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SPECIATION OF SELENOAMINO ACIDS BY LIQUID CHROMATOGRAPHY-MASS SPECTROMETRIC METHODS

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ABSTRACT

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Tiivistelmä – Referat – Abstract

Literature review of the thesis introduces the characteristics of selenium and its importance in human diet. It also gives an overview on different analytical methods used in speciation of selenoamino acids. The aim of this study was to develop a simple and rapid mass spectrometric method which could be used to detect and specify low molecular weight selenoamino acids from different food materials in order to find and quantify probable cancer protective species.

Total selenium content was determined by GFAAS from garlic and Brazil nut samples. The selenium concentration in garlic was 0.1 µg/g and 4.4 µg/g in Brazil nuts. Also, the precipitates and supernatants from the sample extractions (hot water, diluted HCl and proteinase K) were analyzed. There were only about 10% of the total selenium in the supernatants (which were further used in analysis).

Samples were derivatized by AccQ·Tag reagent (AQC) and analyzed with UHPLC-ESI-MS method. Even though the method was easy and fast to use, it was applicable only for selenoamino acid standards (MeSeCys and SeMet). No results were obtained from the real samples. Therefore, a more sensitive piece of equipment, HPLC-ICP-MS was applied with Hamilton PRP-X100 column and 5 mmol/l ammonium citrate buffer (pH 5.2). Hot water and diluted HCl extracted samples showed no signs of selenium. At last, proteinase K digested Brazil nut sample showed a small peak of SeMet which was identified by retention time matching with the standard and quantified semi quantitatively from standard curve (0,06 µg SeMet /g Brazil nut).

This study showed that the sensitivity of the UHPLC-ESI-MS method was not sufficient to detect such low concentrations of selenoamino acids in garlic and Brazil nut samples. However, the AQC derivatization together with UHPLC-ESI-MS offers a fast, linear and repeatable method for future amino acid analysis.

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Selenium, selenoamino acids, extraction, garlic, Brazil nut, liquid chromatography, mass spectrometry

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Selenoaminohappojen määrittäminen nestekromatokrafia-massaspektrometrisin menetelmin

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Tutkielman kirjallisuusosuus käsittelee seleenin yleisiä ominaisuuksia ja merkitystä ihmisen ruokavaliossa. Se antaa myös yleiskuvan erilaisista analyysimenetelmistä, joita käytetään selenoaminohappojen määrittämisessä. Tämän tutkimuksen tavoitteena oli kehittää yksinkertainen ja nopea massaspektrometrinen menetelmä, jolla voitaisiin havaita ja tunnistaa pienimolekyylisiä selenoaminohappoja erilaisista elintarvikkeista ja näin ollen löytää ja määrittää mahdollisia syöpää ehkäiseviä yhdisteitä.

Kokonaisseleenipitoisuus määritettiin GFAAS-menetelmällä valkosipulista ja parapähkinästä sekä niiden uutteista. Seleenipitoisuus oli valkosipulissa 0.1 µg/g ja parapähkinässä 4.4 µg/g. Myös uutesakka ja supernatantit (kuumavesi-, laimennettu HCl- ja proteinaasi K-uutoista) analysoitiin. Vain 10% kokonaisseleenistä päätyi näytteiden supernatantteihin, joita käytettiin jatkoanalyyseissä.

Näytteet derivatisoitiin AccQ·Tag reagenssilla (AQC) ja analysoitiin UHPLC-ESI-MS menetelmällä. Vaikka menetelmä oli helppo ja nopea käyttää, se soveltui vain selenoaminohappostandardeille (MeSeCys ja SeMet). Varsinaisista näytteistä ei saatu tuloksia. Tämän takia herkempi laite, HPLC-ICP-MS otettiin käyttöön, hyödyntäen Hamilton PRP-X100 -kolonnia ja 5 mmol / l ammoniumsitraattipuskuria (pH 5,2). Kuumalla vedellä ja laimennetulla HCl:llä uutetuissa näytteissä ei näkynyt merkkejä seleenistä. Lopulta proteinaasi K:lla uutetussa parapähkinänäytteessä näkyi pieni SeMet-piikki, joka tunnistettiin standardin retentioajan perusteella. Pitoisuus määritettiin semikvantitatiivisesti standardikäyrältä (0,06 µg SeMet /g parapähkinää).

