolf & Goldschmidt, 2004

(c) Smrkolj et al., 2006

(d) Mazej et al., 2006

The matrix of samples can change elution times of compounds. Standards prepared in enzymatic extract are usually shorter than standards prepared in water. In all samples, the presence of selenium compounds was checked with a standard addition method (Figure 4.11), whereby peak areas after standard addition increased linearly, elution times of compounds stayed the same and the shape of peaks were symmetrical. Good agreement was between found and expected peak areas in all selenium compounds (data not shown).

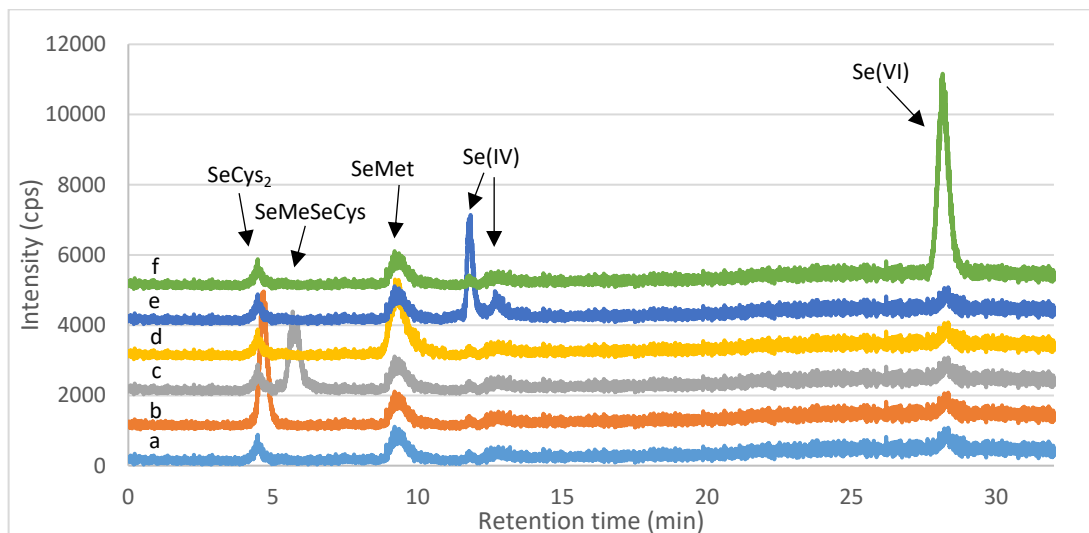


Figure 4.11: Additions of standards: a) sample of chicory Se(IV)+I(-I) b) standard addition of 20 ng/g SeCys₂, c) standard addition of 20 ng/g SeMeSeCys, d) standard addition of 20 ng/g SeMet, e) standard addition of 20 ng/g Se(IV) and f) standard addition of 20 ng/g Se(VI) separated on a Hamilton PRP-X100 by ICP-QQQ.

4.1.2.2.1 Identification of selenium compounds in plant extracts with other methods

To confirmed the optimized separation method with a Hamilton PRP-X100 anion exchange column (marked as JSI in this chapter), selected pea sprout samples were separated on a Thermo IonPac AS11 anion exchange column (Table 3.5; Figure 4.12), marked as HZM in this chapter. Because of the high selenium content, samples were previously diluted with acetate buffer. We confirmed SeMet and Se(VI), but not Se(IV) as we had expected. Standard addition of Se(IV) was performed and a peak at retention time 13 min appeared. SeMeSeCys was present in some samples, but identification was not completely reliable due to similar retention times as SeMet. In almost all samples, a peak at retention time around 10 min was also present, but identification is not possible only with anion exchange column. There were also some unknown peaks at retention times 6.5 and 8.4 min.

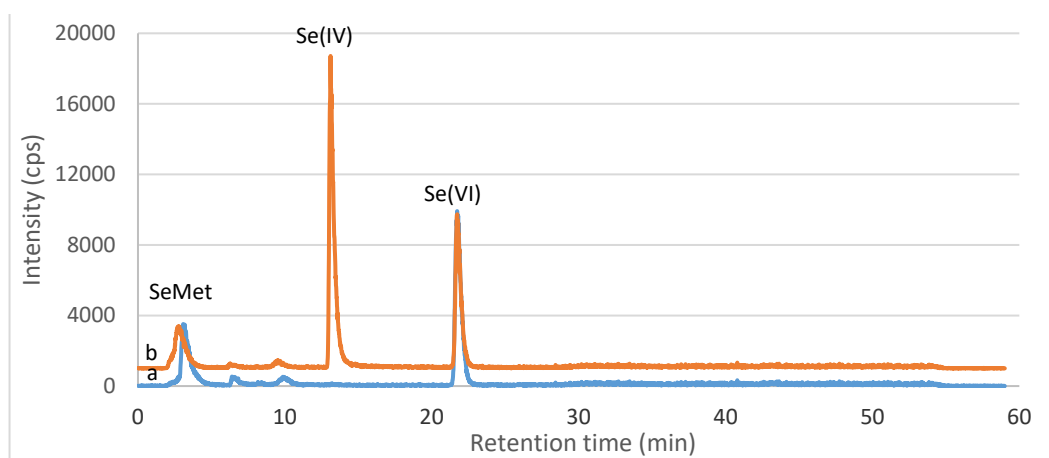


Figure 4.12: Separation of selenium compounds in pea sprout Se(IV) on Thermo IonPac AS11: a) sample and b) sample + standard addition of Se(IV) measured by ICP-MS.

To find out why Se(IV) was not found with the Thermo IonPac AS11 column (separation in ammonium acetate), we decided to separate selenium compounds with a Hamilton PRP-X100 column using ammonium acetate buffer with two different programs: i) program of separation at JSI (Table 3.3), ii) program of separation at HZM (Table 3.5).

Figure 4.13 shows 100 ng/g selenium standards (SeMet, SeMeSeCys, Se(IV), Se(VI), SeCys₂) separated with ammonium acetate buffer with two different separation programs. Standards were not well separated, only Se(IV) was identified with standard addition to the control sample (Figure 4.14). Other peaks were unidentified. Se(IV) was identified at retention time 15 min with the JSI separation program and at 12.3 min when separated with HZM program. To confirm the peak of standard Se(IV), standard was added to a control sample of pea sprouts at concentration 10 ng/g (Figure 4.14). Selenium compounds in pea sprout Se(IV)+I(V) were separated with a Hamilton PRP-X100 column, using ammonium acetate buffer and the HZM program (Figure 4.15). When Se(IV) was added to the sample, additional peaks appeared at retention times 12.5 and 16 min, while peak at the retention time 15 min disappeared. The peak at retention time 12 min was present at the retention time of Se(IV) standard. It can be concluded that an interaction occurred between the Thermo IonPac AS11 column and the matrix of pea sprout samples.

Determination of selenium compounds in pea sprouts and reference material Wheat gluten (NIST 8418) are presented in Table

4.5. Despite different elution programs and lengths of time approximately the same amounts of SeMet and Se(VI) were determined in the selected samples. Se(IV) compounds were not identified when the HZM elution program was used.

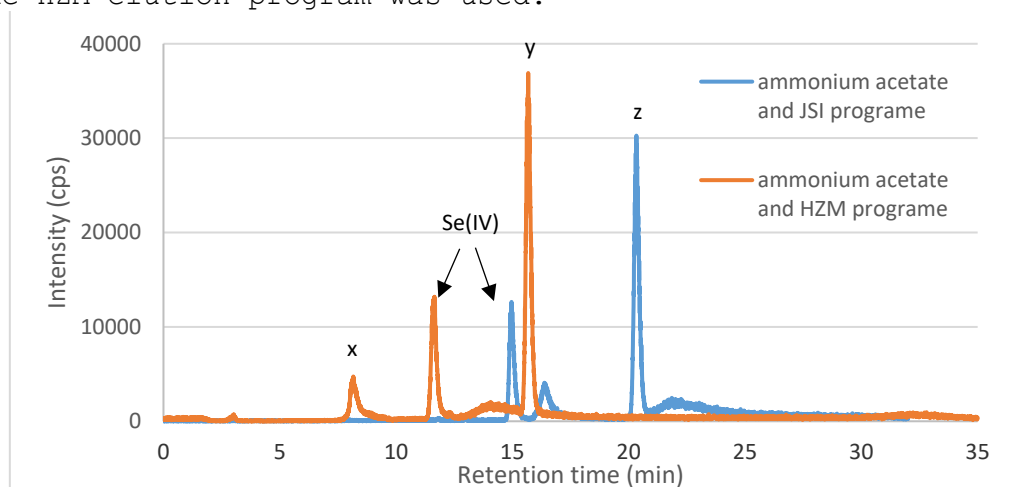


Figure 4.13: Chromatogram of 100 ng/g selenium standards in enzyme Protease XIV solution: a) performed with JSI elution program with Hamilton PRP-X100 column and ammonium acetate buffer (blue color) and b) buffer ammonium acetate and HZM elution program (red color).

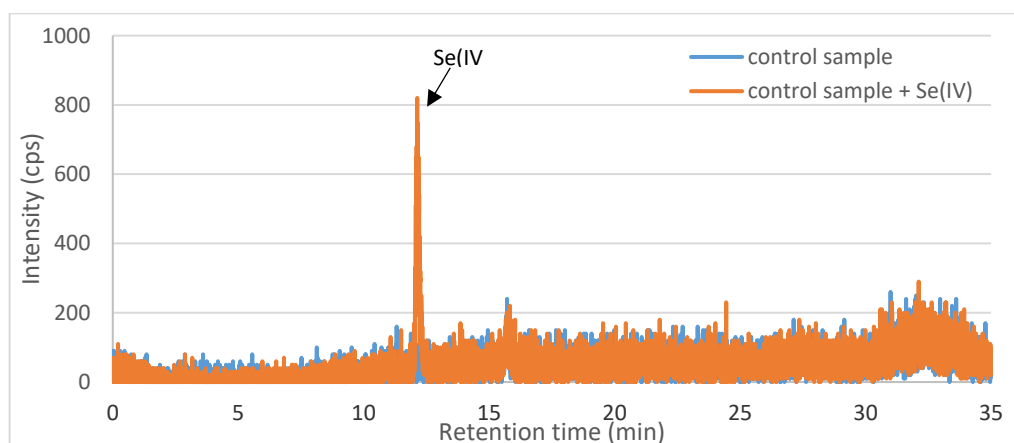


Figure 4.14: Chromatogram of extract control pea sprout sample and with addition of 10 ng/g Se(IV) with HZM elution program and ammonium acetate buffer, separated on Hamilton PRP-X100 at JSI.

Table 4.6: Comparison of identified selenium compounds in pea sprouts and reference material Wheat gluten with two independent methods.

Sample	Extraction date	Institute	Se as SeMet (ng/g)	Se as Se (IV) (ng/g)	Se as Se (VI) (ng/g)
pea sprout Se(VI)	6.9.2016	HZM	2477	/	1490
	25.9.2016	HZM	2395	/	1109
	25.9.2016	JSI	2479	260	1711
	7.11.2016	JSI	2801	385	2040
pea sprout Se (VI) +I (V)	6.9.2016	HZM	2851	/	1453
	6.9.2016	JSI	2620	332	1623
	25.9.2016	JSI	2514	259	1498
	7.11.2016	JSI	2438	268	1511
pea sprout Se (IV)	3.2015	JSI	1174	507	105
	25.9.2016	HZM	1179	/	114
	25.9.2016	JSI	1191	399	50
	7.11.2016	JSI	1129	332	54
pea sprout Se (IV) +I (-I)	3.2015	JSI	1448	407	46
	6.9.2016	HZM	1214	/	83
	25.9.2016	HZM	979	/	27
pea sprout Se (IV) +I (V)	3.2015	JSI	1757	364	55
	6.9.2016	HZM	1620	/	56
	6.9.2016	JSI	1127	432	45
	25.9.2016	JSI	1138	402	44
	7.11.2016	JSI	1210	431	51
buckwheat Se (VI)	25.7.2016	JSI	203	/	/
	25.9.2016	HZM	160	14	11
buckwheat Se (VI) +I (V)	25.7.2016	JSI	96	/	/
	25.9.2016	HZM	148	17	7
Wheat gluten (NIST 8418) (c=1210 ng/g)	1.3.2015	JSI	938	/	/
	25.7.2016	JSI	1293	/	/
	6.9.2016	HZM	920	/	/
	25.9.2016	HZM	1190	/	/
	7.11.2016	JSI	1305	/	/

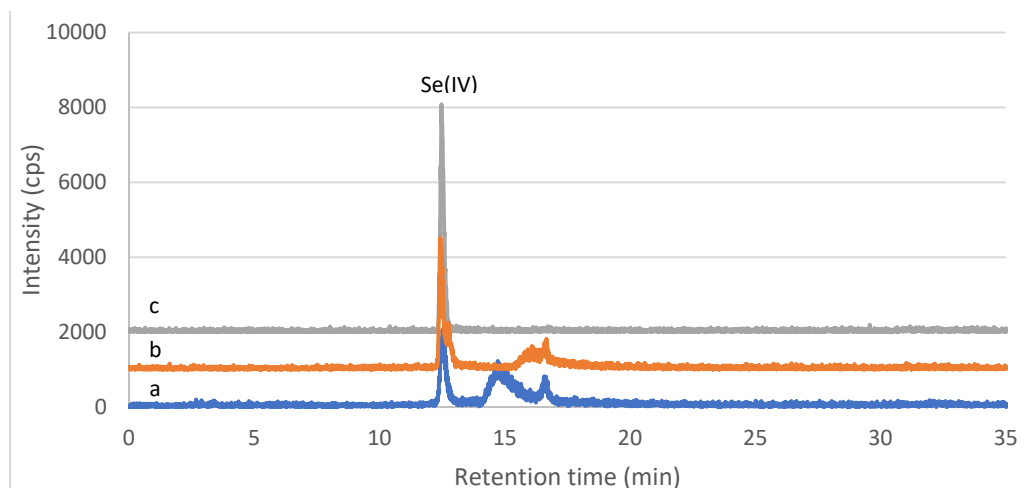


Figure 4.15: Chromatogram of a) pea sprout Se(IV)+I(V), b) with addition of 10 ng/g Se(IV) and c) standard 10 ng/g Se(IV) separated on Hamilton PRP-X100 with elution program from HZM and ammonium acetate buffer.

Selenium compounds in selected pea sprout samples were additionally separated on a HiTrap Heparin 5 mL affinity column (Table 3.7) to confirm the possible presence of SePP and GPx. In all analyzed extracts, selenium compounds were eluted until retention time 10 min, which means that there were no SePP and GPx in our samples (Figure 4.16).

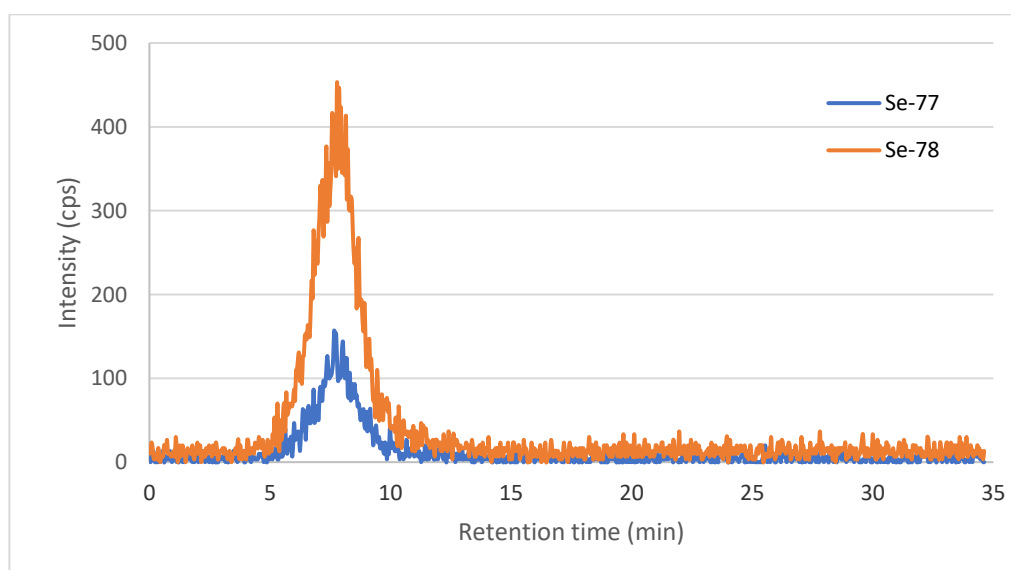


Figure 4.16: Separation of selenium compounds in pea sprout Se(VI) on a HiTrap Heparin 5 mL affinity column.

In addition, size exclusion chromatography showed elution of Se(VI) and SeMet at the same retention times in all analyzed samples (Figure 4.17). Se(VI) is a smaller molecule than SeMet, but was eluted earlier. This may be because of the binding of Se(VI) to proteins, with molecular weight around 60 kDa (such as albumin). Retention times of SeMet and Se(VI) were covered with the retention times of albumin.

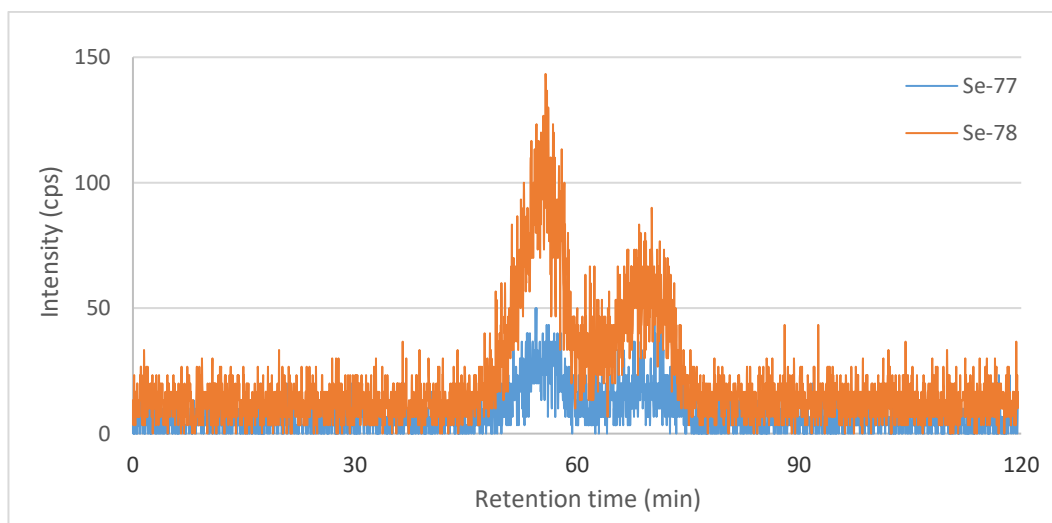


Figure 4.17: Separation of selenium compounds in pea sprout Se(VI) on size exclusion columns.

4.2 Selenium and Its Compounds in Enriched Sprouts

4.2.1 Pea sprouts

No visible toxic signs such as leaf chlorosis or plant drying were observed during the growth of seedlings. Soaking in Se(IV) solution significantly increased sprout height in comparison with control plants. On the other hand, the presence of Se(VI) had no effect. The addition of iodine to the selenium soaking solutions significantly decreased sprout height (Jerše et al., 2017).

4.2.1.1 Selenium and its compounds

Selenium content was measured in three parts of a pea plant: sprouts (leaves and stems), roots and seed residues (Table 4.7). The uptake of selenium was calculated as the mass of selenium in the plant parts divided by the mass of selenium in the seeds after soaking. We presumed that selenium and water passed from the soaking solution into seeds together. Mass portion (sprouts 38.3%, roots 33.4% and seed residues 33.4%) and dry masses (roots 8.4%, sprouts 10.4% and seed residues 16.8%) were taken into account.

Table 4.7: Selenium content and uptake in selenium and selenium-iodine enriched pea sprouts.

Seed soaking solution	Sprouts		Roots		Seed residues	
	content (µg/g DW)	uptake (n) (%)	content (µg/g DW)	uptake (n) (%)	content (µg/g DW)	remaining (n) (%)
Control	0.11 ± 0.03 (4)	/	0.13 ± 0.05 (4)	/	0.10 ± 0.05 (4)	/
Se(IV)	5.60 ± 0.21 (2)	15	10.13 ± 3.17 (2)	19	12.50 ± 2.64 (2)	43
Se(IV) + I(-I)	5.07 ± 0.63 (2)	11	11.03 ± 0.60 (2)	16	9.31 ± 3.17 (2)	40
Se(IV) + I(V)	3.87 ± 0.02 (2)	8	4.95 ± 0.53 (2)	7	9.73 ± 3.87 (2)	41
Se(VI)	14.73 ± 1.96 (2)	38	7.07 ± 1.85 (2)	12	7.35 ± 1.83 (2)	22
Se(VI) + I(-I)	14.08 ± 1.39 (2)	27	6.95 ± 0.76 (2)	10	8.62 ± 1.91 (2)	35
Se(VI) + I(V)	10.50 ± 0.20 (2)	20	5.51 ± 0.71 (2)	9	7.62 ± 0.01 (2)	30

(n) number of samples

^a sum of selenium taken up from soaking solution

Results are given as average values with standard error or standard deviation

Regardless of the selenium form, pea sprouts accumulated a large amount of selenium from soaking media. The sum of calculated selenium uptake into sprouts, roots and the remaining part in the seeds was up to 77%. When Se(IV) was present in the soaking solution, a larger amount of selenium was present in the roots, while in the case of Se(VI) main part of selenium was present in sprouts. It can also be observed that uptake of selenium to sprouts is more than two-folds higher in the case of Se(VI). The same trend has been observed with buckwheat sprouts (Cuderman & Stibilj, 2010; Štrekelj, Stibilj, Gaberščik, Germ, & Kreft, 2014), rice, broccoli and Indian Mustard sprouts (Zayed, Lytle, & Terry, 1998). Fertilization with Se(VI) causes translocation of selenium to the shoot tissue and is sequestered in the vacuoles, the accumulating organ for selenium in most plants (White, 2015). The

main part of selenium from seeds soaked in a solution of Se(IV) remained in the seed residues (43%).