Tutkimus osoitti, että UHPLC-ESI-MS –menetelmä ei ollut riittävän herkkä, jotta sillä olisi voinut havaita näin pieniä selenoaminohappopitoisuuksia valkosipulista ja parapähkinästä. Kaikesta huolimatta, AQC-derivointi yhdistettynä UHPLC-ESI-MS -menetelmään mahdollistaa nopean, lineaarisen ja toistettavan menetelmän tulevaisuuden aminohappoanalyyseihin.

Avainsanat — Nyckelord — Keywords

Seleeni, selenoaminohappo, uutto, valkosipuli, parapähkinä, nestekromatografia, massaspektrometria

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PREFACE

This master thesis was performed at the Department of Food and Environmental Sciences, Food Chemistry Division, at the University of Helsinki. The experimental phase of the study was started already in the summer 2011 and through different stages of life, the writing was completed in the fall 2020.

First, I would like to give my deepest gratitude to Professor Marina Heinonen. Your inspiring words and encouragement motivated me to complete the thesis. Thank you also for the valuable comments during the writing process. I would like to extend my sincere thanks and deep gratitude go to my supervisors Velimatti Ollilainen and Päivi Ekholm for all the advice and support in the beginning of the thesis and throughout the experimental phase. Especial thanks to Miikka Olin, Bhawani Chamlagain and Petri Kylli for their technical support in the lab work. Big thanks also to The Chemistry and Toxicology Research Unit of the Finnish Food Safety Authority and Kirsti Risunen for offering your valuable time, expertise and equipment.

Finally, I would like to express my heartfelt appreciation to my parents and siblings for their endless encouragement throughout my life. The biggest gratitude goes to my beloved husband who is always there for me and children who are the reason I pursued this degree. You have made me stronger, better and more fulfilled than I could have ever imagined. I love you to the moon and back.

Hyvinkää, October 2020

Elisa Lehto

LIST OF ABBVERIATIONS

AAS	Atomic absorption spectroscopy
AI	Adequate Intake
AMQ	6-aminoquinoline
AQC	6-aminoquinolyl-N-hydroxsuccinimidyl carbamate
ESI-MS	Electrospray ionization mass spectrometry
GC	Gas chromatography
GFAAS	Graphite furnace atomic absorption spectroscopy
HPLC	High performance liquid chromatography
ICP-MS	inductively coupled plasma mass spectrometry
IDA	Isotope dilution analysis
IEC	Ion-exchange chromatography
LC	Liquid chromatography
LOD	Limit of detection
MeSeCys	Seleno-methyl-seleno-cysteine
MeSeH	Methyl selenol
MS	Mass spectrometry
NHS	N-hydroxy succinimide
PDA	Photodiode-Array
RDA	Recommended Dietary Allowance
RPIP	Reversed-phase chromatography
RPIP	Reversed-phase ion-pairing chromatography
Se	Selenium
SEC	Size-exclusion chromatography
SeMet	Selenomethionine
UHPLC	Ultra-high performance liquid chromatography
UL	Tolerable Upper Intake Level
γ-G-Se-MeSeCys	γ-glutamyl-Se-methyl-Selenocysteine

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1 INTRODUCTION

Selenium is an essential trace element for humans and its importance in the diet and health has been recognized for many decades (Rayman 2012). Selenium has contradictory behavior from being both essential and toxic, depending on the species, concentration and oxidation state. Food is the major source of selenium although the intake is highly influenced by the geographical location. In several areas of the world the content of selenium in diet has been estimated insufficient. In these regions the use of Se-enriched fertilizers, spraying the crops or treating the seeds with selenium salts has been found an effective way to increase selenium content in plant-based foods (Lv et al. 2017). In Finland, selenium supplementation via fertilization has been in use since 1984.

After the publication of the landmark trial of Clark et al. (1996) suggesting that dietary supplementation with selenium enriched yeast decreased cancer incidence and mortality rate by nearly 50%, the interest on selenium and the importance of selenium to human health has outstandingly increased. Selenium has many impacts on human body through at least 25 selenoproteins (eg. glutathione peroxidase) covering for example antioxidant, anti-inflammatory and anti-viral effects, fertility and reproduction, type 2 diabetes, cardiovascular disease, cognitive decline and thyroid disease (Rayman 2012).