After enzymatic extraction with the enzyme Protease XIV, approximately 60% of the total selenium content in pea sprouts (upper plants) was obtained as soluble selenium, regardless of the selenium form (Table 4.8). The amount of soluble selenium in roots was approximately 40% of the total selenium content, regardless of the selenium form in the soaking solution. Soluble selenium in seed residues was lower when seeds were soaked in a solution of Se(IV) (49%) than in a solution of Se(VI) (59%). In the presence of Se(IV), we predicted a lower amount of soluble selenium, due to lower uptake from the soaking solution. In research by Funes-Collado et al. (2013), soluble selenium in soy sprouts (legume) was obtained after enzymatic extraction with Protease XIV. Seeds were soaked in a mixture of 1 mg Se(IV)/L and 1 mg Se(VI)/L. Soluble selenium was determined in roots (72%) and shoot tissue (84%). A higher amount of selenium was extracted than in this study.

Selenium compounds were determined in parts of pea sprouts to discover the transport, distribution and metabolic pathways of selenium soaked in a solution of Se(IV) or Se(VI) (Table 4.8, Figure 4.18, Figure 4.19). The main selenium compound in enriched pea sprouts and roots was SeMet, regardless of the selenium form present in the soaking solution. In Se(IV) enriched pea sprouts and roots, selenium was mainly metabolized to SeMet (up to 79%), while Se(IV) and SeMeSeCys were present in lower amounts. According to the literature, Se(IV) is rapidly converted to organoselenium compounds in the roots (Li, McGrath, & Zhao, 2008; White, 2015; Ximénez-Embún et al., 2004).

On the other hand, approximately 50% of SeMet was present in Se(VI) enriched pea sprouts (sprouts, roots and seed residues). This is not in agreement with the study by Cuderman et al. (2010), in which a smaller amount of metabolized selenium was determined in buckwheat sprouts. According to the literature, the rate of transformation of Se(VI) to organoselenium compounds is lower than the assimilation of Se(IV), due to a rate-limiting step of reduction of Se(VI) to Se(IV). Moreover, Se(VI) is mainly accumulated as Se(VI) in leaves, in which it is assimilated in the leaf chloroplasts (El-Ramady, Abdalla, Alshaal, et al., 2015). Ximénez-Embún et al. (2004) found Se(VI) in different parts of mustard, lupine and sunflower, while in present study small amount of Se(VI) was observed. We assumed that reduction of Se(VI) was possible due to presence of a reducing agent, such as quercetin, a flavonol compound present in many vegetables, fruits and grains (Cuderman & Stibilj, 2010).

In research by Smoleń et al. (2016), SeMet content was measured in selenium and iodine fortified carrots. The research was performed over 3 years with a broad percentage of SeMet in terms of total selenium content. On average, the SeMet content in Se(VI) treated carrot was lower than in Se(IV) treated carrot. In carrot leaves, the SeMet content was 70% in Se(IV) and 35% in Se(VI) treated carrots, while in roots, content was only 40% (Se(IV)) and 20% (Se(VI)) in terms of total selenium content. The results of the present study can be compared with the study by Smoleń et al. (2016), in terms of the expected percentage of identified SeMet content in roots. In pea sprouts samples, 58% and 52% of SeMet were determined in Se(IV) and Se(VI) treated pea seeds, respectively. The percentage of SeMet in roots samples was lower, 16% in Se(IV) treated and 4% in Se(VI) treated pea seeds. The different percentages of SeMet in roots may be caused by the different structures of pea and carrot roots.

The main selenium compounds in Se(IV) and Se(VI) treated seeds residues were Se(IV) and Se(VI), respectively. In seed residues,

reduction and oxidation had occurred. In Se(VI) treated seed residues, selenium was present in a higher amount in Se(IV) forms, which indicated reduction of Se(VI) to the Se(IV) form (Cuderman & Stibilj, 2010).

Table 4.8: Selenium compounds in selenium enriched pea sprouts.

Seed soaking solution	Plant part	Soluble Se $\mu\text{g Se/g DW}$ (% ^a)	X ^b	SeMeSeCys	SeMet	Se (IV)	Y ^c	Z ^d	Se (VI)	Sum
						ng Se/g DW				
								%		
Se (IV)	sprouts	3.27 $\pm$ 0.54 (60)	82 $\pm$ 29	183 $\pm$ 63	1149 $\pm$ 177	507 $\pm$ 189	20 $\pm$ 3	<LOD	105 $\pm$ 71	37
	roots	4.27 $\pm$ 0.64 (36)	52 $\pm$ 1	133 $\pm$ 59	2726 $\pm$ 588	475 $\pm$ 55	25	14	16 $\pm$ 1	34
	seed	6.68 $\pm$ 0.48	108 $\pm$			2179 $\pm$				
	residues	(49)	15	<LOD	528 $\pm$ 85	13	179 $\pm$ 56	110 $\pm$ 9	95 $\pm$ 1	27
Se (VI)	sprouts	7.81 $\pm$ 1.54 (55)	98 $\pm$ 13	<LOD	3457 $\pm$ 635	284 $\pm$ 98	<LOD	<LOD	2702 $\pm$ 12	45
	roots	2.61 $\pm$ 0.46 (38)	47 $\pm$ 16	<LOD	1113 $\pm$ 161	76 $\pm$ 6	40 $\pm$ 32	<LOD	308 $\pm$ 81	23
	seed	4.70 $\pm$ 1.68								
	residues	(59)	29 $\pm$ 5	<LOD	67 $\pm$ 9	703 $\pm$ 61	53 $\pm$ 13	<LOD	1792 $\pm$ 917	31

^a percentage of soluble Se regarding to its content in sample^b unknown Se compounds with retention time 4.7 min (calculated as SeCys₂)^c unknown Se compounds with retention time 16.6 min (calculated as Se(IV))^d unknown Se compounds with retention time 28.2 min (calculated as Se(VI))^e percentage of identified Se compounds regarding to their content in sample

Results are given as average value of two determinations with absolute error

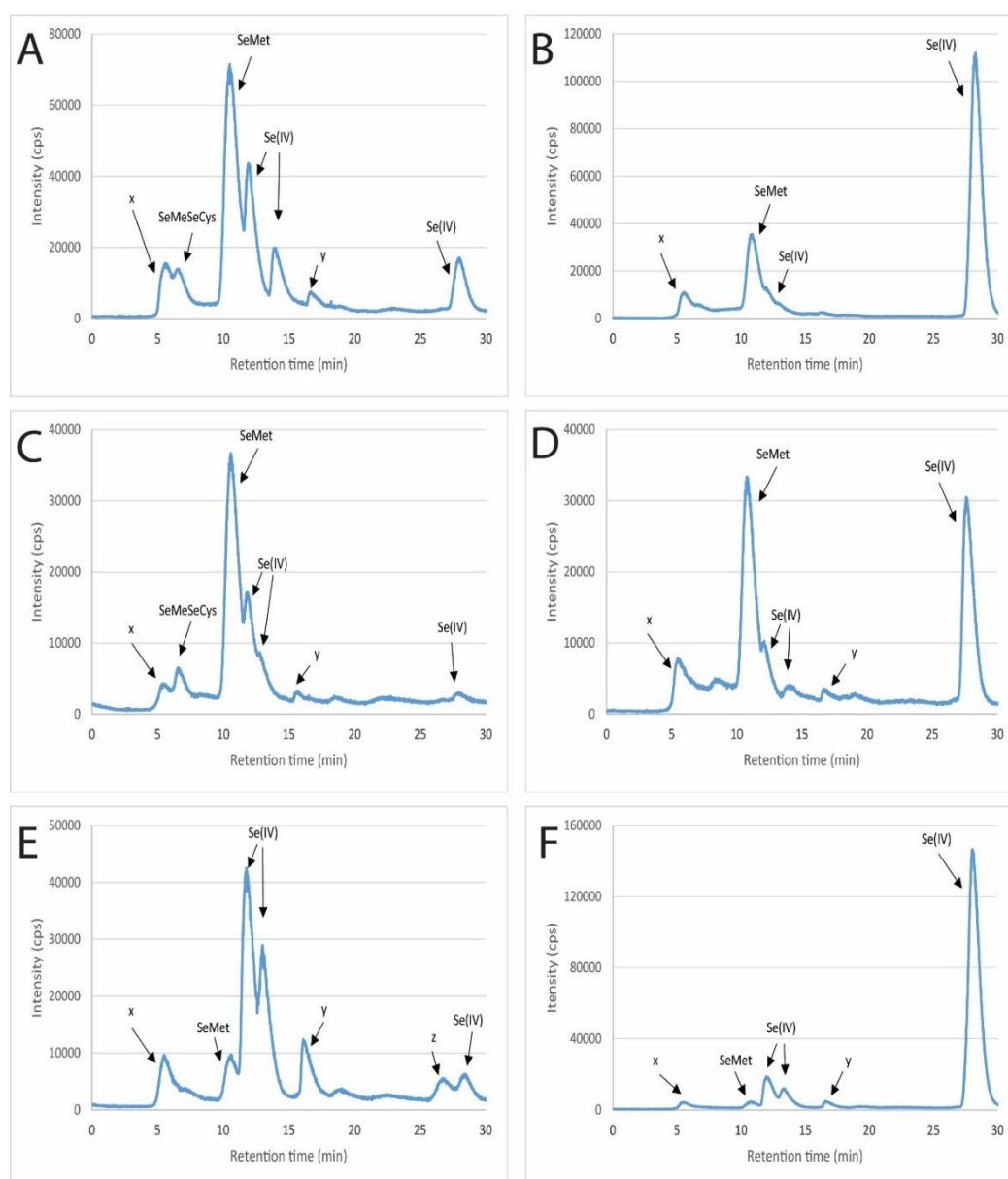


Figure 4.18: Separation of selenium compounds with a Hamilton PRP-X100 anion exchange column in the following parts of selenium enriched pea sprouts: A) sprouts Se(IV), B) sprouts Se(VI), C) roots Se(IV), D) roots Se(VI), E) seed residues Se(IV) and F) seed residues Se(VI).

In addition, identified selenium compounds were confirmed with a Zorbax 300-SCX cation exchange column (data not shown). A peak for SeCys_2 was not obtained at the known retention time for this compound. For further selenium compound determination, therefore, the compound at the retention time of SeCys_2 was marked on the anion column as unknown compound X in all other extracts.

4.2.1.2 Iodine effect on selenium and its compounds

The effect of iodine on selenium content is presented in Table 4.7. $\text{I}(-\text{I})$ had no impact on the uptake of selenium in either form of selenium in roots, nor on the uptake of selenium in sprouts. The presence of $\text{I}(\text{V})$ in solution resulted in an antagonistic

effect of selenium uptake, while the selenium content decreased by 40 – 70% in all plant parts, regardless of selenium form with exception of seeds residues. Selenium and iodine are elements transported from roots to leaves through xylem (White & Broadley, 2009), where I(V) might inhibit transporters of selenium present in both forms.

The iodine effect on selenium compounds distribution in all three parts of a pea sprouts was obtained (Table 4.9; Figure 4.19). The presence of iodine in the soaking solution slightly effected the amount of soluble selenium in pea sprouts and the distribution of selenium compounds (SeMeSeCys, SeMet, Se(IV), Se(VI)). In a study by Smoleń et al. (2016), in which carrot plants were treated with selenium and iodine solutions, iodine did not affect the percentage of SeMet in carrot leaves in Se(IV) and Se(VI) treated plants. On the other hand, the percentage of SeMet in roots from selenium and iodine treated plants, was higher when iodine was present (Smoleń et al., 2016). The SeMet content in carrot leaves is comparable to that of pea sprouts in the present study with no effect of iodine on SeMet content.

Table 4.9: Iodine effect on identified selenium compounds in selenium and selenium-iodine enriched pea sprouts.

Plant part	Seed soaking solution	Soluble Se $\mu\text{g/g DW}$	Sum % ^a	Sum % ^b	Sum % ^c
Sprouts	Se (IV)	3.27 $\pm$ 0.54	60	59	37
	Se (IV) + I (-I)	2.64 $\pm$ 0.20	49	84	43
	Se (IV) + I (V)	2.27 $\pm$ 0.57	59	86	52
	Se (VI)	7.81 $\pm$ 1.54	55	82	45
	Se (VI) + I (-I)	7.83 $\pm$ 1.58	55	89	51
	Se (VI) + I (V)	7.58 $\pm$ 1.68	72	77	56
	Se (IV)	4.27 $\pm$ 0.64	36	78	34
	Se (IV) + I (-I)	5.28 $\pm$ 1.90	49	44	23
Roots	Se (IV) + I (V)	2.10 $\pm$ 0.13	43	71	33
	Se (VI)	2.61 $\pm$ 0.46	38	57	23
	Se (VI) + I (-I)	3.64 $\pm$ 1.01	51	28	15
	Se (VI) + I (V)	2.38 $\pm$ 2.17	44	65	29
	Se (IV)	6.68 $\pm$ 0.48	49	42	27
	Se (IV) + I (-I)	/	/	/	/
Seed residues	Se (IV) + I (V)	5.69 $\pm$ 1.10	55	41	29
	Se (VI)	4.70 $\pm$ 1.68	59	53	31
	Se (VI) + I (-I)	7.09 $\pm$ 1.06	77	58	46
	Se (VI) + I (V)	4.60 ^d	60	79	29

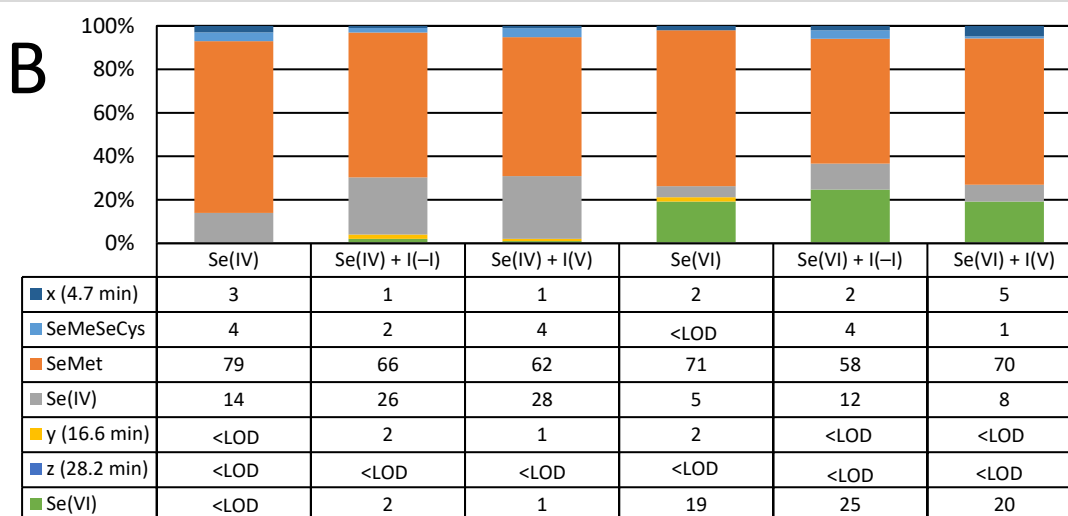
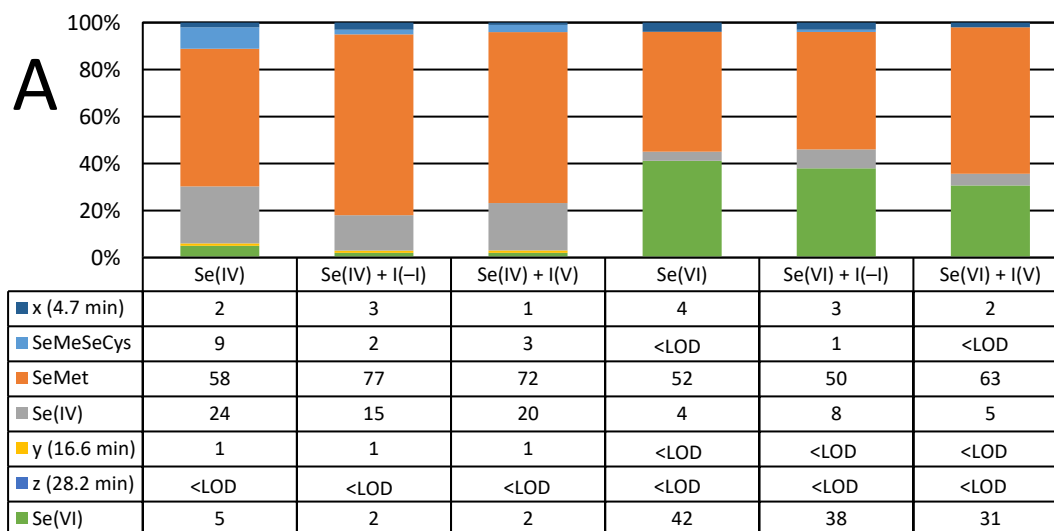
^a percentage of soluble Se regarding to its content in sample

^b percentage of identified Se compounds (SeMeSeCys, SeMet, Se(IV), Se(VI)) regarding to soluble Se

^c percentage of identified Se compounds regarding to their content in sample

^d performed only in one replicate

Results are given as value of one determination or average value of two determinations with standard error



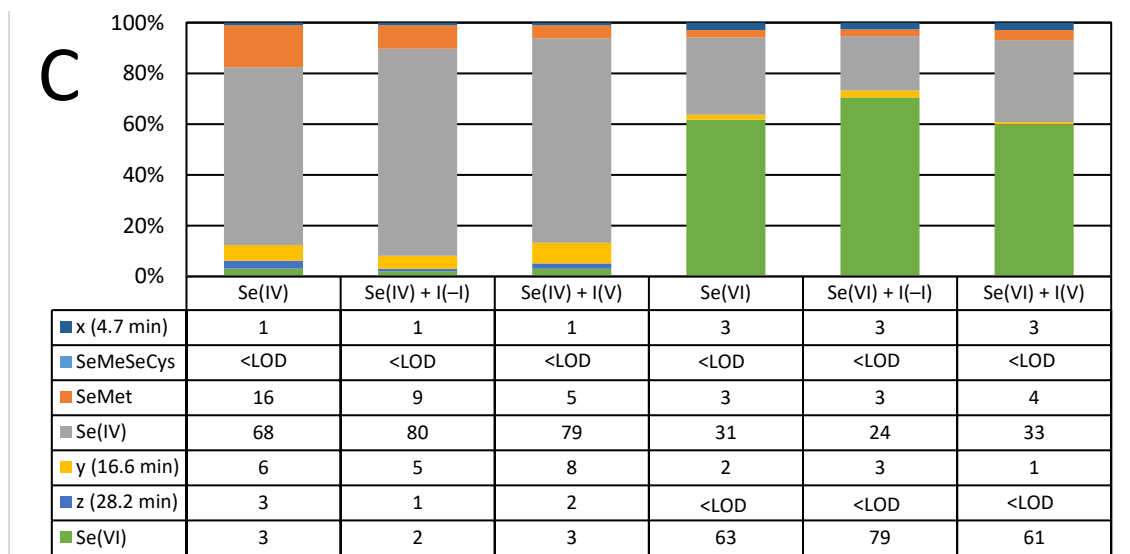


Figure 4.19: Selenium compounds (%) in plant parts of selenium and selenium-iodine treated pea sprouts: A) sprouts, B) roots and C) seed residues (<LOD - under limit of detection).

4.2.2 Pumpkin sprouts

Different selenium and iodine treatments of pumpkin sprouts led to lower germination, between 33 and 60%. No visible toxic signs and no significant differences in the germination rate of the sprouts were observed. Comparison of control, Se(IV) and Se(VI) treated sprouts masses showed significant differences among them. When seeds were soaked in a solution of Se(IV) at both concentrations (2.5 and 10 mg/L), masses were significantly increased over control sprouts, while the presence of Se(VI) had an opposite effect. Simultaneous selenium and iodine treatments did not affect the masses of sprouts (data not shown).

4.2.2.1 Selenium and its compounds

The selenium content in pumpkin sprouts treated with different concentrations of Se(IV) and Se(VI) showed that, regardless of form and concentration in the soaking solution, the selenium content in pumpkin sprouts was increased (Table 4.10). Up to 12-fold higher content was obtained when pumpkin seeds were soaked in a 10 mg/L solution of Se(VI) compared to control sprouts. Se(VI) is a well soluble compound, easily available for plant uptake (Zayed et al., 1998). The selenium content was less increased when seeds were soaked in a 2.5 mg/L solution (in both forms). The percentage of selenium taken up into pumpkin sprouts was 2.1 - 11.1%. To the best of our knowledge, there are no literature data for the selenium content in enriched pumpkin sprouts. Comparison with pea sprouts showed a much lower uptake of selenium. This may be because of the husk of pumpkin seeds, which protects seeds from water incursion.

Table 4.10: Selenium content and uptake of selenium enriched pumpkin sprouts.

Seed soaking solution	c (Se) mg/L	Se content (µg/g DW) (n)	Se uptake (%)
Control		0.037 ± 0.005 (3)	/

Se(IV)	2.5	0.068 ± 0.009 (3)	2.4
	10	0.197 ± 0.009 (3)	5.0
Se(VI)	2.5	0.189 ± 0.016 (3)	4.4
	10	0.453 ± 0.013 (3)	11.1

(n) number of samples

Results are given as average values with standard deviation

The percentage of soluble selenium was similar in all treated pumpkin sprouts (Table 4.11). The amount of soluble selenium was from 67% in control plants to 79% in 2.5 mg Se(IV)/L treated pumpkin sprouts. The percentage of the identified selenium compounds varied from 42 - 73%. Five selenium compounds were identified in all samples: SeMeSeCys, SeMet, Se(IV), Se(VI) and an unknown compound at retention time 4.7 min (Figure 4.20). In control and pumpkin sprout Se(VI), selenium was obtained as SeMet and Se(VI) in similar percentages. SeMet (main compound) and Se(IV) were present in Se(IV) treated sprouts. The same selenium compounds were present in pea sprouts, soaked in Se(IV) and Se(VI) solutions in the present study. Cuderman et al., (2010) soaked buckwheat seeds in a solution of 10 mg Se(IV)/L and 10 mg Se(VI)/L. The enzyme Protease XIV was used for extraction. The main compound in Se(IV) fortified seeds was SeMet, while Se(VI) was mainly present in Se(VI) fortified buckwheat sprouts. The treatment of buckwheat sprouts with Se(IV) in the literature was in agreement with our study, in which selenium was mainly present in the form of SeMet.

Table 4.11: Selenium compounds in selenium enriched pumpkin sprouts.

Seed soaking solution	c(Se) mg/L	Soluble Se µg/g DW (% ^a)	X ^b	SeMeSeCy s	SeMet ng Se/g DW	Se (IV)	Se (VI)	Sum % ^c	Sum % ^d
control	0	0.120 ± 0.008 (76)	11 ± 2	8 ± 1	36 ± 6	13 ± 3	38 ± 1	89	59
Se (IV)	2.5	0.054 ± 0.012 (79)	9 ± 3	6 ± 1	20 ± 5	15 ± 2	tr ^f	92	72
	10	0.150 ± 0.012 (76)	10 ± 1	6 ± 1	50 ± 3	19 ± 1	tr	57	43
Se (VI)	2.5	0.146 ± 0.061 (76)	10 ± 1	5 ± 2	44 ± 27	17 ± 6	26 ± 10	73	54
	10	0.717 (72) ^e	25	14	213	30	155	61	42

^a percentage of soluble Se regarding to its content in sample^b unknown Se compounds with retention time 4.7 min (calculated as SeCys₂)^c percentage of identified Se compounds regarding to soluble Se^d percentage of identified Se compounds regarding to their content in sample^e performed only in one replicate^f trace levels

Results are given as value of one determination or average value of two determinations with standard error

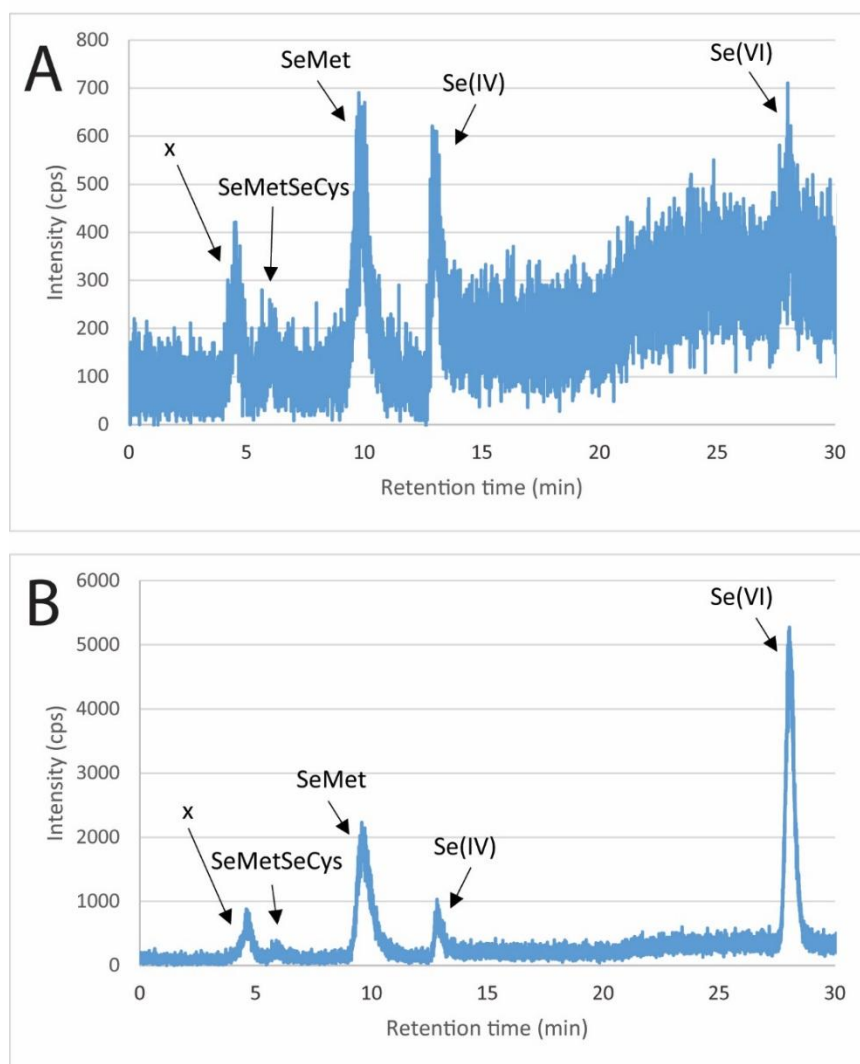


Figure 4.20: Separation of selenium compounds with a Hamilton PRP-X100 anion exchange column in pumpkin sprouts, soaked in a solution of: A) Se(IV) and B) Se(VI).

4.2.2.2 Iodine effect on selenium and its compounds

The presence of 1000 mg I/L (in both forms) in the soaking solution significantly increased the selenium content in pumpkin sprouts (Figure 4.21). The selenium content was the highest (2.2 $\mu\text{g/g}$ DW) in pumpkin sprouts Se(VI)+I(V). In the pumpkin sprout Se(IV)+I(-I), the selenium content was 3-fold higher than in the presence of Se(IV) alone, while the presence of I(V) increased the selenium content for 5-fold in pumpkin sprout Se(VI)+I(V). Both elements are transported from the roots to vital organs through xylem (Lenz & Lens, 2009). The presence of iodine amplified the selenium uptake and transport it to vital organs.

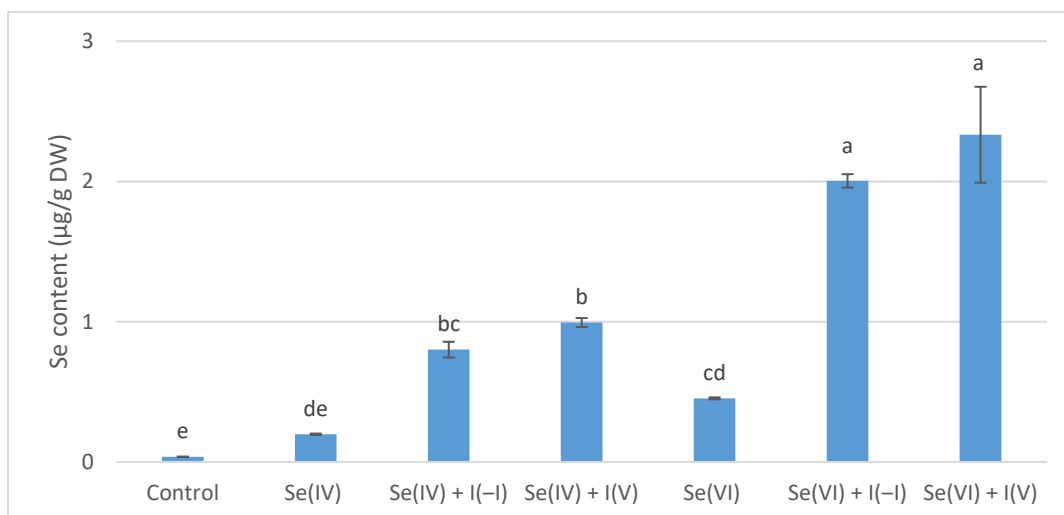


Figure 4.21: Iodine effect on selenium content in selenium and selenium-iodine enriched pumpkin sprouts.

In selenium and iodine enriched pumpkin sprouts, the percentage of soluble selenium was slightly affected by iodine presence (Table 4.12, Figure 4.22). The lowest amount of soluble selenium (63%) was present in pumpkin sprout Se(IV)+I(V), in which only 24% of selenium compounds were identified in terms of total selenium content. In all selenium and iodine treated pumpkin sprouts, an unknown selenium compound at retention time 15.5 min occurred. The presence of iodine in the Se(IV) soaking solution had minimal impact on the composition of selenium compounds. I(-I) influenced the metabolism of Se(VI) to SeMet in pumpkin sprout Se(VI)+I(-I), in which a lower amount of SeMet was present. A slight effect of I(V) on selenium compounds in Se(VI)+I(V) treated sprouts was observed. Our results with pumpkin sprouts Se(IV)+I(-I) are comparable to those in a study by Smoleń et al. (2016), in which the same iodine effect was observed in roots of carrot plants.

Table 4.12: Iodine effect on soluble selenium in selenium and selenium-iodine enriched pumpkin sprouts.

Seed soaking solution	Soluble Se µg/g DW	Sum % ^a	Sum % ^b	Sum % ^c
Se (IV)	0.150 ± 0.012	76	57	43
Se (IV) + I (-I)	0.598 ± 0.127	75	47	35
Se (IV) + I (V)	0.695 ^d	63	39	24
Se (VI)	0.717^d	72	61	42
Se (VI) + I (-I)	1.419 ^d	73	65	59
Se (VI) + I (V)	1.726 ± 0.219	81	59	47

^a percentage of soluble Se regarding to its content in sample

^b percentage of identified Se compounds regarding to soluble Se

^c percentage of identified Se compounds regarding to their content in sample

^d performed only in one replicate

Results are given as values of one determination or average values of two determinations with absolute error

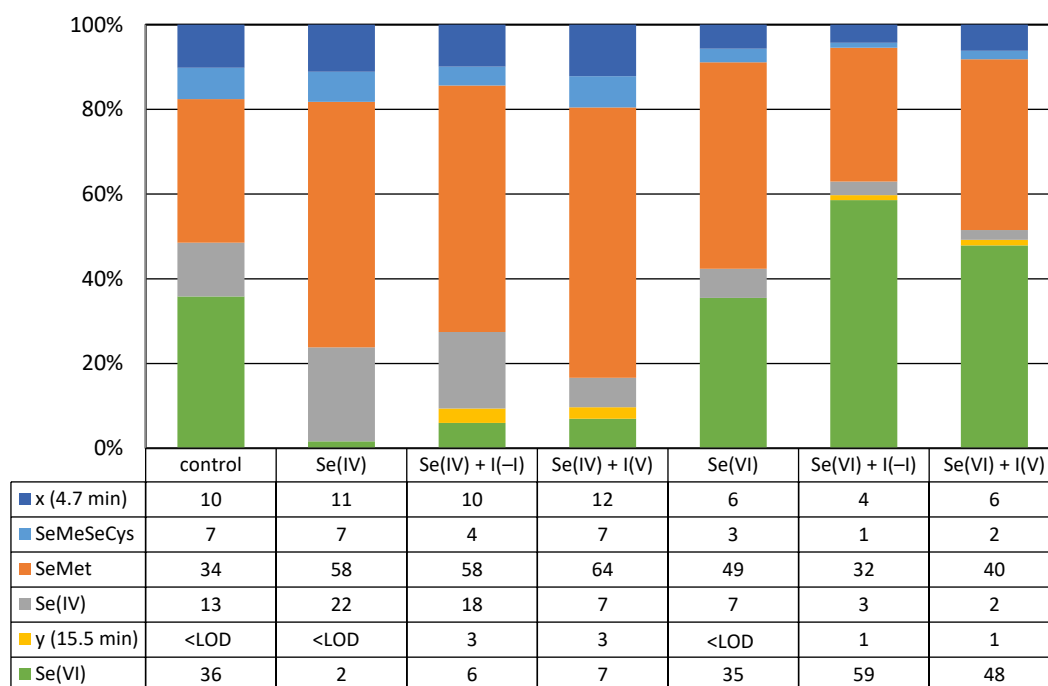


Figure 4.22: Selenium compounds (%) in selenium and selenium-iodine enriched pumpkin sprouts (<LOD – under limit of detection).

4.2.3 Buckwheat sprouts

Soaking of achenes in Se(VI) solution did not affect the amount of germinated sprouts in comparison with control buckwheat sprouts. In contrast, soaking of achenes in Se(IV) solution significantly decreased the amount of germinated sprouts. Soaking achenes in selenium solution (in both forms) did not effect on sprout weight and height. The presence of iodine in the soaking solution did not affect the amount of germinated sprouts and weight of sprouts, but had an effect on sprout height. The presence of 1000 mg I(V)/L in the selenium soaking solution in both forms significantly increased sprout height, while the other treatment did not significantly affect it (Germ et al., submitted).

4.2.3.1 Selenium and its compounds

Selenium content was increased over that in control sprouts (Table 4.13). The rate of uptake was similar for both forms with no significant difference in selenium content. Cuderman et al. (2010) published results of similar research, in which buckwheat achenes were soaked in a solution of 10 mg Se(IV)/L or 10 mg Se(VI)/L. Selenium content was 0.078 ± 0.004 , 1.87 ± 0.06 , 7.08 ± 0.18 ($\mu\text{g/g}$) in control, Se(IV) and Se(VI) enriched sprouts, respectively (Cuderman et al., 2010). In enriched sprouts in our study, the selenium content was 10- to 50-fold lower. Selenium probably remained in the husk or may have remained on the filter paper, which could explain the low uptake (lower than 1%).

Table 4.13: Selenium content and uptake in selenium enriched buckwheat sprouts.

Seed soaking solution	Se content ($\mu\text{g/g DW}$) (n)	Se uptake (%)
Control	0.029 ± 0.013 (3)	/
Se(IV)	0.161 ± 0.035 (3)	0.88
Se(VI)	0.140 ± 0.029 (3)	0.81

(n) number of samples

Results are given as average values with standard deviation

In selenium enriched buckwheat sprouts, the amount of soluble selenium varied from 11% in Se(IV) to 26% in Se(VI) sprouts (Table 4.14). A higher amount of soluble selenium in enzymatic extracts of Se(VI) enriched sprouts was found in research by Cuderman et al. (2010), in which buckwheat sprouts were enriched with 10 mg/L Se(IV) or Se(VI) solutions.

In enriched sprouts, only 8 % in Se(IV) and 23% in Se(VI) of selenium compounds were identified. All selenium compounds were present in low content. SeMeSeCys and Se(VI) were the main selenium compounds in buckwheat sprout Se(IV). In buckwheat sprout Se(VI), Se(VI) was the predominant compound, but also SeMeSeCys, SeMet and Se(IV) occurred. In a study by Cuderman et al. (2010), SeMet and SeMeSeCys or Se(VI) and SeMet were identified in Se(IV) and Se(VI) enriched sprouts, respectively.

Table 4.14: Selenium compounds in selenium enriched buckwheat sprouts.

Seed soaking solution	Soluble Se ($\mu\text{g/g DW}$) (% ^a)	x ^b	SeMeSeCys (ng Se/g DW)	SeMet	Se(IV)	Se(VI)	Sum (%) ^c	Sum (%) ^d
Se(IV)	21 ± 6 (11)	5 ± 1	5 ± 1	trace	trace	3 ± 1	63	8
Se(VI)	85 (32)	7	6	8	9	31	71	23

^a percentage of soluble Se regarding to its content

^b unknown Se compounds with retention time 4.7 min (calculated as SeCys₂)

^c percentage of identified Se compounds regarding to soluble Se

^d percentage of identified Se compounds regarding to their content in sample

Results are given as average of one determination or average of two determinations with standard error

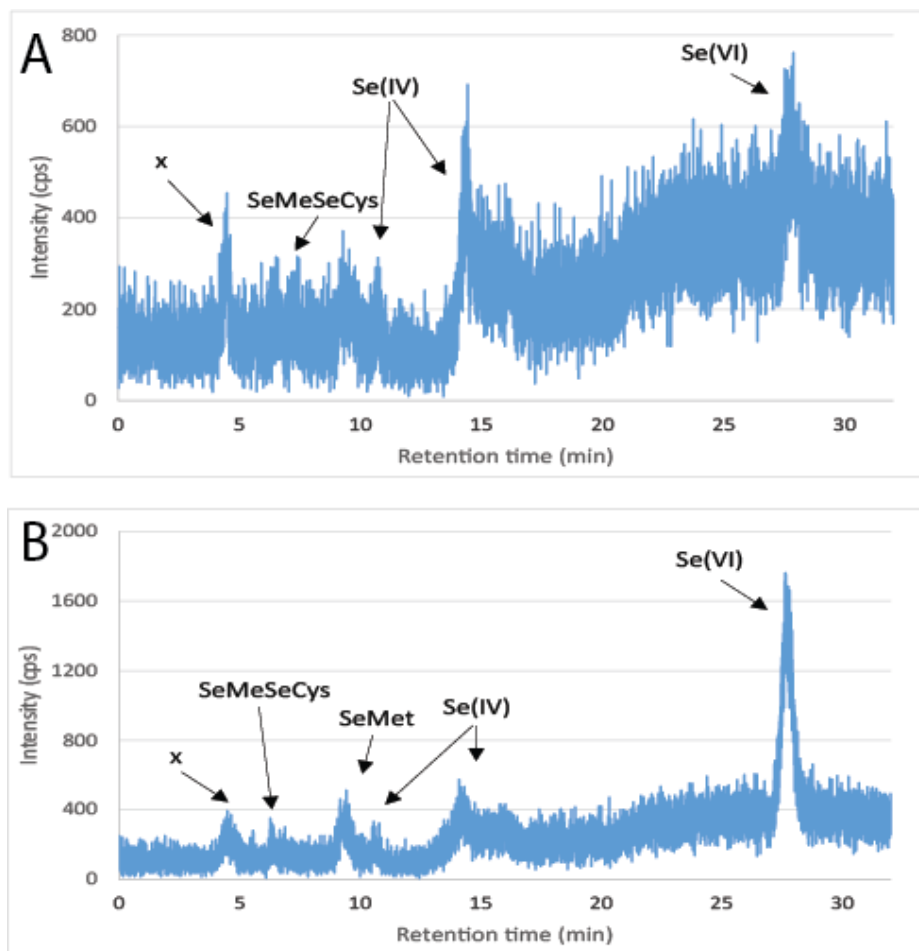


Figure 4.23: Separation of selenium compounds with a Hamilton PRP-X100 anion exchange column in A) Se(IV) treated and B) Se(VI) treated buckwheat sprouts.

4.2.3.2 Iodine effect on selenium and its compounds

On the basis of the results, iodine had a positive impact on selenium content (Figure 4.24). The content of selenium was increased in all treatments with the exception of Se(IV)+I(V), with which the selenium content stayed at the same level as with Se(IV) enriched buckwheat sprouts. In the Se(VI)+I(-I) treatment, the selenium content was lower than with treatment with Se(VI) alone. The reason may be the exchange of selenium and iodine solution with Milli-Q water (control solution). Differences between treatments with 1000 mg I/L and 1500 mg I/L were observed. When iodine was present at a higher concentration (1500 mg/L) in the soaking solution, a higher selenium content was obtained. The highest content was obtained in the combinations of Se(VI)+I(-I) (0.96 $\mu\text{g/g}$ DW) and Se(VI)+1500 I(V) (1.35 $\mu\text{g/g}$ DW). Results are comparable with the results of pumpkin sprouts, with which the highest selenium content was present in Se(VI) enriched sprouts in the presence of I(V). We assume that there is a synergistic effect of iodine on selenium content in sprouts, while increasing the iodine concentration in the soaking solution increased selenium uptake in buckwheat sprouts.

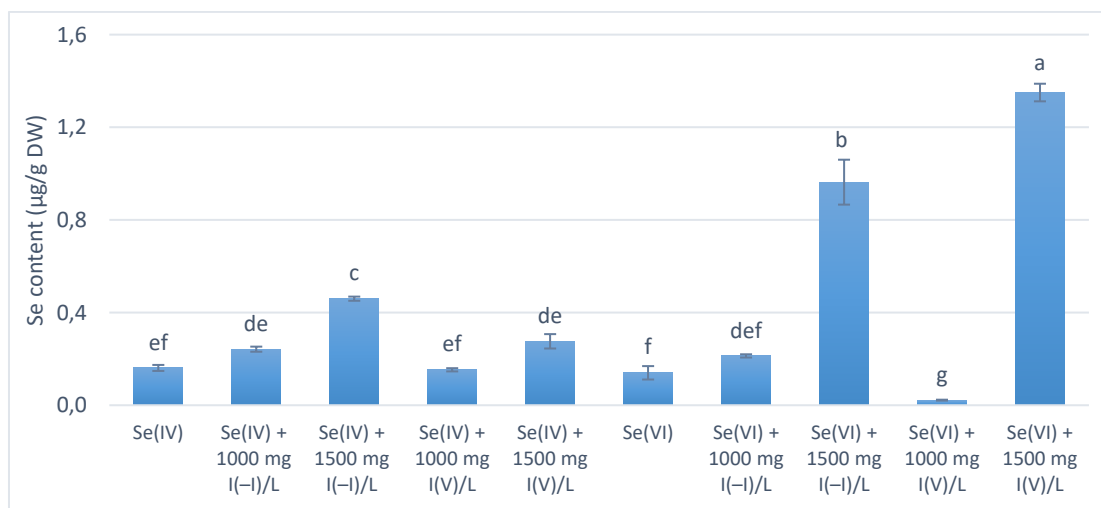


Figure 4.24: Iodine effect on selenium content in selenium and selenium-iodine enriched buckwheat sprouts.