Some selenium compounds, especially the low molecular weight species are considered to have antioxidant and cancer-protective characteristics. It has been stated that especially the methylated selenoamino acids such as Se-methyl-seleno-L-cysteine (MeSeCys) and γ-glutamyl-Se-methyl-Selenocysteine (γ-G-Se-MeSeCys) are more efficient in cancer prevention because they are not metabolized the same pathways as selenium when it is incorporated in selenoproteins. (Ip et al. 2000; Gammelgaard et al. 2008). Instead, they can be metabolized to methylselenol (MeSeH) when enzymes like β-lyase are present. MeSeH is considered to be the key intermediate and the most active species in cancer reduction. Several studies have shown cancer protective effects in selenium-accumulating plants such as *Allium* family, including garlic. These have been attributed to the presence of the methylated selenoamino acids (Gammelgaard et al. 2008). In natural garlic the predominant forms are SeMet, MeSeCys and γ-G-Se-MeSeCys (Kotrebai 2000). Brazil nuts are considered as the richest food source of selenium and the tree (*Bertholletia excelsa)* is regarded as selenium accumulator. The study by Kannamkumarath et al. (2002) showed that

the major selenium species in Brazil nuts is SeMet and 88% of total selenium is firmly bound to proteins, unlike the other accumulator plants.

Several applications have been developed for selenium determination. The most acknowledged and applied analytical approach is liquid chromatography (LC) in combination with mass spectrometer (MS). From different LC-MS methods, inductively coupled plasma mass spectrometry (ICP-MS) is considered as the most potential and realistic analytical tool for determining selenium in biological samples (Cardoso et al. 2019). However, ICP-MS has also its vulnerabilities: it can suffer from spectral interferences which are produced by atomic or molecular species that have the same mass than selenium and also other interferences caused by sample matrix (Pedrero and Madrid 2009). Whereas ICP-MS identification of analytes is based on retention time comparison with standards, ESI-MS provides mass-based confirmation of the analyte structure. The identification is possible thanks to the characteristic isotope pattern of selenium. By using tandem MS, detailed information of fragment losses can be obtained (Gammelgaard et al. 2008). The use of ESI-MS has also its problems as samples have to be precisely purified before identification. The purified sample should only contain the unknown species because all compounds are detected by MS. Thus, different chromatographic purification methods and preconcentration of samples is often needed. Liquid chromatography is usually applied prior to MS methods in order to separate nonvolatile selenium species. Different modes of chromatography have been used, e.g. size-exclusion, ion-exchange and reversed-phase ion-pair. Ultrahigh performance liquid chromatography can also be used as it can provide separations at high pressure using particle diameters of 1,7 µm, which increases the efficiency, speed of the separation and the resolution (Cardoso et al. 2019).

This study aimed to develop a simple and rapid mass spectrometric method which could be used to detect and specify low molecular weight selenoamino acids from different food materials in order to find and quantify probable cancer protective species. In the first part of the thesis, the scientific literature on different methods used in selenium speciation is reviewed together with the overall characteristics of selenium and its importance in human health. In the second part of the thesis, the materials, derivatization, UHPLC-ESI-MS and HPLC-ICP-MS methods for determining selenoamino acids are explained. In the final sections, the results from the experiments and evaluation of the methods are presented and discussed.

2 LITERATURE REVIEW

2.1 Introduction to selenium

2.1.1 Chemistry of selenium

The element selenium was first found over 200 years ago by the Swedish scientist Jöns Jacob Berzelius in Uppsala 1817 (Reilly 2006). Berzelius found the unknown substance with properties very much like those of tellurium. He named selenium after *selene*, which signifies the Greek Goddess of the moon, while *tellus* is the name of our planet. It is one of the rarest elements in the world, placing around 70th in abundance among the 88 naturally occurring elements. Selenium concentration in the earth's crust is about 0.05 ppm which is similar to silver (Ag) and mercury (Hg), being about 0.08 ppm each (Greenwood and Earnshaw 1998).

Selenium has an atomic number 34 and weight of 78.96. It is located between arsenic and bromine in period 4, and along with oxygen, sulfur, tellurium and polonium in Group 16 of the Periodic Table of the elements. This location makes it prone to the biological interactions with sulfur and arsenic (Reilly 2006).