The effect of iodine on the composition of selenium compounds is shown in Table 4.15 and Figure 4.25. Selenium compounds were determined only in Se+1500 I enriched buckwheat sprouts. The presence of iodine in the Se(IV) soaking solution increased the amount of soluble selenium and percentage of identified selenium compounds. The amount of identified selenium compounds regarding to total selenium content varied from 8 - 23% in the analyzed enriched buckwheat sprouts.

Table 4.15: Iodine effect on soluble selenium in selenium and selenium-iodine enriched buckwheat sprouts.

Seed soaking solution	Soluble Se ng/g DW	Sum % ^a	Sum % ^b	Sum % ^c
Se (IV)	21 ± 6	11	63	8
Se(IV) + 1500 mg I (-I) /L	111 ± 22	24	76	18
Se(IV) + 1500 mg I (V) /L	68 ± 11	27	70	18
Se (VI)	85^d	32	71	23
Se(VI) + 1500 mg I (-I) /L	482 ± 108	41	41	17
Se(VI) + 1500 mg I (V) /L	496 ± 104	37	31	11

^a percentage of soluble Se regarding to its content in sample

^b percentage of identified Se compounds regarding to soluble Se

^c percentage of identified Se compounds regarding to their content in sample

^d performed only in one replicate

Results are given as value of one determination or average values of two determinations with standard error

Different iodine treatments had an effect on the metabolism of selenium in enriched buckwheat sprouts (Figure 4.25). The presence of iodine increased Se(IV) and SeMet content and decreased SeMeSeCys content in Se(IV)+I treated achenes. We hypothesized that iodine influences the transformation of Se(IV) to SeMet

(White, 2015). On the other hand, soaking achenes in Se(VI)+I solution did not affect the SeMeSeCys content. In the presence of I(V), non-metabolized Se(VI) was present in a higher amount than in Se(VI) treated buckwheat sprouts. We assumed that iodine at the higher concentration influences on the transformation of Se(VI) to a less oxidized selenium form.

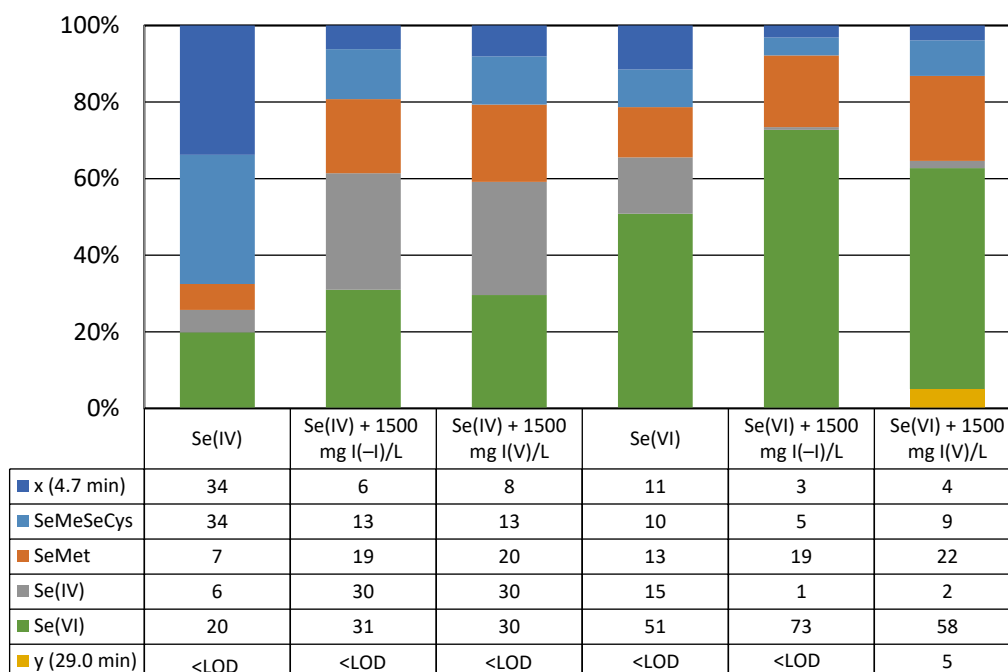


Figure 4.25: Selenium compounds (%) in selenium and selenium-iodine enriched buckwheat sprouts (<LOD - under limit of detection).

4.3 Selenium and its Compounds in Enriched Adult Plants

4.3.1 Pea plant

No significant differences were found regarding to selenium treatment for the agronomic characteristics of pea plants, such as the number of pods per plant, pod mass per plants, mass of fresh seed per plant, pod and fresh seed yield and the yield of dry matter of pea seeds. Nineteen days after foliar application, the yield of pea seeds was lower than 29 days after foliar spraying, with no statistical difference. Treatments with selenium and iodine influenced pod mass and length. Pod mass was decreased in Se(IV)+I(-I) and Se(VI)+I(V) treatments, while pods had a shorter length in all treatments with the exception of Se(IV)+I(V) (longer pods) (Jerše et al., 2018).

4.3.1.1 Selenium and its compounds

Selenium content was measured in all parts of pea plant: leaves, stems, roots and pods (Table 4.16). Pods (seeds and pod shells) were sampled in the first sampling, and other parts of pea plants (leaves, stems, roots) in the second one. Sampling of pea plant parts was performed: i) to found out the effect of foliar application of different selenium forms, ii) to determine the distribution of selenium among plant parts and iii) to evaluate differences in transport mechanisms when they were treated with different selenium forms.

Table 4.16: Selenium content in parts of a selenium enriched pea plant.

Foliar solution	Se content (ng/g DW)				
	Seeds (n)	Pod shells (n)	Leaves (n)	Stems (n)	Roots (n)
Control	15.2 ± 0.9 (3)	5.7 ± 2.1 (3)	25.3 ± 3.1 (3)	18.1 ± 2.8 (3)	96.5 ± 13.9 (3)
Se(IV)	55.8 ± 18.3 (3)	37.1 ± 10.2 (3)	81.1 ± 8.2 (3)	43.4 ± 2.3 (3)	168.8 ± 86.0 (3)
Se(VI)	172.8 ± 7.4 (3)	61.3 ± 9.6 (3)	326.5 ± 4.6 (3)	221.1 ± 1.21 (3)	407.8 ± 63.4 (3)

(n) number of samples

^a Results are calculated as average value with standard deviation

Compared to the control plant, selenium content was enhanced in all parts of the pea plant. The content in all parts of the pea plant, when treated with Se(VI) (roots > leaves > stems > seeds > pod shells), was 3-fold higher than with Se(IV) (roots > leaves > seeds > stems > pod shells) foliar application. Selenium was transported from leaves to roots, because the highest selenium content was found in root tissue. Accumulation in roots may be linked to the biosynthesis of phytochelatin – proteins responsible for detoxification of selenium (Simmons & Wallschläger, 2005). Selenium was also transported to the seeds, in which a higher content (12-fold) was observed when the pea plant was fortified with Se(VI) solution than with control one. According to literature data, Se(VI) and organoselenium compounds can be redistributed within the plant with water through phloem (Smoleń et al., 2014; White, Broadley, Bowen, & Johnson, 2007).

With foliar spraying, selenium ions diffuse from the surface of the leaves to epidermal cells (Ježek et al., 2012). There is a

difference between transport of Se(VI) and Se(IV). It has been suggested that Se(VI) is transported apoplastically (in inert transport) without infiltration into the cell and without selenium compounds transformation. Se(IV) is transported sympastically (selenium crosses the cell membrane), whereby it is reduced to seleno amino acids. (Bulska et al., 2006).

The results from the present study are comparable to those from the study by Thavarajah et al. (2015), in which lentil was foliar sprayed with Se(IV) and Se(VI) in different concentrations (10 – 30 µg/g). They found a significantly higher selenium content when Se(VI) was present in the solution. In research by Garousi et al. (2017) pea plant was enriched with Se(IV) (up to 90 µg/g) and Se(VI) (up to 30 µg/g) solution in different concentrations, by soil fortification. When Se(IV) was present in the solution, selenium distribution within the pea plant (roots, shoots, pods, seeds) was similar to our study (1000-fold lower content). A higher selenium content was found in roots and lower in pods despite different enrichment method.

Selenium compounds were extracted with enzymatic extraction from seeds and leaves, and different rates of extractability were found (Table 4.17; Figure 4.26). From seeds, 58 – 64% of selenium was extracted, while from leaves the rate was from 41 – 76%. In the literature, higher amount of selenium (approximately 92%) was extracted with proteolytic hydrolysis (Protease XIV in phosphate buffer) from pea seeds and young leaves (Smrkolj, Germ, Kreft, & Stibilj, 2006).

Foliar application of pea plants with Se(IV) solution resulted in a high amount of SeMet (26%) and compounds similar to SeCys₂ at retention time 4.7 min (62%) in seeds. SeMet (62%) was the main compound in Se(VI) treated seeds. Selenium compounds present in leaves foliar sprayed with Se(IV) solution mainly stayed in un-metabolized selenium form (51%), while SeMet, Se(VI) and unknown compounds at retention times 4.7 and 6.8 min were obtained in smaller amounts. Treatment with Se(VI) metabolized approximately 25% of Se(VI) to SeMet, while 63% was obtained in un-metabolized Se(VI) form.

Ježek et al. (2012) reported that metabolization of Se(VI) to organoselenium forms occurred in leaves, which is not in agreement with our data. The reason for un-metabolized forms may be the low uptake of selenium from foliar solutions, and consequentially, the presence of selenium mainly in forms of Se(VI). Smrkolj et al. (2006) reported that selenium compounds were determined after enzymatic extraction in seeds and young leaves of pea plants treated with 10 mg/L Se(VI) whereby only SeMet was obtained. In research by Lyubenova et al. (2015), chickpea was enriched with Se(VI) solution in a semi-hydroponic system. Selenium was mainly present in forms of SeMet and SeCys, depending on the length of the experiment and concentration of selenium in the solution (Lyubenova et al., 2015).

Table 4.17: Selenium compounds in leaves and seeds of selenium enriched pea plant.

Foliar solution	Plant part	Soluble Se µg/g DW (%) ^a	X ^b	SeMeSeCys	Y (6.8 min)	SeMet ng Se/g DW	Se (IV)	Se (VI)	Sum % ^c	Sum % ^d
Se (IV)	seeds	41 ± 2 (58)	16 ± 2	<LOD	<LOD	7 ± 7	trace	2 ± 1	59	34
	leaves	31 ± 12 (41)	9 ± 6	<LOD	5 ± 5	5 ± 3	24 ± 19	5 ± 2	/	71
Se (VI)	seeds	113 ± 2 (64)	11 ± 1	9 ± 3	<LOD	49 ± 13	7 ± 3	8 ± 7	51	32
	leaves	272 ± 10 (76)	12 ± 3	2 ± 1	7 ± 2	41 ± 3	<LOD	102 ± 13	58	44

^a percentage of soluble Se regarding to Se in sample^b unknown Se compounds with retention time 4.7 min (calculated as SeCys₂)^c percentage of identified Se compounds regarding to soluble Se^d percentage of identified Se compounds regarding to total Se in sample

Results are given as average values of three determinations with standard deviation

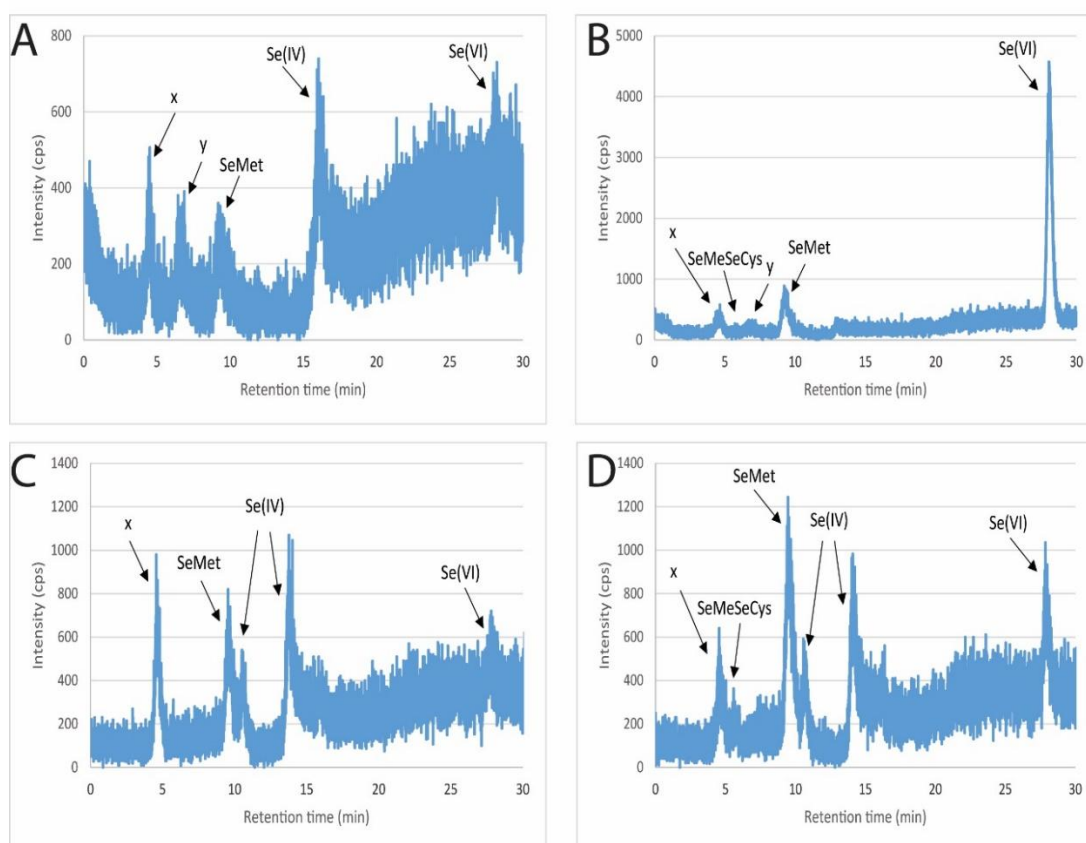


Figure 4.26: Separation of selenium compounds with a Hamilton PRP-X100 anion exchange column in selenium enriched parts of pea plant: A) pea leaves Se(IV), B) pea leaves Se(VI), C) pea seeds Se(IV) and D) pea seeds Se(VI).

4.3.1.2 Iodine effect on selenium and its compounds

Iodine had a varying effect on selenium uptake after foliar application of pea plants (Table 4.18). I(-I) significantly increased selenium content when present in the Se(IV) solution, with the exception of the content in pod shells. The presence of I(V) did not contribute to a higher selenium content. Various effects of different iodine forms were observed in Se(VI) treated pea plant. The higher selenium uptake was observed when Se(IV)+I(-I) was present in the foliar solution. The reason for a higher Se(IV) in the presence of I(-I) may be: a) interaction of iodine in the cuticle structure of leaves, b) synergistic effect of I(-I) on Se(IV) uptake or c) effect of iodine on selenium accumulation in leaves.

The presence of iodine in the foliar solution enhanced the amount of soluble selenium, irrespective of iodine and selenium form (Table 4.19; Figure 4.27). The only exception was in Se(VI)+I(-I) sprayed pea leaves. Iodine had various effects on the distribution of selenium compounds in foliar treated seeds and leaves. The amount of SeMet and SeMeSeCys was increased in Se(IV)+I enriched seeds. In Se(VI) enriched seeds, the presence of iodine had a minimal impact on selenium compounds. In leaves, iodine affected the metabolism of Se(IV) and Se(VI). Se(IV) was metabolized to SeMet and Se(VI) and one unknown compound at retention time 28.8 min (near elution of Se(VI)). The metabolism of Se(VI) to SeMet and unknown compounds at retention time 6.8 min in pea leaves was enhanced in iodine presence. The present study is not in agreement with study by Smoleń et al. (2016), in

which a negative impact of iodine presence in Se(IV) foliar solution was observed.

Table 4.18: Iodine effect on selenium content in selenium and selenium-iodine enriched pea plant.

Foliar solution	n	Se content (ng/g DW)				
		Seeds	Pod shell	Leaves	Stems	Roots
Control	3	15.2 ± 0.9 ^e	5.7 ± 2.1 ^c	25.3 ± 3.1 ^c	18.1 ± 2.8 ^c	96.5 ± 13.9 ^d
Se (IV)	3	55.8 ± 18.3^d	37.1 ± 10.2^{ab}	81.1 ± 8.2^c	43.4 ± 2.3^c	168.8 ± 86.0^{bc}
Se(IV) + I (-I)	3	194.5 ± 16.7 ^a	82.7 ± 12.0 ^a	300.7 ± 8.0 ^a	210.6 ± 3.10 ^a	372.3 ± 119.1 ^a
Se(IV) + I (V)	3	76.7 ± 6.3 ^d	38.4 ± 9.5 ^b	69.1 ± 5.5 ^c	49.0 ± 6.9 ^c	126.1 ± 24.6 ^{cd}
Se (VI)	3	172.8 ± 7.4^b	61.3 ± 9.6^b	326.5 ± 4.6^{ab}	221.1 ± 1.21^{ab}	407.8 ± 63.4^a
Se(VI) + I (-I)	3	179.2 ± 17.3 ^{ab}	51.2 ± 12.0 ^b	180.7 ± 3.0 ^b	123.2 ± 2.23 ^b	214.4 ± 109.8 ^{bc}
Se(VI) + I (V)	3	132.8 ± 11.1 ^c	40.0 ± 3.7 ^b	229.8 ± 2.7 ^b	143.5 ± 2.43 ^b	298.1 ± 58.1 ^{ab}

n number of samples

Results are given as average values with standard deviation

The superscripts indicate statistically significant in the each group of results

Table 4.19: Iodine effect on soluble selenium and percentage of identified compounds in seeds and leaves of selenium and selenium-iodine enriched pea plant.

Plant part	Foliar solution	Number of samples	Soluble Se ng/g DW	Sum % ^a	Sum % ^b	Sum % ^c
seeds	Se (IV)	3	41 ± 2	58	63	36
	Se(IV) + I (-I)	3	138 ± 8	68	65	44
	Se(IV) + I (V)	3	65 ± 4	78	71	55
	Se (VI)	3	113 ± 2	64	51	32
	Se(VI) + I (-I)	3	142 ± 17	71	67	48
	Se(VI) + I (V)	3	117 ± 25	72	60	49
leaves	Se (IV)	2	31 ± 12	41	/	/
	Se(IV) + I (-I)	2	213 ± 27	69	87	57
	Se(IV) + I (V)	3	65 ± 12	74	101	51
	Se (VI)	2	272 ± 10	76	58	44
	Se(VI) + I (-I)	2	104 ± 3	66	77	51
	Se(VI) + I (V)	3	198 ± 17	80	66	55

^a percentage of soluble Se regarding to Se in sample

^b percentage of identified Se compounds regarding to soluble Se

^c percentage of identified Se compounds regarding to total Se in sample

Results are given as average values with standard error or deviation

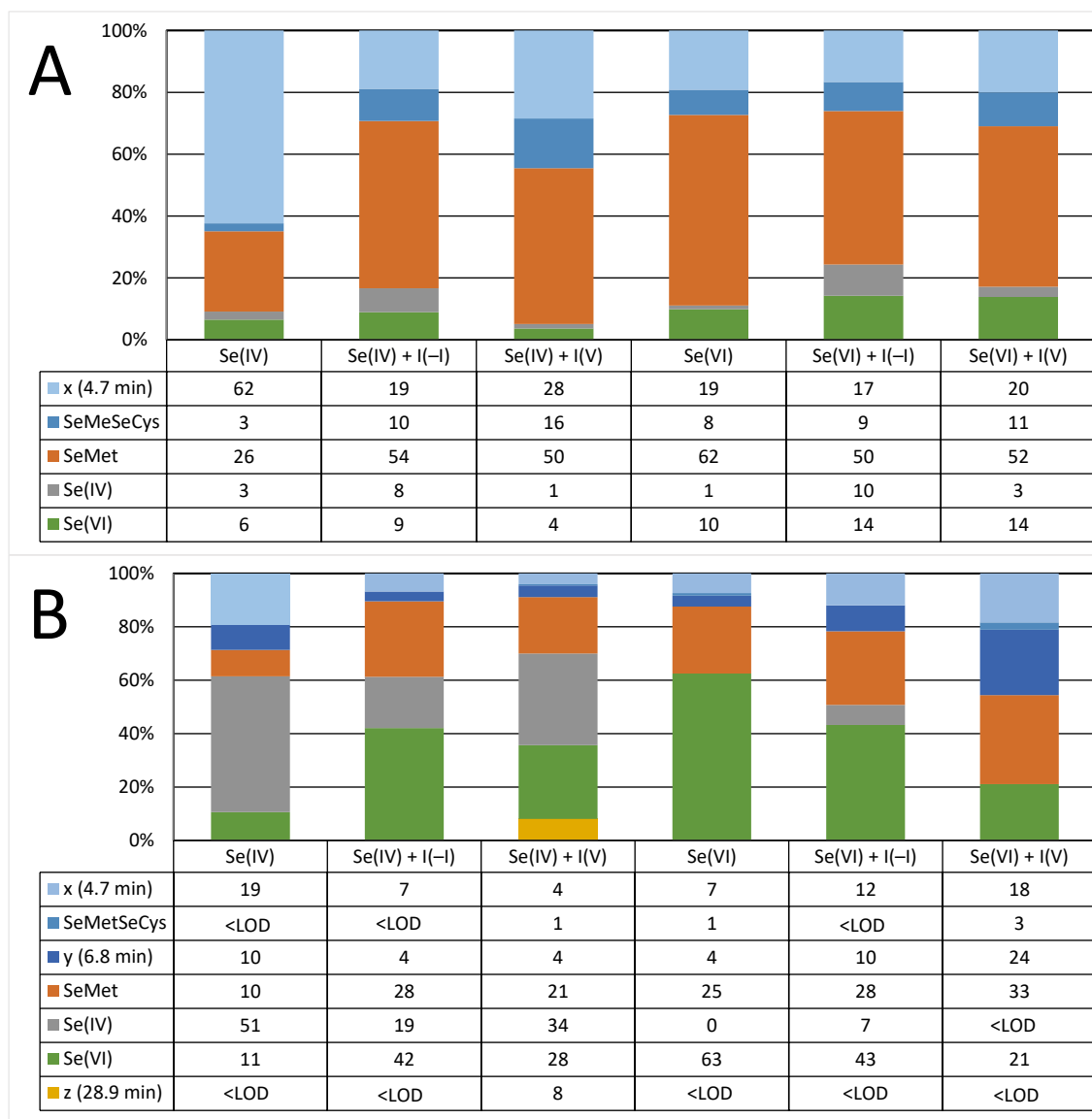


Figure 4.27: Selenium compounds (%) in A) seeds and B) leaves of selenium and selenium-iodine enriched pea plant.