Selenium occurs naturally in six stable isotopes ^{74}Se, ^{76}Se, ^{77}Se, ^{78}Se, ^{80}Se and ^{82}Se, of which ^{80}Se (49.82%) and ^{78}Se (23.52%) are the most abundant (Greenwood and Earnshaw 1998). It is important to understand the isotopic pattern of selenium in order to understand the mass spectrometric capabilities to discriminate between isotopes in ICP-MS and the search for the characteristic isotopic pattern in ESI-MS. For example, Ar $^{2+}$ has the same mass than ^{80}Se which causes interference especially in ICP-MS.

Like sulfur, selenium also has allotropy, existing in an amorphous state or in any of three crystalline forms; alpha-monoclinic, beta-monoclinic, and hexagonal (Reilly 2006). The most common allotrope is hexagonal grey selenium (also called black or metallic) which is stable at ordinary temperatures (Subcommittee on Selenium et al. 1983). Selenium is rarely found in its elemental form in nature and can only be found in a few minerals, for example in sulfide ores such as pyrite, where selenium partially substitutes sulfur. Selenium can react with metals and non-metals, gaining electrons to form ionic compounds such as naturally

occurring; -2 (e.g. sodium selenide Na_2Se), +4 (e.g. sodium selenite Na_2SeO_3) and +6 (e.g sodium selenate Na_2SeO_4) (Reilly 2006).

2.1.2 Selenium in food

Selenium is an essential trace element for humans and its importance in the diet has been recognized for many decades (Pyrzynska and Sentkowska 2020). Food is the major source of selenium although the intake is highly influenced by the geographical location (Reilly 2006). Therefore, selenium concentration in the soil usually reflects its occurrence in the food. The concentration in the soil usually ranges from 0.01–2.0 mg/kg but some seleniferous soils in Ireland, India, China and the United States can contain more than 5 mg/kg (Saha et al. 2017).

Selenium enters the food chain by plant uptake from soil where it is usually in the inorganic forms (mainly selenite or selenite). Selenium is more easily available from alkaline soils than from acidic soils. Selenium occurs in foods in many different forms such as selenite Se(IV) and selenate Se(VI) and several selenoaminoacids including selenomethionine (SeMet), selenocysteine (SeCys), selenocystine ($SeCys_2$), selenohomocysteine ($SeHoCys_2$), Se-methylselenocysteine (MeSeCys) and ɣ-glutamyl-Se-methylselenocysteine (ɣ-Glu-MeSeCys) (Rayman 2012). The presence of these forms depends on the food in question and the overall selenium content, and also on the amount of selenium used for the enrichment of plants.

In several areas of the world the content of selenium in diet has been estimated insufficient (middle and northern Europe, part of China and New Zealand). In the regions with low selenium content the use of Se-enriched fertilizers, spraying the crops or treating the seeds with selenium salts has been found an effective way to increase selenium content in plant-based foods (Lv et al. 2017). In Finland, selenium supplementation via fertilization (in the form of sodium selenite) has been in use already since 1984 as it was shown by Mutanen and Koivistoinen (1983) that dietary selenium intakes were among the lowest in the world (25 to 60 µg/day).

Selenium is a vital trace element for humans but with too high values it can be toxic. The limit between the adequate amount and the toxic level is narrow. The recommended levels

differ depending on the country/organization giving the recommendations and, on the age, sex or group of the individual. Table 1 combines the data from the United States, European Food Safety authority and The National Nutrition Council of Finland. In the USA, The Recommended Dietary Allowance (RDA) for adult is 55 µg/day (Institute of Medicine (US) Panel on Dietary Antioxidants and Related Compounds 2000). The Dietary Reference Values for selenium in Europe were discussed in 2014 and evidence from human studies on the relationship between selenium intake and plasma selenoprotein P concentration was reviewed. The Adequate Intake (AI) was set a bit higher, 70 µg/day for adults (EFSA Panel on Dietetic Products, Nutrition and Allergies,(NDA) 2014). In Finland, The National Nutrition Council has published nutrition recommendations also in 2014 advising the level to be 50 µg/day for women and 60 µg/day for men (Fogelholm et al. 2014). For children, the levels change according to age, being quite similar in all recommendations. For pregnant and lactating women limits are a bit higher than for adults. The Tolerable Upper Intake Levels (UL) are 400 µg/day in USA and 300 µg/day in Finland and EFSA.

Table 1 Reference values for selenium intake (Institute of Medicine (US) Panel on Dietary Antioxidants and Related Compounds 2000; Fogelholm et al. 2014; EFSA Panel on Dietetic Products, Nutrition and Allergies,(NDA) 2014).