4.3.2 Pumpkin seeds

No visual symptoms of selenium toxicity appeared in pumpkin plants enhanced with selenium. Treatment of pumpkin plants with a solution of selenium caused a higher yield of pumpkin seeds (2.4 – 3.8 kg/ha) than in the control plant (0.7 kg/ha). The yield of pumpkin fruit and pumpkin seeds was not affected by iodine presence (data not shown).

4.3.2.1 Selenium and its compounds

Selenium content was measured in seeds, which are an edible part of pumpkin plant, previously treated with foliar solutions (Table 4.20). The content in foliar sprayed pumpkin seeds was significantly enriched over that of control seeds. There were no significant differences between the selenium contents in Se(IV) or Se(VI) enriched pumpkin seeds. Kreft et al. (2002) published that the selenium content in untreated pumpkin seeds (*Cucurbita pepo* L.) was around 30 ng/g DW. A study by Smrkolj et al. (2005) on treated pumpkin (*Cucurbita pepo* L.) seeds shown, that foliar

spraying of pumpkin plants with 1.5 mg/L in Se(VI) form causes an 8-fold higher selenium content than in untreated plants. A similar study of foliar application with 1 mg Se/L in Se(VI) form was made on the same species of pumpkin plant. The selenium content in enriched pumpkin seeds was about 3.5-fold higher (about 380 ng/g DW) than in untreated seeds (about 108 ng/g DW) (Stibilj et al., 2004). The reason for the low selenium content in our study, may be at (i) small absorption of foliar solution, (ii) higher yield of pumpkin seeds in selenium treatment or (iii) distribution of selenium uptake within the pumpkin plant.

Table 4.20: Selenium content in selenium enriched pumpkin seeds.

Foliar solution	Se content ng/g DW (n)
Control	35.2 ± 2.8 (3)
Se(IV)	51.1 ± 7.5 (3)
Se(VI)	44.0 ± 11.2 (3)

(n) number of samples

Results are given as average values with standard deviation

Due to the presence of oils in pumpkin seeds, they were extracted with diethyl ether before determination. We extracted from 15% of oils in Se(IV) and Se(VI) enriched seeds to 18% in control one (As with selenium sprayed pumpkin plants, selenium compounds were determined in selenium and iodine enriched pumpkin seeds with the previous extraction of oils (Table 4.21).

Table 4.21. The amount of soluble selenium in control and selenium sprayed pumpkin seeds was approximately the same (53 – 69%) (Table 4.22). In all pumpkin seeds, only SeMet was obtained (other peaks belong to enzyme Protease XIV) (Figure 4.28). Another selenium compounds with unknown identity was present at the retention time of SeCys₂ (component X). The results from the present study are comparable to those in the study by Smrkolj et al. (2005), in which pumpkin plants were foliar treated with Se(VI) solution.

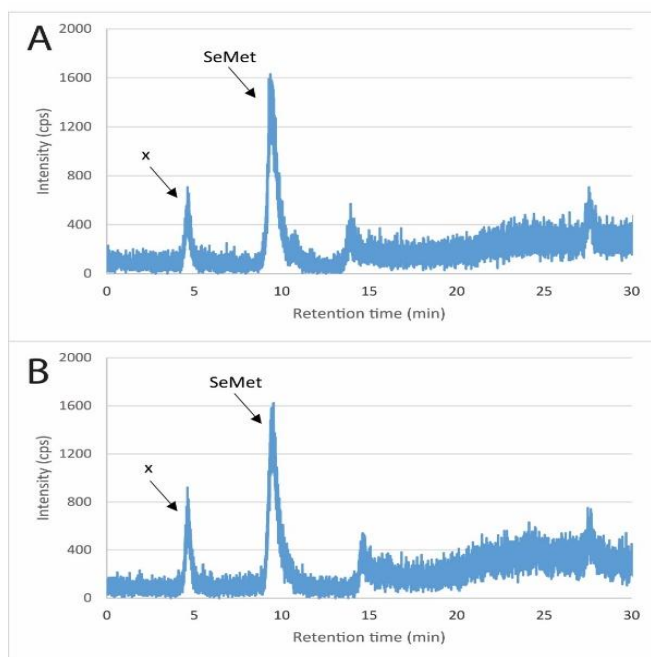


Figure 4.28: Separation of selenium compounds with a Hamilton PRP-X100 anion exchange column in selenium enriched pumpkin seeds, sprayed with A) Se(IV) and B) Se(VI) solution.

4.3.2.2 Iodine effect on selenium and its compounds

Foliar application of pumpkin plants with selenium and iodine solution increased the selenium content up to 2.5-fold in comparison with control seeds (Figure 4.29). The presence of iodine (both forms) in the Se(IV) solution slightly decreased the content of selenium in pumpkin seeds. Significantly higher contents were obtained when the pumpkins were sprayed with a solution of Se(VI)+I(V) (up to 87 ng/g DW) and Se(VI)+I(-I) (up to 74 ng/g DW). The results showed that iodine had a positive impact on the transport of Se(VI) from leaves to seeds.

The results are comparable with Se(VI) and I(V) treated lettuce (Smoleń et al., 2014). They observed a higher uptake of Se(VI) in the presence of I(V) than with only Se(VI) treatment. In a study by Smoleń et al., (2016), the presence of iodine increased selenium content in the Se(VI) treatment, but decreased it in Se(IV) treatments. Our results are in agreement with those obtained by Smoleń et al. (2016). Because of a higher uptake of selenium in the presence of iodine we suggest, that the transport mechanism for the two elements is bounded or connected.

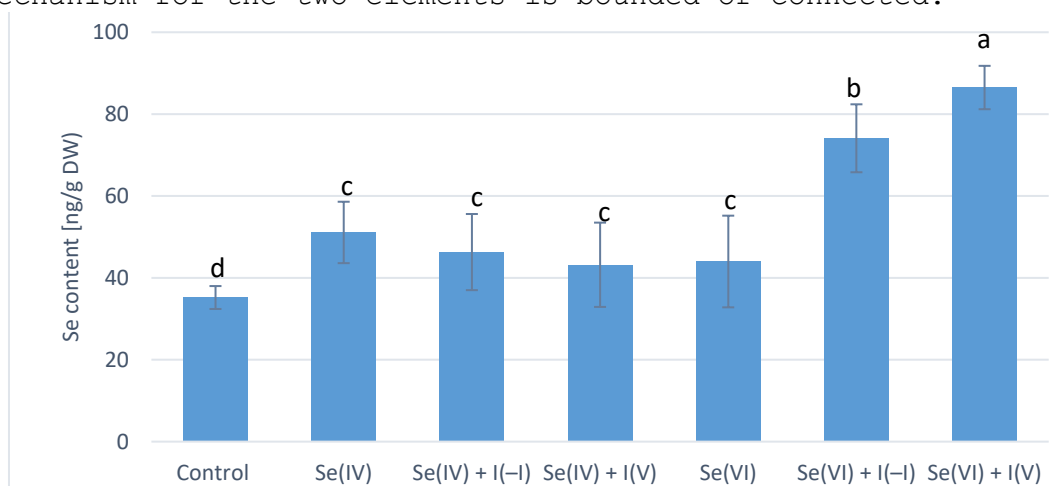


Figure 4.29: Iodine effect on selenium content in selenium and selenium-iodine enriched pumpkin seeds.

As with selenium sprayed pumpkin plants, selenium compounds were determined in selenium and iodine enriched pumpkin seeds with the previous extraction of oils (Table 4.21).

Table 4.21: Selenium content (%) in selenium and selenium-iodine enriched pumpkin seeds after extraction with diethyl ether.

Foliar solution	Percentage of Se content (%)	
	in residue	in seeds
Control	83	17
Se (IV)	83	17
Se (IV) + I (-I)	92	8
Se (IV) + I (V)	87	13
Se (VI)	84	16
Se (VI) + I (-I)	77	23

Se(VI) + I(V) 75 25

The amount of soluble selenium was not effected in the presence of iodine in the foliar solution (Table 4.22). In comparison with selenium treated pumpkin seeds, iodine did not affect the amount of SeMet in selenium and iodine treated pumpkin seeds (Figure 4.30). The results of the present study are in agreement with those of a study by Smoleń et al. (2016), in which carrot leaves were treated with selenium and iodine solutions.

Table 4.22: Iodine effect on selenium compounds in selenium and selenium-iodine enriched pumpkin seeds.

Foliar solution	Soluble Se ng/g DW (% ^a)	x ^b ng/g DW	SeMet	Sum % ^c	Sum % ^d
Control	25 (69)	2	9	37	25
Se(IV)	28 (53)	1	10	35	19
Se(IV) + I(-I)	28 (50)	1	17	60	30
Se(IV) + I(V)	17 (36)	1	12	70	25
Se(VI)	19 (56)	2	12	63	35
Se(VI) + I(-I)	34 (43)	2	24	70	30
Se(VI) + I(V)	52 (57)	2	32	62	35

^a percentage of soluble Se regarding to Se in sample

^b unknown Se compounds with retention time 4.7 min (calculated as SeCys₂)

^c percentage of identified Se compounds regarding to soluble Se

^d percentage of identified Se compounds regarding to total Se in sample

Measurement performed only in one replicate.

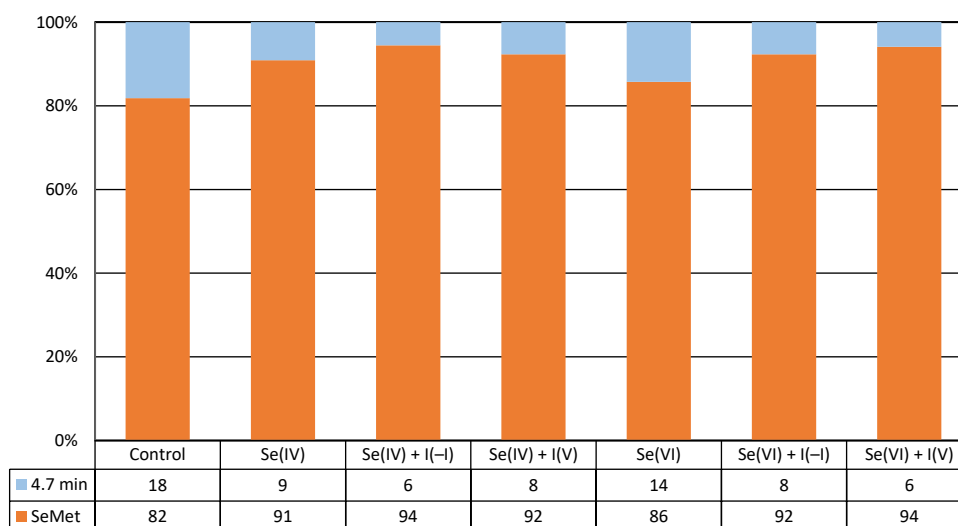


Figure 4.30: Selenium compounds (%) in selenium and selenium-iodine enriched pumpkin seeds.

4.3.3 Buckwheat achene

The yield of buckwheat achene was not significantly influenced by selenium biofortification. Plants treated with selenium (in both forms) had a lower yield than the control plants. The addition of iodine (I(V) and I(-I)) to selenium (Se(IV) and Se(VI)) decreased the yield of buckwheat achene compared to the treatment with individual selenium solution, but the differences were not significant (Germ et al., submitted).

4.3.3.1 Selenium and its compounds

Selenium content was enhanced in both treatments compared to control achenes (Table 4.23). A higher selenium content was present when the buckwheat was foliar sprayed with Se(VI) than when sprayed with Se(IV) solution. Golob et al. (2016) determined the selenium content in 20 mg Se(VI)/L treated Tartary and hybrid buckwheat achenes. In hybrid achenes, the selenium content was 2-fold higher than in our research, while in Tartary achenes up to 6-fold (Golob et al., 2016). In another study, buckwheat plants were fortified with 1 mg Se(VI)/L, whereby a 2-fold lower content was found (Stibilj et al., 2004). As in our study, the selenium content was also measured in research by Vogrinčič et al. (2009), in which buckwheat plants were sprayed with 10 mg Se(VI)/L. They found a 5-fold higher content of selenium in achenes than was found in our study (Vogrinčič et al., 2009). The lower selenium content in our research may be consequence of (i) climate conditions during growth on the experimental field (low absorption of foliar solution), (ii) different enrichment methods (manual and automatic) or (iii) use of different watering cans.

Table 4.23: Selenium content in selenium enriched buckwheat achenes.

Foliar solution	Se content ng/g DW (n)
Control	29 ± 4 (3)
Se(IV)	115 ± 8 (3)
Se(VI)	653 ± 16 (3)

(n) number of samples

Results are given as average values with standard deviation

Selenium compounds were determined in selenium enriched buckwheat achenes (Table 4.24, Figure 4.21). The amount of soluble selenium from buckwheat achene varied from 39% in treated achenes to 55% in control ones. We identified SeMet (84 - 94%), a small amount of SeMeSeCys and an unknown compound at retention time 4.7 min. Other peaks belong to enzyme Protease XIV. Identified selenium compounds from our study were in agreement with a study by Vogrinčič et al., (2009), in which buckwheat plants were foliar treated with 10 mg Se(IV)/L. We think that selenium was transported from leaves through phloem to achenes, in which metabolism of selenium took place (Smoleń et al., 2014; White et al., 2007).

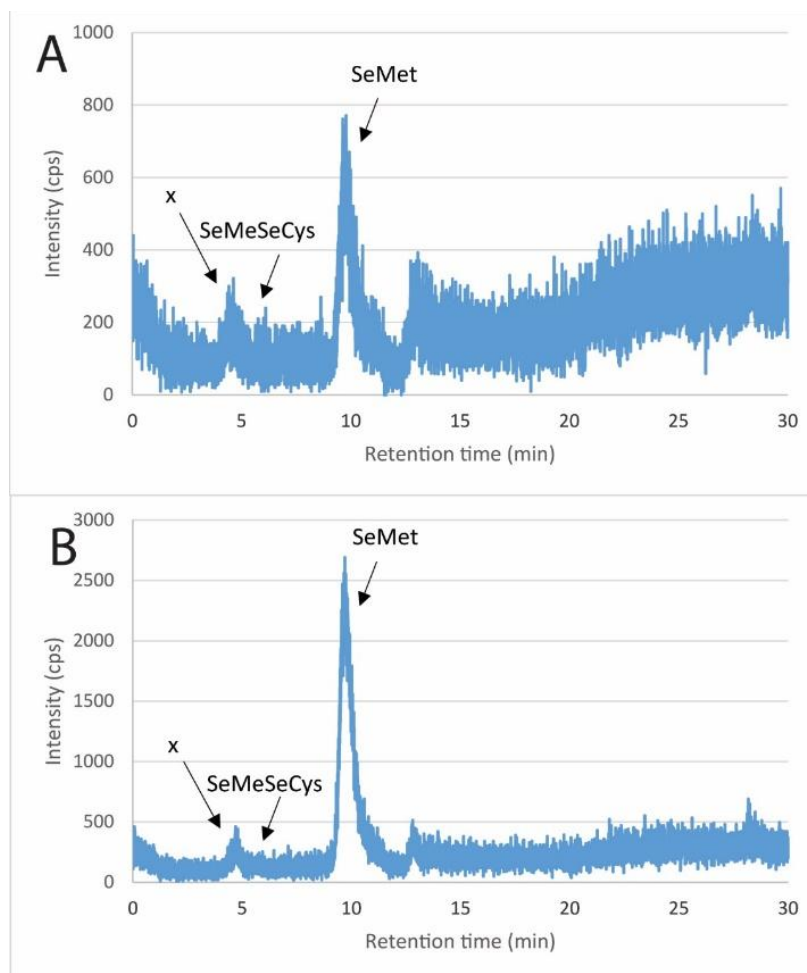


Figure 4.31: Separation of selenium compounds with a Hamilton PRP-X100 anion exchange column in selenium enriched buckwheat achenes, sprayed with A) Se(IV) and B) Se(VI) solution.

4.3.3.2 Iodine effect on selenium and its compounds

The selenium content was significantly increased in Se(IV)+I(V) treated buckwheat achenes, while I(-I) significantly decreased selenium content in Se(IV)+I(-I) treated achenes (Figure 4.32). The presence of iodine in the Se(VI) solution had a negative effect on selenium content. The highest selenium content in selenium and iodine enriched achenes was found when plants were sprayed with Se(VI)+I(-I) solution (up to 460 ng/g DW). Smoleń et al. (2016) found a higher selenium uptake in carrot leaves and roots when Se(VI) and I(-I) were present in the foliar solution. The lowest selenium uptake was observed when Se(IV) and I(-I) were present, which is comparable with our results.

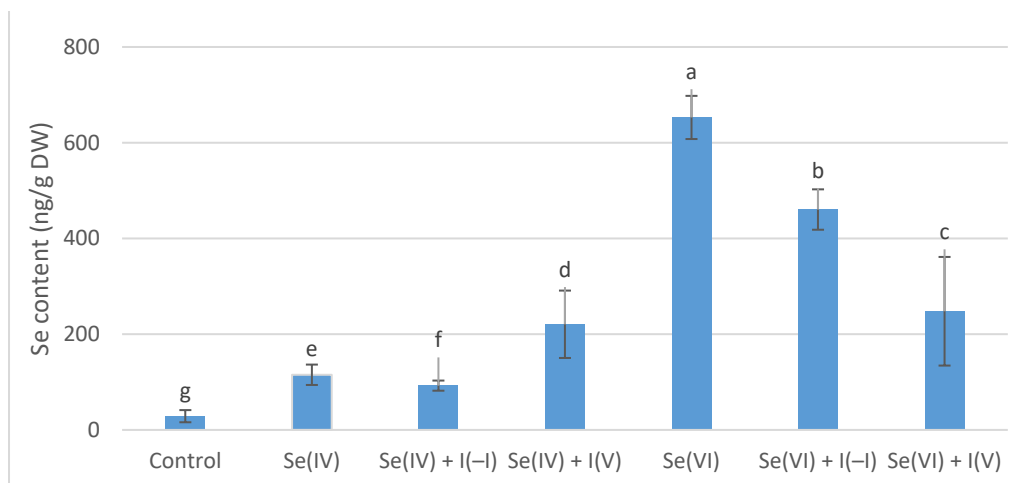


Figure 4.32: Iodine effect on selenium content in selenium and selenium-iodine enriched buckwheat achenes.

The soluble selenium content of buckwheat achenes was not affected by the presence of iodine in the foliar application (23 – 45%) (Table 4.24). We identified from 22 – 31% of selenium compounds regarding to total selenium content, regardless of the presence of iodine. The main share of identified selenium compounds belonged to SeMet and was not affected by iodine presence, with the exception of Se(IV)+I(-I), in which a higher share of identified selenium compounds belonged to an unknown selenium compound at retention time 4.7 min (Figure 4.33). The highest SeMeSeCys content was present in Se(IV)+I(-I) treated buckwheat achenes, while in other treatments the content varied from 2 – 6%. The results of the present study were in agreement with those of the study by Smoleń et al. (2016) of carrot leaves treated with selenium and iodine solutions.

Table 4.24: Iodine effect on selenium compounds in selenium and selenium-iodine enriched buckwheat achenes.

Foliar solution	Soluble Se ng/g DW (% ^a)	X ^b	SeMeSeCys ng Se/g DW	SeMet	Sum % ^c	Sum % ^d
Control	20 (55)	6 ± 1	<LOD	7 ± 1	75	36
Se (IV)	57 ± 6 (39)	4 ± 1	3 ± 1	34 ± 3	71	28
Se(IV) + I(-I)	23 (23)	8 ± 2	3 ± 1	14 ± 0	80	31
Se(IV) + I(V)	130 ± 3 (45)	6 ± 1	2 ± 0	89 ± 16	90	22
Se (VI)	270 ± 2 (39)	7 ± 2	7 ± 2	203 ± 6	75	34
Se(VI) + I(-I)	178 ± 5 (36)	5 ± 1	7 ± 2	129 ± 13	79	28
Se(VI) + I(V)	129 ± 17 (37)	7 ± 1	4 ± 2	96 ± 4	82	30

^a percentage of soluble Se regarding to Se in sample

^b unknown Se compounds with retention time 4.7 min (calculated as SeCys₂)

^c percentage of identified Se compounds regarding to soluble Se

^d percentage of identified Se compounds regarding to total Se in sample

Results are given as average values of two determinations with standard error

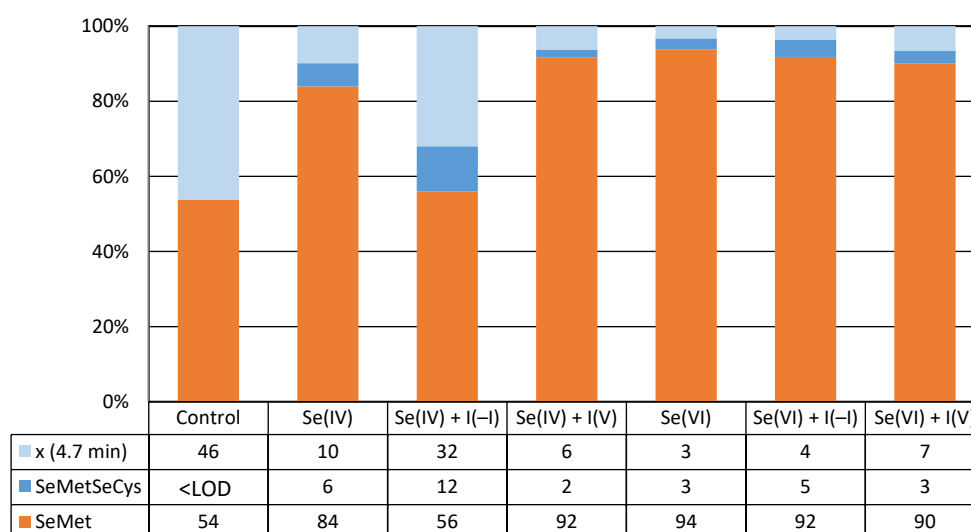


Figure 4.33: Selenium compounds (%) in selenium and selenium-iodine enriched buckwheat achenes.

4.3.4 Chicory

There were no significant differences in the yield and mass of the head of chicory plant between all treatments (Kroflič et al., 2017).

4.3.4.1 Selenium and its compounds

The selenium content was enriched in both selenium treatments (up to 6-fold) regarding to control plants (Table 4.25). In the outer leaves, the contents were approximately the same in both selenium treatments (86 – 100 ng/g DW). In the inner leaves, the selenium content was higher, when a solution of Se(VI) was sprayed onto the chicory leaves. The content of selenium after Se(IV) treatment was 2-fold lower in the outer leaves than in the inner leaves. The reason may be at (i) growth of the heads (ii) oxidation of selenium or (iii) transport of selenium to roots. Se(IV) is metabolized into other forms in root tissue (Ježek et al., 2012). The selenium content after Se(VI) biofortification stayed at the same level. Selenium metabolism of Se(VI) into organic compounds take place in plant leaves (Ježek et al., 2012), which may be a reason for not translocating to roots. Mazej, Osvald, & Stibilj (2008) aeroponically cultivated four cultivars of chicory with 7 mg Se(IV)/L, in which the selenium contents were in a range from 167 to 480 µg/g DW. Germ et al. (2007) foliar sprayed chicory plant twice with 1 mg Se(VI)/L. The selenium content was 2-fold higher (43 – 46 ng/g DW) than in to control plants (21 – 24 ng/g DW). We did not find any data on a comparison between Se(IV) and Se(VI) foliar treated chicory leaves.

Table 4.25: Selenium content in selenium enriched chicory leaves.

Foliar solution	Se content (ng/g DW)	
	Outer leaves (n)	Inner leaves (n)
Control	26 ± 7 (3)	14 ± 7 (3)
Se(IV)	100 ± 28 (3)	51 ± 12 (3)

Se(VI)	86 ± 22 (3)	87 ± 16 (3)
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(n) number of samples

Results are given as average values with standard deviation

Selenium compounds were determined in the outer leaves of selenium treated chicory plants, while the selenium content in inner leaves was too low (Table 4.26; Figure 4.34). The amount of soluble selenium and identified compounds in outer chicory leaves was around 30%, regardless of the selenium form in the foliar solution. The main selenium compounds was SeMet (approximately 50%), regardless of the selenium form. We also identified an unknown selenium compound at retention time 4.5 min (approximately 18%). Mazej et al., (2008) published a study in which chicory plant was aeroponically treated with Se(VI): 75 – 91% of selenium was present in soluble form after enzymatic extraction, and selenium was mainly present in non-metabolized Se(VI) form.

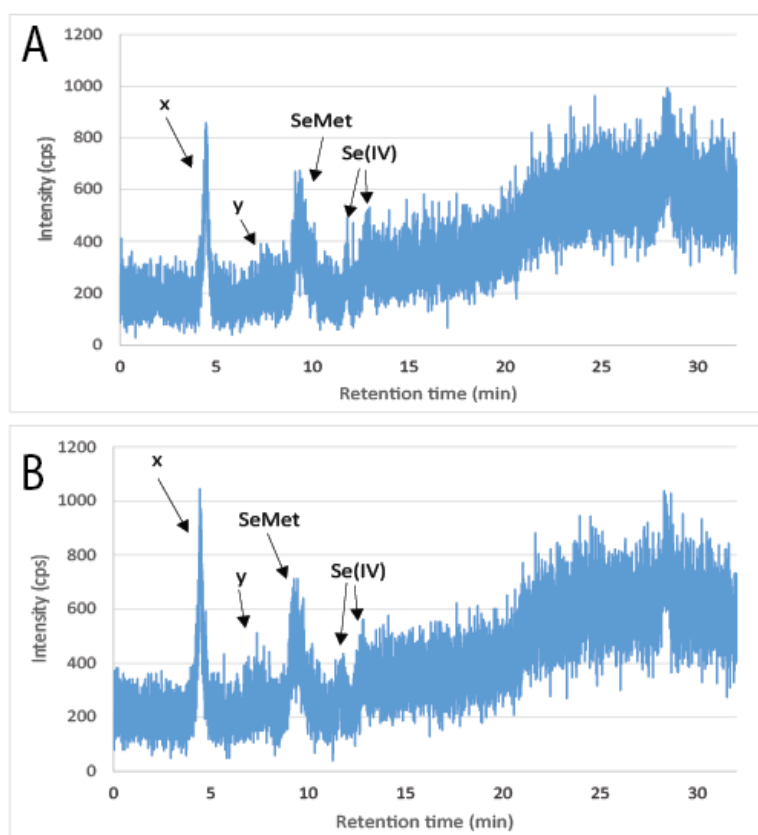


Figure 4.34: Separation of selenium compounds with a Hamilton PRP-X100 anion exchange column in selenium enriched outer chicory leaves with A) Se(IV) and B) Se(VI) foliar solution.

4.3.4.2 Iodine effect on selenium and its compounds

Iodine had a varying impact on selenium content in selenium and iodine treated chicory leaves (Figure 4.35). The selenium content in inner leaves was enhanced in Se(VI)+I treated chicory. There was an opposite trend in outer leaves, in which the selenium content was decreased up to 6.5-fold. I(V) presence in the solution of Se(IV) had an effect on selenium content in comparison with a solution of Se(IV) in both samplings. The highest selenium uptake was observed in the outer leaves when iodine was present in the foliar solution. In the literature, the highest uptake of selenium in carrot leaves was obtained when Se(VI)+I(-I) was present in the solution (Smoleń et al., 2016). This is in agreement

with our results for outer leaves. A lower selenium content was observed in the inner leaves, especially in the presence of iodine. Iodine probably plays an important role in transport mechanisms, because the selenium content in the presence of iodine decreased in the second sampling phase.

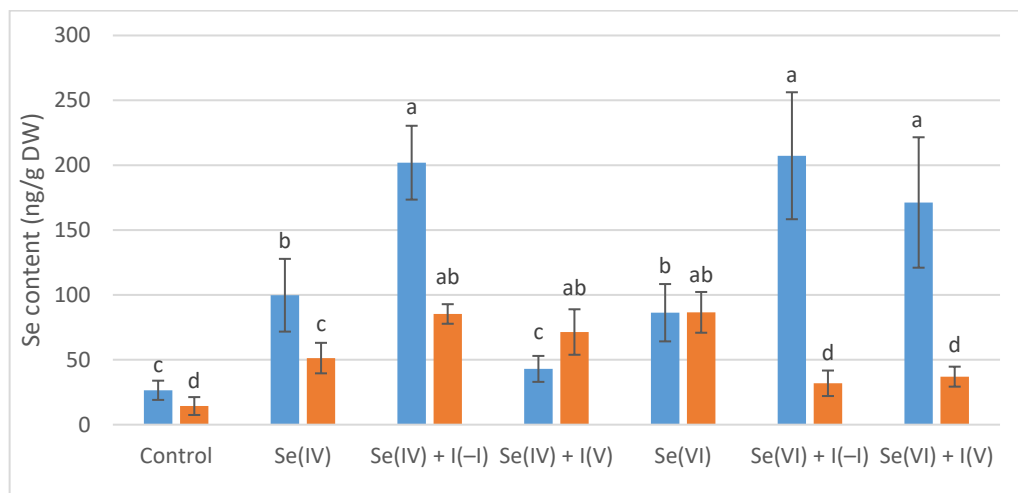


Figure 4.35: Iodine effect on selenium content in selenium and selenium-iodine enriched chicory outer (blue color) and inner leaves (orange color).

Simultaneous treatment of chicory leaves with selenium and iodine increased the amount of soluble selenium up to 2.5-fold in comparison with selenium treatment alone (Table 4.26). The main selenium compounds in all treatments was SeMet, the amount of which varied from 39% in Se(IV)+I(V) treated leaves up to 85% in Se(VI)+I(V) treated chicory leaves. Un-metabolized selenium form (Se(IV)) was present in all Se(IV) foliar treated leaves, while in Se(VI) foliar treated leaves, un-metabolized selenium form (Se(VI)) was not present in Se(VI)+I(V) enriched leaves. In all treatments, selenium compounds in retention times 4.5 min and 7.7 min were present. Overall, iodine had a minimal impact on the distribution of selenium among the identified selenium compounds in Se(IV) enriched chicory plant. This is in agreement with research, in which carrot leaves were simultaneously treated with selenium and iodine (Smoleń et al., 2016). On the other hand, SeMet content was enhanced when iodine was present in Se(VI) foliar solution.

Table 4.26: Iodine effect on selenium compounds in selenium and selenium-iodine enriched outer chicory leaves.

Foliar application	Soluble Se ng/g DW (% ^a)	Identified Se compounds	Sum % ^b
Se (IV)	33 ± 2 (32)	SeMet^c, Se (IV)	41
Se (IV) + I (-I)	83 ± 1 (37)	SeMet ^c , Se (IV), Se (VI)	40
Se (IV) + I (V)	22 ± 5 (50)	SeMet ^c , Se (IV), Se (VI)	68
Se (VI)	20 ± 8 (31)	SeMet^c, Se (VI)	42
Se (VI) + I (-I)	110 ± 48 (70)	SeMet ^c , Se (IV), Se (VI)	37
Se (VI) + I (V)	98 ± 3 (64)	SeMet ^c	36

^a percentage of soluble Se regarding to Se in sample

^b percentage of identified Se compounds regarding to
total Se in sample

^c the most abundant Se compound

Results are given as average values of two
determinations with standard error

4.4 Comparison of Selenium and Its Compounds in Sprouts and Adult Plants

4.4.1 Sprouts

A comparison of selenium content and content of selenium compounds was performed to found out the similarities and differences in content and compounds among pea, pumpkin and buckwheat sprouts. To provide similar growing conditions for all researched sprouts, soaking in solutions of 10 mg/L Se(IV) and Se(VI) alone or in combination with 1000 mg/L I(-I) and I(V) were compared.

4.4.1.1 Selenium content

The highest selenium content in control sprouts was measured in pea sprouts (Table 4.27). The content in pumpkin and buckwheat sprouts was approximately the same. Soaking of seeds or achenes in a solution of Se(IV) and Se(VI) provided different uptake in the investigated sprouts. When pea seeds were soaked in Se(IV) and Se(VI) solutions, the enriched selenium content was obtained at different rates. The uptake was 2-fold higher when Se(VI) was present in the soaking solution than with Se(IV) solution alone. The uptake of Se(IV) and Se(VI) revealed no significant differences between pumpkin and buckwheat sprouts. Comparison of the selenium content in pea, pumpkin and buckwheat sprouts revealed up to 88-fold higher content in Se(VI) enriched pea sprouts than in pumpkin and buckwheat sprouts.

The presence of iodine in the soaking solution had different effects on the obtained selenium content in sprouts in comparison with selenium soaking alone (Table 4.27). Simultaneous soaking of pea seeds in selenium and I(V) solution decreased the selenium content in pea sprouts, while the content was increased in pumpkin sprouts. I(-I) presence in selenium enriched buckwheat sprouts slightly enhanced the selenium content, as in pumpkin sprouts.

Table 4.27: Iodine effect on selenium content in selenium and selenium-iodine enriched sprouts.

	I form	Control	Se(IV)	Se(VI)
		Se content (ng/g DW)		
Pea sprouts	0	110 ± 30	5600 ± 210	14730 ± 1960
	I(-I)	/	=	=
	I(V)	/	↓	↓
Pumpkin sprouts	0	37 ± 5	197 ± 9	453 ± 13
	I(-I)	/	↑	↑
	I(V)	/	↑	↑
Buckwheat sprouts	0	29 ± 13	161 ± 35	140 ± 29
	I(-I)	/	↑	↑
	I(V)	/	=	-

/ not determined

Results are given as determined values with standard deviation

↓ means decrease, ↑ increase and = no changes in Se content regarding to Se(IV) and Se(VI) groups

4.4.1.2 Selenium compounds

Amount of soluble selenium was lowest in selenium enriched buckwheat sprouts and the highest in pumpkin sprouts (Table 4.28). The percentage of identified selenium compounds was lowest in buckwheat sprouts (8 - 23%), while it was at the same level in pea and pumpkin sprouts. Selenium compounds (SeMeSeCys, SeMet, Se(IV), Se(VI)) were identified in all selenium enriched sprouts, with the exception of SeMeSeCys in Se(VI) enriched pea sprouts. The main selenium compound in pea and pumpkin sprouts was SeMet. There was the highest rate of enrichment of buckwheat achenes with Se(IV) taken up and metabolized to SeMeSeCys. Se(VI) was main compound in Se(VI) enriched buckwheat sprouts.

Table 4.28: Iodine effect on selenium compounds in selenium and selenium-iodine enriched sprouts.

Sprouts	Seed soaking solution	Soluble Se %	Present Se compound (%)				Sum ^a %
			SeMeSeCys	SeMet	Se(IV)	Se(VI)	
Pea	Se(IV)	59	9	58^b	24	5	37
	Se(IV)+I(-I)	84	↓	↑	↓	=	43
	Se(IV)+I(V)	86	↓	↑	↓	=	52
	Se(VI)	82	/	52	4	42	45
	Se(VI)+I(-I)	89	=	=	=	=	51
	Se(VI)+I(V)	77	/	↑	=	↓	56
Pumpkin	Se(IV)	76	7	34	13	36	43
	Se(IV)+I(-I)	75	=	=	=	↑	35
	Se(IV)+I(V)	63	=	↑	↓	↑	24
	Se(VI)	72	3	49	7	35	42
	Se(VI)+I(-I)	73	=	↓	=	↑	59
	Se(VI)+I(V)	81	=	↓	=	↑	47
Buckwheat	Se(IV)	11	34	7	6	20	8
	Se(IV)+I(-I)	/			/		
	Se(IV)+I(V)	/			/		
	Se(VI)	26	10	13	15	51	23
	Se(VI)+I(-I)	/			/		
	Se(VI)+I(V)	/			/		

^a Percentage of identified Se compounds regarding to Se content in sample

^b text in bold shows most abundant Se compound in sprouts

/ not determined

↓ means decrease, ↑ increase and = no changes in Se content regarding to Se(IV) and Se(VI) groups

Iodine had a minimal impact on the amount of soluble selenium in all sprouts. The presence of selenium and iodine in enriched pea sprouts increased the amount of SeMet (main compound) with the exception of Se(VI)+I(-I) with which it stayed at the same level. The main selenium compound in Se(IV)+I treated sprouts was SeMet, as in selenium treatment alone. The effect of iodine on selenium compounds in pumpkin sprouts was an increase in the

amount of Se(VI) in all treatments, while the amount of other compounds depended on the form of selenium and iodine in solution. Selenium compounds in selenium and iodine enriched buckwheat sprouts were not identified, because of too low amount of soluble selenium.

To conclude, the highest selenium content was obtained in selenium treated pea sprouts. SeMet was the main selenium compound in selenium enriched pea and pumpkin sprouts, while the main compound in buckwheat sprouts depended on the selenium form in the soaking solution. The effect of iodine on the presence of selenium compounds depended on the enriched plant species and the presence of selenium and iodine forms in the soaking solutions.

4.4.2 Adult plants

As with sprout plants, the selenium content and compounds were compared in edible parts of adult plants - pea seeds, pumpkin seeds, buckwheat achenes and chicory leaves. Plants were foliar treated with 10 mg/L Se(IV) and Se(VI) alone or in combination with 1000 mg/L I(-I) and I(V) to compare selenium uptake and metabolism in the edible parts of the plants.

4.4.2.1 Selenium content

The selenium content in edible parts of control plants was similar (Table 4.29). Foliar application with selenium did not enrich its content in pea and pumpkin seeds to the same levels as seed soaking. The reasons for different selenium uptakes may be: i) the distribution of selenium within the adult plant and its storage in other parts of the plants or ii) the foliar solution did not penetrate the leaf surface due to weather conditions (rain may have washed off the solution). The selenium content in all enriched edible parts of plants was significantly higher in comparison with control plants. Pumpkin seeds took up the smallest amount of all the edible parts of plants (up to 1.5-fold), while the highest was observed in buckwheat achenes (up to 22-fold). Foliar application with Se(VI) enriched the edible parts of plants at higher rates than Se(IV) application, with the exception of pumpkin seeds, in which the uptake level with Se(IV) and Se(VI) was not significantly different. The highest selenium uptake was obtained in buckwheat achenes, foliar sprayed with Se(VI).

Table 4.29: Iodine effect on selenium content in control and selenium and selenium-iodine enriched pea seeds, pumpkin seeds, buckwheat achene and chicory leaves.

	I form	Control	Se(IV)	Se(VI)
		Se content (ng/g DW)		
Pea seeds	0	15 ± 1	56 ± 18	173 ± 7
	I(-I)	/	↑	=
	I(V)	/	=	↓
Pumpkin seeds	0	35 ± 3	51 ± 8	44. ± 11
	I(-I)	/	=	↑
	I(V)	/	=	↑
Buckwheat achene	0	29 ± 4	115 ± 8	653 ± 16
	I(-I)	/	↓	↓
	I(V)	/	↑	↓
	0	14 ± 7	51 ± 12	87 ± 16

Chicory	I(-I)	/	↑	↓
leaves				
(inner)	I(V)	/	↑	↓

/ not determined

Results are given as determined values with standard deviation

↓ means decrease, ↑ increase and = no changes in Se content regarding to Se(IV) and Se(VI) groups

The effect of iodine on selenium content in the edible parts of plants depended on plant species and forms of selenium and iodine in the foliar solution with no general role. The selenium content was enriched, regardless of the iodine form, in Se(VI) treated pumpkin seeds and Se(IV) treated chicory heads. In contrast, iodine had a negative impact on the selenium content in Se(VI) treated buckwheat achenes and chicory leaves, regardless of iodine form.

4.4.2.2 Selenium compounds

Selenium compounds in pea seeds, pumpkin seeds and buckwheat achenes were compared (Table 4.30). The amount of soluble selenium was lowest in buckwheat achenes, while it was approximately the same in pea and pumpkin seeds. We identified from 19 - 55% of selenium compounds in all edible parts of treated plants regarding to the total selenium content in edible parts of plants. The main selenium compound in all treated seeds/achenes was SeMet regardless of foliar solution. In pea seeds, in addition to SeMet, also SeMeSeCys, Se(IV) and Se(VI) were present in lower amounts. The only obtained selenium compounds in pumpkin seeds was SeMet, while a small amount of SeMeSeCys was also present in buckwheat achenes.