EFSA		USA		FINLAND	
Age/group	Selenium µg/day	Age/group	Selenium µg/day	Age/group	Selenium µg/day
7-11 months	15	*0-6 months	15	6-11 months	15
1-3	15	*7-11 months	20	12-23 months	20
4-6	20	1-3	20	2-5	25
7-10	35	4-8	30	6-9	30
11-14	55	9-13	40	10-13	40
≥15	70	≥14	55	≥14	50 W/60 M
Pregnancy	70	Pregnancy	60	Pregnancy	60
Lactation	85	Lactation	70	Lactation	60

*For infants from birth to 12 months, an AI was established for selenium that is equivalent to the mean intake of selenium in healthy, breastfed infants

Selenium enters the food chain through plants and can be found in different concentrations for human consumption depending on the area. Selenium content of foods varies normally as follows: organ meats and seafood, 0.4 to 1.5 µg/g; muscle meats, 0.1 to 0.4 µg/g; most agricultural crops <1 µg/g dry weight; dairy products < 0.1 to 0.3 µg/g; fruits and vegetables <0.1 µg/g (Rayman 2008). Variation can be a lot higher when considering the selenium accumulator plants.

Plants differ greatly in their ability to accumulate selenium from soils and therefore could be divided into three major groups: (1) hyperaccumulators are plants that are able to accumulate high levels of selenium, exceeding the threshold of 1000 mg Se/kg; (2) secondary accumulators can accumulate from 100–1000 mg Se/kg and (3) non-accumulators do not exceed 100 mg Se/kg (Lima et al. 2018)

Members of the *Allium* family, including garlic (*A. Sativum*), can accumulate high amounts of selenium, especially if grown in seleniferous soils. Garlic is considered as hyperaccumulator as it can accumulate more than 1000 µg Se/g. It is also a widely consumed food product which makes it a significant source of selenium. The overall selenium concentration in garlic has been found to be around 0.02 to 0.25 µg/g (dry sample) grown on normal soil, 68 µg/g to 1355 µg/g (dry sample) in enriched soil (Cai et al. 1995; Block 1998; Reilly 2006).These plants contain only small amounts of protein-bound selenium and mainly monomethylated species of selenium (Block 1998; Kotrebai, Birringer et al. 2000a). Main species of selenium which were found in natural garlic using HPLC-ICP-MS by Kotrebai et al. (2000a) were ɣ-glutamyl-Se-methyl-selenocysteine (31%), SeMet (53%), Se-methyl selenocysteine (12%) and selenite (4%).

Brazil nut is not as widely consumed as garlic, but it is considered as one of the richest sources of natural dietary selenium (Dumont et al. 2006a). Significantly high levels can be found as the proteins of these nuts are rich in sulfur amino acids. One single Brazil nut could exceed the RDA or AI for selenium. However, Brazil nuts originated from different areas of Brazil and even the individual nuts from the same batch can show great variation between their selenium content (Vonderheide et al. 2002). Their research also revealed that there is a great difference of the selenium content between nuts analyzed with and without shells, 3510 µg/100 g compared to 830 µg/100 g without shells. Large variations (30 - 51200 µg/100 g) among selenium content of different Brazil nuts have also been reported by other researchers (Table 2). Several reasons have been suggested to explain the variations in selenium content among Brazil nuts. These include difference in soil types, efficiency of selenium taken up by the roots system from soil and the availability of selenium as determined by soil type, moisture content, and other factors (Reilly 1999).

Rayman et al. (2008) concluded in their review article that the major Se species found in Brazil nuts and other nuts is selenomethionine. Kannamkumarath et al (2002) studied the

total selenium distribution in Brazil nuts and revealed that 88% of total selenium is firmly bound to proteins which means that the amount of free selenoamino acids is quite small.

Table 2 Selenium content in Brazil nuts.