The effect of the presence of iodine on selenium compounds depended on plant species and forms of selenium and iodine in the foliar solution. The main compound (SeMet) in enriched pea seeds was increased in the iodine presence in Se(IV) and decreased in Se(VI) enriched pea seeds. The presence of the main compound SeMet was not affected by iodine presence almost in all enriched pumpkin and buckwheat seeds with expect in the Se(IV)+I(-I) treatment of buckwheat plants where amount was decreased. The presence of iodine in the Se(IV) solution increased SeMeSeCys content in treated pea seeds. At the same time, total selenium content was increased.

Overall, buckwheat took up the higher amount of selenium than other edible parts of the studied plants. The main compound in all treatments was SeMet, regardless of solution and plant species. In pea seeds, four selenium compounds were found, while in pumpkin seeds only SeMet was present. The effect of iodine on selenium and its compounds depended on the plant species and selenium and iodine presence in the foliar solution. To ensure reliable effect of iodine on content of selenium and its compounds, experiment over three or more years is needed due to different climate conditions in each year of the experiment.

Table 4.30: Iodine effect on selenium compounds in selenium and iodine enriched pea seeds, pumpkin seeds and buckwheat achene.

Plant parts	Foliar solution	Soluble Se %	Present Se compound (%)				Sum ^a %
			SeMeSeCys	SeMet	Se (IV)	Se (VI)	
Pea seeds	Se (IV)	58	3	26^b	3	6	36
	Se (IV)+I (-I)	68	↑	↑	=	=	44
	Se (IV)+I (V)	78	↑	↑	=	=	55
	Se (VI)	64	8	62	1	10	32
	Se (VI)+I (-I)	71	=	↓	↑	=	48
	Se (VI)+I (V)	72	=	↓	=	=	49
Pumpkin seeds	Se (IV)	53		82			19
	Se (IV)+I (-I)	50		=			30
	Se (IV)+I (V)	36		=			25
	Se (VI)	56		86			35
	Se (VI)+I (-I)	43		=			30
	Se (VI)+I (V)	57		=			35
Buckwheat achene	Se (IV)	39	6	84			28
	Se (IV)+I (-I)	23	↑	↓			31
	Se (IV)+I (V)	45	=	=			22
	Se (VI)	39	3	94			34
	Se (VI)+I (-I)	36	=	=			28
	Se (VI)+I (V)	37	=	=			30

^a Percentage of identified Se compounds regarding to total Se content in sample

^b the most abundant Se compound

↓ means decrease, ↑ increase and = no changes in Se content regarding to Se(IV) and Se(VI) groups

4.4.3 Comparison between selenium in its compounds in sprouts and adult plants

Selenium and its compounds were compared between sprouts and seeds or achenes, after different enrichment methods (Table 4.31). Sprouts were developed from seeds soaked in solutions, while seeds of adult plants were developed after foliar application with the same solutions. Selenium was present in the solution in a concentration 10 mg/L in Se(VI) or Se(IV) forms. In the addition, 1000 mg/L of iodine was added in the forms of I(-I) or I(V).

Table 4.31: Comparison of selenium content, soluble selenium and selenium compounds in selenium and selenium-iodine treated sprouts, seeds and achenes of pea, pumpkin and buckwheat.

		Se content (ng/g DW)	Soluble Se (%)	Se compounds
Pea	Sprouts	3870 (Se(IV)+I(V)) -14730 (Se(VI))	59 - 89	SeMeSeCys, SeMet, Se(IV), Se(VI)
	Seeds	56 (Se(IV)) -195 (Se(IV)+I(-I))	58 - 78	SeMeSeCys, SeMet, Se(IV), Se(VI)
Pumpkin	Sprouts	68 (Se(IV)) -2333 (Se(VI)+I(V))	63 - 81	SeMeSeCys, SeMet, Se(IV), Se(VI)
	Seeds	43 (Se(IV)+I(V)) - 87 (Se(VI)+I(V))	36 - 75	SeMet
Buckwheat	Sprouts	161 (Se(IV)) - 242 (Se(IV)+I(-I))	11 - 26	SeMeSeCys, SeMet, Se(IV), Se(VI)
	Achenes	93 (Se(IV)+I(-I)) - 653 (Se(VI))	23 - 39	SeMeSeCys, SeMet

4.4.3.1 Pea

The highest selenium content in selenium treated sprouts or seeds was obtained in pea sprouts (leaves and stems), in which the content was enhanced up to 14730 ng/g DW in Se(VI) treated sprouts. The same treatment enriched the selenium content of pea seeds up to 173 ng/g DW with a different enrichment method. Treatment with Se(VI) enriched the selenium content at higher rates than Se(IV) treatment in sprouts and seeds.

In pea sprouts, in which the highest selenium content was obtained, the presence of iodine in I(V) form had a negative impact on the selenium content in selenium treated sprouts. In contrast, the presence of iodine increased the selenium content in Se(IV) treated pea seeds.

There was approximately the same amount of soluble selenium in pea sprouts and seeds. SeMet was present at the highest rate in investigated sprouts and seeds, regardless of selenium form and enrichment method. In addition to SeMet, also SeMeSeCys, Se(IV) and Se(VI) were present in small amounts in sprouts and seeds.

The influence of iodine presence on selenium compounds was different in pea sprouts and seeds. Iodine enhanced the SeMet content in Se(IV) enriched pea sprouts. In contrast, the SeMet content stay approximately the same in pea seeds, while the SeMeSeCys content was increased. The effect of iodine on the selenium content in Se(VI) enriched pea sprouts, depended on the form of iodine in the soaking solution. However, the presence of iodine in the Se(VI) soaking solution decreased the SeMet content in enriched pea seeds.

4.4.3.2 Pumpkin

The selenium content in pumpkin sprouts was enriched up to 453 ng/g DW, and in pumpkin seeds up to 51 ng/g DW. The uptake of selenium was higher in Se(VI) enriched sprouts than in Se(IV) treated sprouts. The uptake of selenium was similar in Se(IV) and Se(VI) treated pumpkin seeds.

Comparison of iodine effect on selenium content in pumpkin sprouts and seeds showed that iodine increased the selenium content in all treatments of pumpkin sprouts and Se(VI) treated pumpkin seeds, while it had no impact on the content in Se(IV) treated pumpkin seeds. The highest selenium content was obtained in Se(VI)+I(V) treated pumpkin sprouts (2333 ng /g DW) and seeds (87 ng/g DW).

The amount of soluble selenium was in approximately the same range in sprouts and seeds. In pumpkin sprouts, SeMeSeCys, SeMet, Se(IV) and Se(VI) were determined, with SeMet as the main selenium compound. However, only SeMet was determined in the higher rates in pumpkin seeds.

Iodine had a minimal impact on the distribution of selenium compounds in Se(IV)+I treated sprouts, while an impact of iodine in Se(VI)+I treated sprouts was observed. The amount of Se(VI) was higher when iodine was present in the soaking solution. In pumpkin seeds, SeMet was not influenced by iodine presence in the foliar solution.

4.4.3.3 Buckwheat

The uptake of selenium was approximately the same for buckwheat sprouts and achenes when Se(IV) was present in the soaking or foliar solution. Se(VI) influenced selenium uptake in buckwheat achenes (up to 653 ng/g DW). The selenium content in buckwheat sprouts was not effected by different selenium solutions. In contrast, foliar treated buckwheat achenes took up higher selenium content when Se(VI) was present.

The effect of iodine presence in the fortified solution in the case of buckwheat sprouts and achenes depended on the forms of selenium and iodine, with no general rules. The content in buckwheat sprouts was not influenced by iodine presence, while differences were observed in buckwheat achenes. Iodine increased the selenium content in Se(IV)+I(V) treated achenes. In other combinations, the selenium content decreased.

The percentage of soluble selenium was the lowest in buckwheat sprouts (11 - 23%). In buckwheat achenes, an approximately 3-fold higher selenium content was extracted. The main selenium compound in all treatments was SeMet, with the exception of buckwheat sprouts, with which the presence of compounds depended on the selenium form in the fortified solution. In Se(IV) treated buckwheat sprouts, SeMeSeCys was the main compound while in Se(VI) treated sprouts Se(VI).

Selenium compounds were not identified in buckwheat sprouts soaked in a solution of selenium and iodine, so a comparison with buckwheat achenes could not be made.

4.5 Selenium Enriched Plants for Human Nutrition

4.5.1 Sprouts

According to the European Food Safety Authority (EFSA), the recommended daily selenium intake (RDI) is 70 µg per day for adult men and women, needed to support maximum synthesis of selenoproteins glutathione peroxidase (GPx) ("Dietary Reference Values for selenium," 2014). The daily intake was calculated for a consumption of 1 g of dry sprouts, on the assumption that all selenium would be absorbed in the body. In Table 4.32, RDI for selenium are calculated as the daily intake. In the calculation, all selenium (10 mg Se/L) and iodine (1000 mg I/L) treated sprouts are taken into account.

Despite the high selenium content in pea sprouts (up to 14.73 µg/g DW), consumption of 1 g of dry sprouts provides only 6 – 21% of RDI. Pumpkin and buckwheat sprouts contained smaller amounts of selenium, up to 2.33 µg/g DW and 0.96 µg/g DW, respectively. With 1 g of dry sprouts, only up to 3.3% of selenium recommended intake would be consumed.

Table 4.32: Calculated percentage of RDI (70 µg per day) for selenium in enriched sprouts (1g of dry sprouts).

	Pea sprouts	Pumpkin sprouts	Buckwheat sprouts
Control	0.4%	0.1%	0.1%
Treated ^a	6 – 21%	0.7 – 3.3%	0.2 – 0.4%

^a Selenium and selenium-iodine treatments

4.5.2 Edible parts of adult plants

The daily selenium intake was calculated as the consumption of one portion (100 g) of pea seeds, pumpkin seeds, buckwheat achenes and chicory leaves. Because pumpkin seeds are normally consumed as dry seeds, dry mass was taken into account in the calculation. Other edible parts are consumed as fresh food, so the calculation was based on fresh mass. Dry masses of pea seeds (on average 31%) were measured during our experiment. Dry masses for buckwheat achene and chicory leaves were taken from the literature (Souci, Fachmann, & Krafft, 2000). The amount of dry mass for buckwheat achenes was 86% (data for buckwheat flour) and for chicory leaves 5.9%. In the calculation, all selenium and iodine treated sprouts are taken into account.

Consumption of pea and pumpkin seeds provides approximately the same percentage of RDI for selenium. The highest amount of selenium is provided by consumption of 100 g of buckwheat achenes, in which 11 – 80% of RDI selenium is present. With consumption of chicory leaves only less than 1% RDI is taken in. Based on the calculated percentage of RDI in sprouts and edible parts of selected plants, all parts could be used for consumption.

Table 4.33: Calculated percentage of RDI (70 µg per day) for selenium enriched plants.

	Pea seeds ^b	Pumpkin seeds ^b	Buckwheat achene ^b	Chicory leaves(inner) ^c
Control	1.5%	12%	8%	0.3%
Treated ^a	3 – 9%	6 – 12%	11 – 80%	0.3 – 0.7%

^a Selenium and selenium-iodine treatments

^b 100 g of dry plants

^c 100 g of fresh plants

4.6 Effect of Extensive Agriculture on Selenium Content

4.6.1 Selenium content in river sediments

Sediment samples were sampled simultaneously with the aquatic plant *Veronica anagallis-aquatica*. The content of selenium in the sampled sediments was up to 0.86 µg/g DW in all three watercourses (Table 4.34). The highest content was measured in sediment at Žerovniščica watercourse in 2013. Meadows and fields are located around the sampling site and can affect the selenium content in the sediment. In literature, identified concentrations of selenium was found in 50 sediment samples from the Tagus estuary, Portugal (Pinho et al., 2005). Concentrations were up to 1.17 µg/g. The location with the highest selenium content was a hotspot, because its mean value was three times higher than the contents at other measured locations. The hotspot might be present due to an old pyrite treatment industrial plant (Pinho et al., 2005). Bonanno (2011) measured selenium concentrations in different sediments from the Imera Meridionale River (Sicily, Italy) (Bonanno, 2011). The average selenium content was 1.38 µg/g. The area is mainly affected by massive urbanization and intensive agriculture. In a study by Turner (2013) selenium concentrations in the sediments of four estuaries in southwest England in 2012 were reported to range from 0.36 to 1.49 µg/g. Agricultural land, forest, open moorland and in one estuary mining activities are present in this area. According to our results, approximately 25 years of selenium addition to feedstuff in Slovenian agriculture has shown no impact in the studied area, with various human activities (daily farming, agriculture, cattle and pig farm), because the contents of selenium in sediments were in range of natural background (Pinho et al., 2005).

Table 4.34: Selenium content in sediment samples in three watercourses (µg/g DW).

Watercourse	2009 (n)	2010 (n)	2011 (n)	2013 (n)
Pšata	<LOD ^b (2)	<LOD (1)	<LOD (2)	0.41 ± 0.02 (1) (0.39–0.43) ^a
Žerovniščica	<LOD (2)	<LOD (2)	<LOD (2)	0.65 ± 0.20 (4) (<LOD–0.86)
Lipsenjščica	<LOD (1)	<LOD (1)	0.44 ± 0.17 (2) (<LOD– 0.61)	<LOD (1)

(n) number of samples

^a () concentration range

^b LOD detection limit for Se (0.36 µg/g DW)

4.6.2 Selenium and its compounds in *V. anagallis-aquatica*

4.6.2.1 Selenium content

Selenium content was measured in the aquatic plant *V. anagallis-aquatica* at various sampling sites in 2009, 2010, 2011 and 2013. *V. anagallis-aquatica* was not always present at the same sampling sites (Table 4.35). The concentration range in *V. anagallis-aquatica* in three successive years (2009 – 2011) from the selected watercourses varied from 0.186 to 1.535 µg/g DW. The highest content of selenium was found in plants growing in the Žerovniščica watercourse in July 2010, while the lowest content was measured in the Lipsenjščica watercourse in July 2009. The highest selenium content was expected in plants in the Žerovniščica watercourse because of the nearby village, fields and dairy farms with cattle, while the lowest selenium content was expected in the Lipsenjščica, since it flows through a landscape that is relatively unaffected by human activities. The selenium content in *V. anagallis-aquatica* in Žerovniščica was higher in April and July than in February and October. Seasonal differences were not observed in the Lipsenjščica watercourse. In the Žerovniščica, the highest content in April and July was caused by fertilization with liquid manure and manure (Mihelič et al., 2010) and the lowest in October by leaching of selenium from the fields because of precipitation.

Table 4.35: Concentration range of selenium content in *V. anagallis-aquatica* at each sampling site (µg/g DW).

Plant parts	Year	Pšata (n)	Žerovniščica (n)	Lipsenjščica (n)
Whole plant	2009	April	0.997–1.330 (2)	/
		July	0.562–0.689 (4)	0.186–0.198 (2)
		October	/	/
	2010	February	0.354–0.781 (2)	1.082–1.102 (2)
		April	0.998–1.042 (2)	/
		July	1.304–1.535 (2)	0.464–0.472 (2)
		October	0.270–0.849 (4)	0.515–0.531 (2)
	2011	February	/	/
		April	0.453–1.453 (4)	/
		July	0.907–0.929 (2)	/
		October	/	/
	2013	July	0.292–0.299 (2)	/
		September	0.414–0.430 (2)	/
Leaves	2013	July	0.025–0.185 (6)	0.046–0.051 (2)
		September	0.081–0.084 (2)	0.028–0.032 (2)
Stems		July	0.019–0.077 (6)	0.032–0.033 (2)

	September	0.059-0.060 (2)	0.274-0.529 (6)	0.028-0.029 (2)
Roots	July	/	0.302-0.714 (6)	0.524-0.539 (2)
	September	/	/	0.662-0.727 (2)

(n) number of samples

The selenium content in *V. anagallis-aquatica* sampled in year 2013 was determined in various plant parts: leaves, stems and roots (Table 4.35). The concentration range of selenium in leaves varied from 0.025 - 0.480 µg/g, in stems from 0.019 - 0.529 µg/g and in roots from 0.302 - 0.727 µg/g, all on a dry weight basis. In the Pšata watercourse in July and one sampling site in Žerovniščica in September plants were so small that selenium was determined in the whole plant. The selenium content in *V. anagallis-aquatica* sampled in 2013 was lower than in the years 2009 - 2011. The highest content of selenium in 2013 was found in roots in the Lipsenjščica in September. The lowest selenium content in 2013 was found in stems at all sampling sites with the exception of a sample from the Žerovniščica in September. The content of selenium in different plant parts of aquatic macrophytes were measured in *Potamogeton pectinatus* and *Ruppia maritima* (Wu & Guo, 2002). Higher selenium concentrations were found in roots than in shoot tissues. In the 1930s, it was demonstrated that in non-accumulator plants selenium was mainly present in roots and seeds, with small amounts being found in leaves and stems (Beath, Eppson, & Gilbert, 1937). The reason for the higher selenium content in roots may be in a detoxification mechanism that protects the plant from toxicity (Simmons & Wallschläger, 2005). According to the obtained selenium content in different plant parts and literature data (Chambers et al., 1989), it can be assumed that the higher selenium content in roots is the result of absorption of selenium from the sediment via roots, because the selenium content in water was very low (0.06-0.15 µg Se/L) (Kroflič, Germ, Mechora, & Stibilj, 2016). In our study, *V. anagallis-aquatica* accumulated up to 1.5 µg Se/g DW, without showing symptoms of toxicity. It can therefore be used for evaluation of selenium content in an agricultural area.

The bioconcentration factor (BAF) is an indication of accumulation of a substance in an organism compared to its content in the environment (Travis & Arms, 1988). BAF factors in literature are named differently: from bioconcentration factor BCF (macrophyte/sediment) to enrichment factor (plant/bottom sediments). BAFs for selenium vary from 1126 - 1933 L/kg in the whole plant (the average selenium concentration in the water was in the range 0.06 - 0.15 µg/L, data are not shown). There are no BAF values available for macrophytes, so BAF values for microalgae were used. The reported value for selenium in microalgae was in the range of 10³ L/kg (International Atomic Energy Agency., 2004). BAFs in *V. anagallis-aquatica*, sampled in 2009 - 2011 are in good agreement with reported values.

4.6.2.2 Selenium compounds

To the best of our knowledge, there are scarce data about selenium compounds in aquatic plants. Investigation of selenium compounds in *V. anagallis-aquatica* with enzymatic extraction using the enzyme Protease XIV was performed. The enzymatic fraction of Protease XIV contained 249 ng SeMet/g enzyme, 58 ng Se(IV)/g enzyme and 37 ng Se(VI)/g enzyme (Figure 4.36) and those data were

considered in determination of selenium compounds in plant extracts.

The content of soluble selenium in the enzymatic extract of selected whole plant samples varied from 19 - 28% (Table 4.36). In enzymatic extracts, Se(VI) and Se(IV) were identified (Figure 4.36). Content of Se(VI) in whole plants ranged from 1.0 - 2.6 ng/g DW and Se(IV) varied from 2.7 - 21.1 ng/g DW. A peak at approximately 10 min was identified as SeMet but counts belonged to SeMet in enzyme Protease XIV. Despite the high selenium content in *V. anagallis-aquatica*, seleno amino acids were not present. Mechora et al. (2012a) determined selenium and its compounds in the aquatic moss *F. antipyretica*. The highest selenium content was 2250 ± 170 ng/g. In enzymatic extract, approximately 25% of soluble selenium was found. Se(IV) and Se(VI) in aquatic moss were determined but no seleno amino acids were found (Mechora et al., 2012a). Wu & Guo, (2002) found the seleno amino acids SeCys₂, SeMeSeCys, and SeMet in submerged aquatic macrophytes *P. pectinatus* and *R. maritima* that were exposed for 10 days to 60 µg/L (Wu & Guo, 2002) solution.

Identification of selenium compounds in different plant parts was obtained in selected samples from 2013 (Table 4.36). The amount of soluble selenium in samples varied from 10.5 - 14.5% in roots, 21.0 - 27.9% in stems and 26.4 - 29.6% in leaves. Leaves are vital organs, in which photosynthesis takes place (Sors et al., 2005). That may be the reason for high amounts of soluble selenium in leaves. We identified from 1.3 - 39.1% of selenium compounds in soluble selenium in the different plant parts. We obtained only Se(VI) and Se(IV) in the aquatic plant. The content of Se(IV) was determined in leaves, stems and roots and was up to 16.17 ng/g DW, while the content of Se(VI) in the different plant parts varied from 0.2 to 3.3 ng/g DW. To the best of our knowledge, there has been no research where selenium compounds in different parts of naturally occurring aquatic plants were obtained.