Brazil nut type	Selenium content µg/g	Reference
Whole Brazil nuts (low selenium levels in soil)	0.30–0.317	(Chang et al. 1995)
Whole Brazil nuts (high selenium levels in soil)	1.25–5.12	(Chang et al. 1995)
Whole Brazil nuts	35.0–49.9	(Vonderheide et al. 2002)
Shelled Brazil nuts	2.54–8.30	(Vonderheide et al. 2002)
Whole Brazil nuts	49.9 ± 6.4 %	(Dumont et al. 2006a)
Shelled Brazil nuts	5.1 ± 10.6 %	(Dumont et al. 2006a)
Whole Brazil nuts	38.0 ± 0.15	(Manjusha et al. 2007)
Whole Brazil nuts	3.0 ± 0.17	(Moreda-Piñeiro et al. 2016)
Whole Brazil nuts	0.76 ± 0.67	(Tošić et al. 2015)
Whole Brazil nuts (high selenium levels in soil)	19.8–21.0	(Moreda-Piñeiro et al. 2018)

2.1.3 Health effects, deficiency and toxicity of selenium

Following the publication of the landmark trial of Clark et al. (1996) that appeared to show that dietary supplementation with selenium enriched yeast decreased cancer incidence and mortality rate by nearly 50%, the importance of selenium to human health has outstandingly increased. Selenium has many impacts on human body through at least 25 selenoproteins (eg. glutathione peroxidase) covering for example antioxidant, anti-inflammatory and anti-viral effects, fertility and reproduction, type 2 diabetes, cardiovascular disease, cognitive decline and thyroid disease (Rayman 2012).

The cancer-protective effect of the element is highly dependent on the chemical form of the ingested selenium as different selenium compounds are metabolized via different pathways in the organism (Ip et al. 2000). In vitro experiments have indicated that methylated selenium compounds are more efficient in cancer protection as they are able to provide a constant production of methyl selenol, CH_3SeH (MeSeH), which has been demonstrated to be one of the most active species for cancer prevention in humans (Lü et al. 2016).) They do not enter the normal metabolism pathways where selenium is incorporated in selenoproteins. Instead, they are transformed to the active MeSeH. Low molecular weight selenoamino acids such as Se-methylselenocysteine (MeSeCys) and γ-glutamyl-Se-methylselenocysteine are

precursors of methyl selenol, and therefore attributed to the cancer preventive effects. There is a clear difference between the metabolism of the selenoamino acids MeSeCys and SeMet. MeSeCys is not usually incorporated into selenoproteins and is likely to be readily available for β-lyase cleavage to MeSeH, whereas SeMet is normally incorporated into selenoproteins and the alternative γ-lyase pathway to MeSeH may be only a minor pathway (Ip et al. 2000).

Selenium has contradictory behavior from being both essential and toxic, depending on the species, concentration and oxidation state. Selenium deficiency occurs normally in areas with low selenium content in the soil. The most well-known and serious diseases that selenium deficiency can cause are two endemic diseases: Keshan disease (an endemic cardiomyopathy), and Kashin-Beck disease (a deforming arthritis). Selenium toxicity, on the other hand, can cause acute or chronic symptoms. Acute symptoms include e.g. respiratory, gastrointestinal and cardiovascular effects. Chronic symptoms include e.g. discoloration of the skin, hair loss, deformation of nails and excessive tooth decay (Pedrero and Madrid 2009).

2.2 Analytical methods in selenium speciation

Several applications have been developed for selenium determination. The most acknowledged and applied analytical approach is liquid chromatography (LC) in combination with mass spectrometry (MS). From different LC-MS methods, high performance liquid chromatography inductively coupled plasma mass spectrometry (HPLC-ICP-MS) is considered as the predominant and the most realistic analytical tool for determining selenium in biological samples due to its high sensitivity, ability to discriminate between isotopes and simple sample preparation (Cardoso et al. 2019). However, ICP-MS method provides only elemental information of the analytes as it does not preserve the molecular bonds, and therefore molecule specific techniques, ESI-MS or MALDI-MS are necessary in identifying unknown selenium species by mass-based structure confirmation. The best option for selenium speciation would be to combine molecular ESI-MS in parallel to atomic ICP-MS analysis (Gammelgaard et al. 2011).

2.2.1 Inductively coupled plasma mass spectrometry (ICP-MS)

Inductively coupled plasma mass spectrometer (ICP-MS) coupled with HPLC is probably the most popular analytical technique in selenium speciation. It is a widely applied method due to its multi-elemental capabilities, low detection limits and the possibility of measuring isotope ratios. It allows determination of the element at the sub part per billion levels. When it is hyphenated with HPLC the detection limits are generally from 0.03-1µg Se/l (Gammelgaard et al. 2008; Hildebrand et al. 2020).

The sample for ICP-MS is introduced into the plasma as an aerosol, usually as a liquid sprayed through a nebulizer. The sample is atomized and ionized under high temperature (6 000–10 000 K) in the plasma creating positively charged atomic ions. While the larger aerosol droplets are removed from the gas stream by a spray chamber, the remaining smaller droplets are dried, decomposed and dissociated into individual atoms in the central channel with argon plasma. These atoms are converted to positively charged ions before they are extracted into the vacuum system for the detection. Detection creates the ICP-MS spectrum which represents the elemental composition of the sample. The most important features of ICP-MS, compared to other elemental analysis techniques, are better sensitivity, selectivity, and simultaneous multi-element capability (Cajka et al. 2008). However, ICP-MS is not able to directly measure hydrogen, helium, neon, argon, and fluorine (Todolí and Mermet 2006). Mass analyzers applied for the ICP separation include quadrupole, TOF and sector-field instruments of which quadrupole has been employed the most (Gammelgaard et al. 2008). The identification of selenocompounds in ICP-MS is based on coelution with the standards, but the commercially available standards are limited. Isotope dilution analysis (isotope enrichment) can be used for quantification of unknown structures (Bamonti et al. 2016).

ICP-MS has also its vulnerabilities: It can suffer from spectral interferences which are produced by atomic or molecular species that have the same molecular mass than selenium and also other interferences caused by sample matrix (Ponce De León et al. 2002). The most significant isotopic interference is $^{40}Ar_2^+$ which overlaps with the most abundant Se isotope ^{80}Se. This overlapping can be overcome by using high resolution mass spectrometers or dynamic collision/reaction cell. Oxygen, used as a reaction gas, seems to react with argon dimer working also as an interference neutralization (Połatajko et al. 2006). Selenium has relatively poor sensitivity in ICP compared to other trace elements, however it can be

overcome by addition of carbon-containing solutes (e.g. 2-10% of methanol) to mobile phase. On the other hand, too high amounts of organic solvents will decrease the sensitivity. The amount of organic solvent is dependent on the nebulizer and interface used (Gammelgaard et al. 2008) . ICP-MS cannot be used for molecular identification and therefore, due to the lack of commercial standards, characterisation of selenium-containing fractions produced by LC-ICP-MS requires ESI-MS techniques to identify unknown peaks (Cardoso et al. 2019).

2.2.2 Electrospray ionization mass spectrometry (ESI-MS)

Even though ICP-MS is a highly appreciated element specific detector in selenium speciation it is not able to provide structural information of the species. Therefore, molecule specific techniques, ESI-MS or matrix assisted laser desorption ionization (MALDI-MS) are necessary in identifying unknown selenium species.

ESI-MS is widely used and suitable method for polar and ionic compounds. It is based on the extraction of ions from solution. ESI-MS consists of three main parts: an ion source, a mass analyzer, and a detector. The sample molecules are introduced into the ion source for the ionization process. These ions are then extracted into the analyzer where they are separated according to their m/z. ESI-MS provides usually protonated molecular ions $[M+H]^+$ in the positive mode or deprotonated molecular ions $[M-H]^-$ in the negative mode. In ESI, the sample is first dissolved in a polar solvent and introduced though a narrow stainless-steel nebulizing needle. The polar solvent is coming either from a syringe pump or as an effluent flow from a liquid chromatography with the typical flow rates of 1 ml/min to 1 mL/min (Cajka et al. 2008). A strong electric field is resulted from the use of high voltage (2.5-4 kV) in the tip of the capillary which causes the sample emerging from the tip to disperse into an aerosol consisting of highly charged droplets. Nitrogen is usually used as a nebulising gas around the capillary which leads the aerosol spray towards the mass analyzer. The charged droplets reduce their size by the assistance of a warm nitrogen gas passing across the front part of the ion source. When the surface tension of the droplets cannot stand the charge too high, the droplets are disintegrated. This process continues until analyte ions evaporate from the droplet. The charged analyte ions then pass through a sampling cone into a vacuum region. From the vacuum region ions go through a small gap into the mass analyzer.