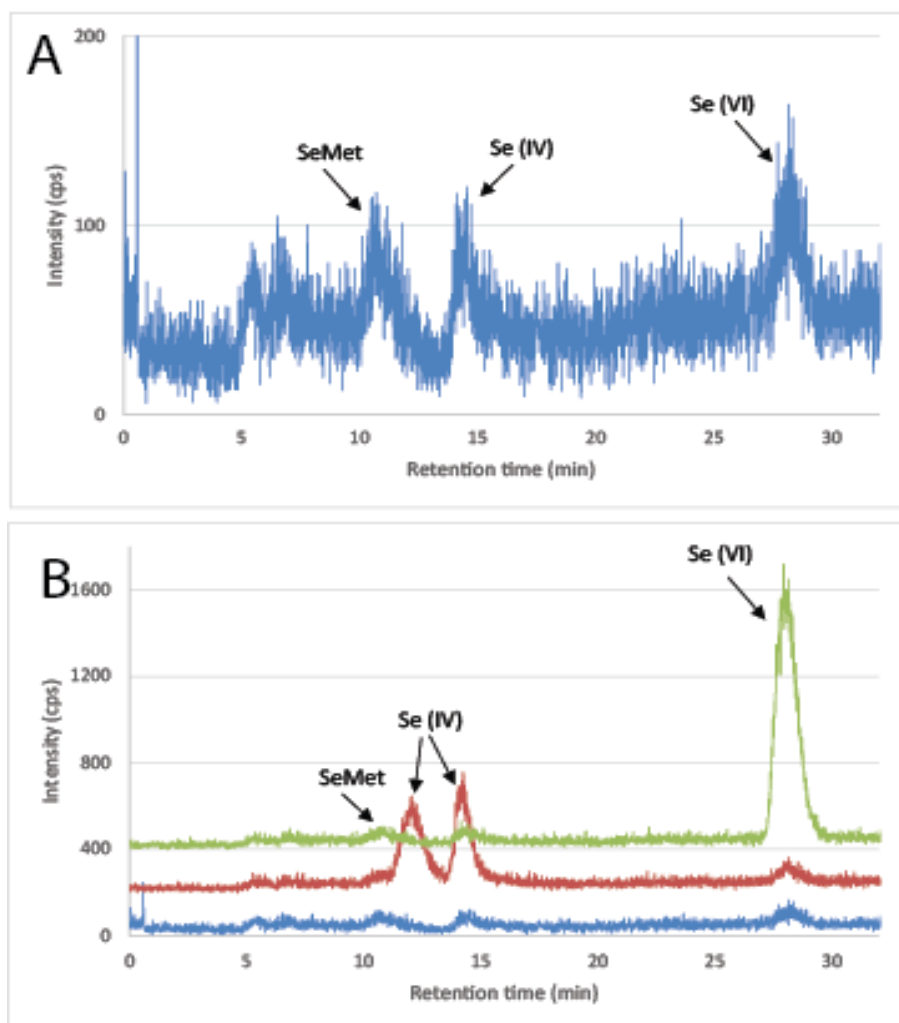


Figure 4.36: A) Chromatogram of the water-soluble fraction of enzyme Protease XIV on a Hamilton PRP-X100 anion exchange column, B) Chromatogram of enzymatic extracts of macrophyte sample without standard addition (blue), with addition of 20 ng/g Se(IV) (red) and addition of 20 ng/g Se(VI) (green).

Table 4.36: Selenium compounds in aquatic plant *V. anagallis-aquatica*.

Plant part	Sampling site	Date	Total Se (ng/g DW)	Soluble Se (%) ^a (ng/g DW)	Se(IV) (ng/g DW)	Se(VI)	Sum (%) ^b
Whole plant	Žerovniščica	July 2009	958.4 ± 51.0	185.5 ± 6.2 (19)	10.1 ± 5.2	2.3 ± 0.3	6.7
		April 2011	818.7 ± 53.7	186.1 ± 4.5 (23)	14.2 ± 3.4	1.7 ± 0.1	8.5
		July 2011	852.5 ± 35.5	239.6 ± 4.2 (28)	16.7 ± 4.4	1.7 ± 0.3	7.7
	Lipsenjščica	October 2010	422.8 ± 27.8	99.8 ± 5.2 (24)	5.5 ± 2.8	1.3 ± 0.3	6.8
	Žerovniščica	July 2013	78.1 ± 0.8	20.6 ± 1.4 (24)	4.1 ± 2.8	2.1 ± 1.2	39.1
Leaves	Lipsenjščica	September 2013	62.1 ± 8.4	18.5 ± 3.3 (26)	5.1	<LOD ^d	27.5
Stems	Žerovniščica	July 2013	58.8 ± 2.8	16.3 ± 1.8 (28)	<LOD ^c	0.2 ± 0.1	1.3
	Lipsenjščica	September 2013	80.8 ± 11.7	17.5 ± 6.1 (21)	1.4	<LOD	8.0
Roots	Žerovniščica	July 2013	703.9 ± 9.9	60.7 ± 7.0 (11)	<LOD	<LOD	
	Lipsenjščica	September 2013	550.2 ± 0.8	79.6 ± 3.5 (15)	15.8 ± 0.4	0.5	20.3

^a percentage of soluble Se regarding to its content in sample

^b percentage of identified Se regarding to soluble Se

^c Limit of detection for Se(IV) is 1.1 ng/g

^d Limit of detection for Se(VI) is 0.1 ng/g

Results are given as average value of two determinations with standard error

4.7 Comparison of Selenium and Its Compounds in Cultivated and Aquatic Plants

A comparison of selenium and its compounds between terrestrial (cultivated) and aquatic plants was made (Table 4.37). In *V. anagallis-aquatica* from an aquatic environment, a higher selenium content was obtained according to content in control pea plant. The highest selenium content was present in the roots. The same trend was seen in pea plants, with approximately 7-fold lower selenium content.

Table 4.37: Selenium content in aquatic and pea plant from present work.

Plant part	Plant	Se content (ng/g DW)
Leaves	Pea (<i>Pisum sativum</i> L.)	22 - 28
	<i>V. anagallis-aquatica</i>	62 - 78
Stems	Pea (<i>Pisum sativum</i> L.)	15 - 21
	<i>V. anagallis-aquatica</i>	59 - 81
Roots	Pea (<i>Pisum sativum</i> L.)	83 - 110
	<i>V. anagallis-aquatica</i>	550 - 704

We compared the selenium content in the aquatic plant *V. anagallis-aquatica* and selenium enriched pea plants and chicory leaves from the present study (Table 4.38). Aquatic plants were exposed to selenium in lower concentrations in water (0.06 - 0.15 µg/L) over a period of time, while pea plants and chicory leaves were foliar sprayed once with 10 mg Se/L. The selenium content was then compared with red cabbage, with which the plant was foliar sprayed twice with 0.5 mg Se/L (Mechora et al., 2012b). A much higher selenium content was obtained in foliar sprayed leaves and stems, than in *V. anagallis-aquatica*.

We wanted to discover whether the metabolism of selenium lead to the same selenium compounds in aquatic and terrestrial plants. Selenium compounds were obtained in enzymatic extract of *V. anagallis-aquatica* and selenium enriched pea plants and chicory leaves from the present study. With the same extraction procedure, the amount of soluble selenium was much higher in pea leaves than in *V. anagallis-aquatica* (up to 3-fold). Selenium is probably present in the aquatic plant in insoluble selenium forms. In *V. anagallis-aquatica* seleno amino acids were not present despite the high selenium content, but were present in inorganic forms (Se(IV) and Se(VI)). Moreover, selenium was present in cultivated plants in the forms of SeMeSeCys, SeMet, Se(IV) and Se(VI).

When *V. anagallis-aquatica* was compared with Se(IV) and Se(VI) treated cultivated plants with a similar or higher selenium content than *V. anagallis-aquatica* from the present study, differences in soluble selenium and identified selenium compounds were seen. The percentage of soluble selenium was higher in selenium treated cultivated plants, regardless of the selenium content. Organic selenium compounds were present in all treated cultivated plants.

The comparison between *V. anagallis-aquatica* and cultivated plants showed i) differences in the accumulation capacity of

selenium from the environment and ii) differences in selenium compounds composition.

Table 4.38: Comparison of selenium and its compounds in *V. anagallis-aquatica* and selenium enriched cultivated plants.

Plant part	Plant	Enrichment	Total Se (ng/g DW)	Soluble Se (%)	Identified Se compounds	Reference
Leaves	<i>V. anagallis-aquatica</i>	/	62-78	25	Se (IV), Se (VI)	present study
	Pea (<i>Pisum sativum</i> L.)	Se (IV)	81	41	SeMet, Se (IV), Se (VI)	present study
	Pea (<i>Pisum sativum</i> L.)	Se (VI)	327	76	SeMeSeCys, SeMet, Se (VI)	present study
	Red cabbage (<i>Brassica oleracea</i> L. cv. Erfurtskorange)	Se (VI)	960	43	SeMet, Se (IV), Se (VI)	(Mechora et al., 2012b)
Stems	<i>V. anagallis-aquatica</i>	/	59-80	24	Se (IV), Se (VI)	present study
	Red cabbage (<i>Brassica oleracea</i> L. cv. Erfurtskorange)	Se (VI)	810	31	SeMet	(Mechora et al., 2012b)
Roots	<i>V. anagallis-aquatica</i>	/	550-704	13	Se (IV), Se (VI)	present study
	Red cabbage (<i>Brassica oleracea</i> L. cv. Erfurtskorange)	Se (VI)	1190	28	SeMet	(Mechora et al., 2012b)
Whole plant	<i>V. anagallis-aquatica</i>	/	423-958	24	Se (IV), Se (VI)	present study
Leaves	Chicory (<i>Cichorium intybus</i> L.)	Se (IV)	100	32	SeMet, Se (IV)	present study
	Chicory (<i>Cichorium intybus</i> L.)	Se (VI)	86	31	SeMet, Se (IV)	present study

Chapter 5

Conclusions

In the doctoral dissertation, selenium and its compounds were investigated in selected cultivated and aquatic plants. Four cultivated plants from different groups were chosen: pea (legume), pumpkin (oil plants), buckwheat (pseudocereals) and chicory (leafy vegetables) due to different agricultural and nutritional parameters and their cultivation in Slovenia. Fortified plants were obtained by seed soaking or foliar spraying of plants with selenium solutions in forms of Se(IV) or Se(VI) (10 mg/L) and in the combination with iodine in forms of I(-I) and I(V) (1000 mg/L). Iodine is known as essential elements for human like selenium. Aquatic plant *V. anagallis-aquatica* was chosen for our research because it is widely distributed in Slovenia, Europe (especially in the Mediterranean area) and Asia and can be used for biomonitoring studies.

In the first part of the dissertation, optimizations of some analytical parameters for determination of selenium content and separation with detection of its compounds were performed. In the microwave digestion procedure for selenium content determination, the following parameters were optimized: (i) temperature of digestion, (ii) volume of HNO₃ and (iii) presence of H₂O₂. The optimal parameters were: 4 mL of HNO₃, no presence of H₂O₂ and digestion at 220 °C. Selenium was detected by ICP-QQQ. To check the accuracy and precision of the method, various certified reference materials (Trace elements in spinach leaves NIST 1570a, Durum wheat flour NIST 8436, Wholemeal flour BCR 189) were used, and good agreement was found between obtained and certified value. The method was also tested by an independent method HG-AFS.

Selenium compounds from selected plants were extracted with the enzyme Protease XIV. Separation and detection were done with HPLC on a Hamilton PRP-X100 anion exchange column coupled by ICP-MS or ICP-QQQ. Identification of selenium compounds in enzymatic plant extracts was performed with five selenium standards (SeCys₂, SeMeSeCys, SeMet, Se(IV) and Se(VI)) in citrate buffer (pH=4.8). Instead of citrate buffer 3 and 10 mM, 1 and 10 mM citrate buffer were used for better separation of SeMet and Se(IV). Stability studies were made of selenium compounds in enzymatic plant extracts over time. We found out, that selenium compounds were stable: (i) for two months stored at - 20 °C, (ii) for 48 h stored at 4 °C and (iii) for 12 h stored at room temperature. The optimized method was checked with reference materials. Only one certified reference material Selenium enriched yeast (SELM-1) have certified values of SeMet. There was a good match for SeMet content between the found result and certified value. Also, the accuracy and precision of SeMet determination was checked with literature data in reference materials: Wheat gluten NIST 8418 and Durum wheat flour NIST 8436, with which good matches were found.

To check the identified selenium compounds in enriched pea sprouts and buckwheat achenes obtained with a Hamilton PRP-X100

anion exchange column, we performed the separation with a Thermo IonPac AS11 anion exchange column performed at Helmholtz Zentrum München (HZM). The presence of SeMet and Se(VI) in the selected enriched plants was confirmed, while no Se(IV) was observed. We assume that possible interaction between the Thermo IonPac AS11 column and the matrix of extracts occurred with the disappearances of the peak for Se(IV). Identification of SeMeSeCys was not completely reliable, due to a similar retention time as SeMet. In addition, separation with an affinity column (HiTrap Heparin 5 mL) was made to determine SePP and GPx in enzymatic extracts of pea sprouts and buckwheat achenes. We found that SePP and GPx were not present. With a size exclusion column (SK 55 S HW, TSK 40 S SW), a possible bond between Se(VI) and proteins was obtained due to its earlier elution than SeMet.

In the second part of the thesis, the selenium content was first determined in pea, pumpkin and buckwheat sprouts grown from the seeds soaked in a solution of selenium, in the form of Se(IV) and Se(VI) in a concentration of 10 mg/L. Enhanced selenium content was obtained in all sprouts regarding to control sprouts. The selenium content in pea sprouts was up to 88-fold higher in Se(VI) treated sprouts regarding to buckwheat and pumpkin sprouts. The highest selenium amount was obtained in Se(VI) enriched pea sprouts, in which the content was up to 14.73 µg/g DW. A higher selenium content in Se(VI) enriched sprouts was determined in pea and pumpkin sprouts. The content in Se(IV) and Se(VI) enriched buckwheat sprouts was approximately the same. The hypothesis of seed soaking increasing selenium content in selected sprouts was confirmed. Selenium compounds and the amount of soluble selenium depend on the sprout species. The highest amount of soluble selenium was found in pumpkin sprouts (59 - 82%), while the lowest was in buckwheat sprouts (11 - 26%). In pea and pumpkin sprouts, approximately the same percentage of selenium compounds was identified, 37 - 45% in pea and 42 - 43% in pumpkin sprouts. The percentage was much lower in buckwheat sprouts (8 - 23%). SeMeSeCys, SeMet, Se(IV) and Se(VI) were present in almost all treatments. The main selenium compounds in pea and pumpkin sprouts was SeMet, while in buckwheat sprouts selenium compounds depended on the form of selenium present in soaking solution. The hypothesis of SeMet as the main selenium compounds, regardless of plant species can confirmed for pea and pumpkin sprouts. In buckwheat sprouts, Se(IV), Se(VI), SeMeSeCys and SeMet were present, but SeMet was not the main selenium compound.

The effect of the presence of iodine in the soaking solution was studied in selenium enriched pea, pumpkin and buckwheat sprouts. In the presence of iodine in the selenium soaking solution a different effect on the determined content of selenium and its compounds was obtained, with no general rule. In the presence of I(V), the selenium content decreased in pea sprouts and iodine increased selenium content in pumpkin sprouts. On the other hand, only a minimal impact of the presence of iodine was observed in terms of the amount of soluble selenium. The hypothesis of the different effect of iodine on selenium and its compound content was confirmed for pea and pumpkin sprouts. Selenium compounds in selenium and iodine enriched buckwheat sprouts could not be identified, because of too low amount of soluble selenium.

Furthermore, adult plants of pea, pumpkin, buckwheat and chicory were foliar sprayed with selenium in the form of Se(IV) and Se(VI), in a concentration of 10 mg/L, and edible parts of selected plants were analyzed. We found approximately the same amount of selenium in pumpkin seeds and chicory leaves, while the selenium content in pea seeds and buckwheat achenes was up to 8-fold higher. The highest selenium content was found in buckwheat

achenes from Se(VI) foliar sprayed buckwheat plants. The hypothesis that foliar application will increase the selenium content regarding to the selenium form in the foliar solution was confirmed. The main selenium compound in edible parts was SeMet, regardless of the composition of the selenium and iodine foliar solution and plant species. In pea seeds, four selenium compounds were found, while in pumpkin seeds only SeMet was present. We also confirmed the hypothesis, that the main selenium compound is SeMet in the edible parts of selected adult plants.

As with sprouts, adult plants were foliar treated with selenium (in both forms) and iodine (I(-I) and I(V)). The effect of iodine on selenium and its compounds depended on the forms of iodine and plant species. Selenium compounds in pumpkin seeds and buckwheat achenes were not affected by iodine. The hypothesis that iodine has an effect on selenium and its compounds content was partly confirmed. The selenium content in pumpkin seeds and buckwheat achenes was not affected by the presence of iodine.

The hypothesis of a higher selenium content in sprouts grown from the seeds soaked in the selenium solution than in edible parts of foliar treated adult plants was confirmed in pea and pumpkin sprouts. The selenium content in buckwheat sprouts was not higher than the content in buckwheat achenes.

In the third part of the thesis, selenium and its compounds were investigated in the aquatic plant *Veronica anagallis-aquatica* and river sediments sampled in three Slovenian watercourses for which different activities were present in the vicinity: dairy farming, stock raising and extensive agriculture. The goal was to evaluate whether agriculture at the different sampling sites affected the selenium content in *V. anagallis-aquatica* and associated river sediments.

For determination of selenium in sediment samples, the microwave digestion procedure published by Ščančar et al. (2007) was modified. Optimized parameters were: (i) time of digestion 1h, (ii) temperature 210 °C in first step of digestion, (iii) addition of 15 mL of H₃BO₃ and (iv) temperature 220 °C in second step of digestion. Using the optimized parameters, we found good agreement between the obtained value and the reference value of certified reference material (Channel sediment BCR 320R) with no residues in the digested solution.

The selenium content in the sediment revealed that approximately 25 years of selenium addition to feedstuff in Slovenian agriculture shows no impact in the research area, because contents were in the range of the natural background (around 1 µg/g). On the other hand, *V. anagallis-aquatica* accumulated a large amount of selenium (up to 1.5 µg/g DW) without showing visual symptoms of toxicity. The highest selenium content was found in roots samples. It can be assumed that the selenium was mainly taken up from the sediment via the roots, because the selenium content in the water was very low (0.06 – 0.15 µg Se/L). The hypothesis of the influence of extensive agriculture on selenium content in *V. anagallis-aquatica* and sediment was not confirmed. In *V. anagallis-aquatica* only Se(IV) and Se(VI) were obtained. Despite the high selenium content in *V. anagallis-aquatica*, seleno amino acids were not present.

Finally, comparison between the selenium content and compounds in the selected cultivated plant and *V. anagallis-aquatica* was performed. The hypothesis of a higher selenium content in the aquatic plant was confirmed: *V. anagallis-aquatica* contained 59 – 704 ng/g DW while there was 15 – 110 ng/g DW in the selected cultivated plants. Comparison of *V. anagallis-aquatica* and 10 mg Se/L foliar sprayed leaves of pea and chicory revealed approximately the same content of selenium and various selenium compounds. Only inorganic selenium was determined in *V. anagallis-*

aquatica, while in foliar sprayed leaves of pea and chicory also organic selenium compounds (SeMet, SeMeSeCys) were also found despite the lower selenium content. The hypothesis of the presence of the same selenium compounds in aquatic and selected cultivated plants was not confirmed.

To conclude, (i) the procedure for determination of selenium content in plant and sediment samples and procedure for determination of selenium compounds in plant samples were optimized, (ii) with both enrichment approaches, seed soaking and foliar application, selenium content was enriched in sprouts (pea, pumpkin, buckwheat) and in edible parts of adult plants (pea seeds, pumpkin seeds, buckwheat achenes, chicory leaves), (iii) selenium content was higher in selenium enriched sprouts in comparison with edible parts of adult plants, (iv) selenium compounds were determined in sprouts and edible parts of adult plants, where SeMet was found as the main compounds in almost all investigated plants, with except of buckwheat sprouts, (v) the highest decrease in selenium content was found in selenium and iodine enriched pea sprouts, (vi) extensive agriculture did not effected on selenium content in aquatic plant *V. anagallis-aquatica* and river sediments and (vii) despite high selenium content, seleno amino acids were not present in *V. anagallis-aquatica*.

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Kroflič, A. (2013). Določanje titana in železa s potenciometrično titracijo. Diplomsko delo. Fakulteta za kemijsko in kemijsko tehnologijo, Univerza v Ljubljani.

Treatise, Preliminary Study, Study

Kroflič, A., Michalke, B., Stibilj, V. (2017). Identification of Se compounds in Se enriched plant samples with spectroscopic methods. *IJS delovno poročilo*,

Submitted Articles Related to the Thesis

Germ, M., Stibilj, V., Šircelj, H., Jerše, A., Kroflič, A., Golob, A., Kacjan-Maršič, N. The application of different forms of Se and I influences the uptake of both elements in common buckwheat sprouts and grains *Plant Biology and Biochemistry*. Revised version resubmitted on February 2018

Editor

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Biography

Ana Kroflič was born on 23th of November 1988 in Celje. She graduated from the Faculty of Chemistry and Chemical Technology, University of Ljubljana with the thesis: "Determination of titanium and iron with potentiometric titration (in Slovene)" and obtained the title Bachelor of Science in Chemistry. The bachelor degree was performed for Cinkarna Celje, d.d., in order to improve determination procedure in their laboratories. During her study, she worked for about 6 months in the laboratory for Quality of Production Procedures at Cinkarna Celje, d.d., where she gained a great deal of laboratory experiences. In 2013, she enrolled as a PhD student at the International Postgraduate School Jožef Stefan in the program Ecotechnology. In the first year of her PhD study, she was a Jožef Stefan Institute internship holder. For the next two and a half years, she was young researcher. Her experimental work was done at Jožef Stefan Institute at the Department of Environmental Sciences, mentored by Prof. Dr. Vekoslava Stibilj. During her PhD study, she published 2 original scientific article as author and 3 as co-author. She provided many scientific contributions in conferences in Slovenia, Serbia and Austria. During her PhD study, a one-month internship at Helmholtz Zentrum München under Prof. Dr. Bernhard Michalke was part of her research work. She will finish her study with the doctoral thesis: "Determination of selenium compounds in selected cultivated and aquatic plants".