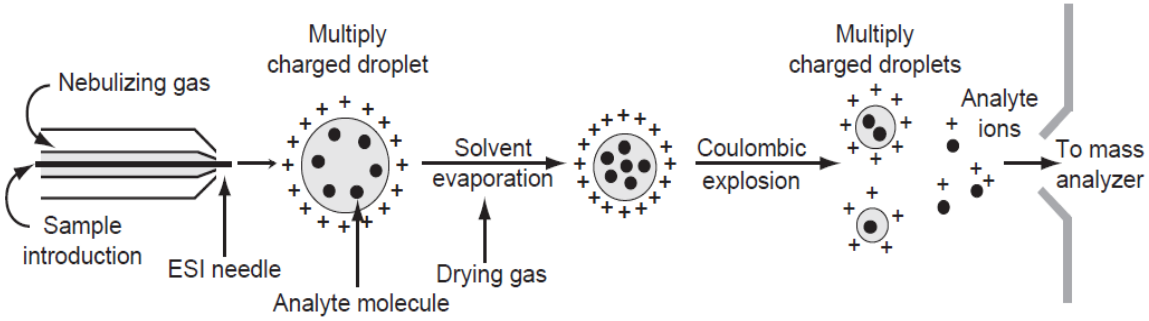


Figure 1 Ion formation in ESI (Cajka et al. 2008).

After the molecules are converted to charged particles, they undergo mass analysis in a mass analyzer. The most frequently used mass analyzers are ion-trap instruments (eg. Quadrupole) for tandem fragmentation (MS^n) or time-of-flight (TOF) instruments for accurate mass determination. There are also combinations of quadrupoles and and TOF instruments, QTOF, providing accurate mass together with structural information. Hybrid ion trap–TOF instruments can offer tandem MS^n and accurate mass analysis of parent and daughter ions (Gammelgaard et al. 2011). In addition, new opportunities have emerged through the method called Orbitrap, which is an electrostatic ion-trap mass analyzer using fast Fourier transformation to obtain mass spectra with high resolution (Perry et al. 2008).

The quadrupole ion trap (QIT) is comprised of three cylindrically symmetric electrodes, two end-caps and a ring. It is able to allow both full scan and SIM acquisition, while better selectivity can be obtained in MS^n mode, which is usually MS/MS (Cajka et al. 2008). The use of MS/MS considerably reduces the chemical noise, originating from different source s (e.g. matrix compounds, co-eluting analytes, column bleed, and contamination from an ion source). Although each MS step causes a loss in the analyte signal intensity, the noise decreases even more rapidly, resulting in better signal to noise ratio. Time-of-flight (TOF) mass analyzer consists of a pulsing electrode, a flight tube and a reflectron. The method is based on the measurement of flight time of the ions. The times of arrival at the detector are proportional to the square root of respective m/z values. The efficiency of TOF-MS is significantly higher compared to quadrupole instruments which enables availability of full spectra information even at ultra-trace levels of a specific compound and easier identification.

Selenocompounds cannot be directly analyzed by ESI-MS because the matrix contains high molecular weight compounds and salts which suppress the signal. Identification is usually preceded by purification of the samples by different chromatographic methods (e.g size exclusion, ion-exhange). Casiot and others (1999) isolated the most abundant selenocompound in yeast extract and identified it by ESI tandem quadrupole MS/MS based on the collision induced dissociation pattern of the protonated molecular ions corresponding to the closest selenium isotopes. Identity of Se-adenosylhomocysteine could be confirmed by comparison of the fragment spectra with the S-containing analogues. The same compound was identified in a study by McSheehy et al. (2001) by SEC-RP-chromatography and ESI-MS. Selenomethionine and Se-adenosylhomocysteine could be identified from an enzymatic yeast extract by Kotrebai et al. (1999). In the same study, γ-glutamyl-Se-methylselenocysteine and possibly γ-glutamyl-Se-methylselenomethionine were also detected from garlic extracts by online HPLC-ESI-MS based on retention time comparison with synthesized standards. In their following study (2000a) ion-pairing reagents (TFA, HFBA) were used in RP-HPLC to optimize the separation of standards accompanied by parallel use of ICP-MS and ESI-MS. Nine selenocompounds could be found from 23 standards in the different samples investigated. The same team of Kotrebai et al. (2000b) also reported detection of more than 20 different selenium compounds using different ion-pairing agents (TFA, PFPA and HFBA) with HPLC separation with the detection of both ESI- and ICP-MS. McSheehy and others (2000) used preparative SEC, RP-HPLC and ICP-MS in selenium analysis. They obtained one single peak which was further analyzed by ESI-MS and MS/MS. No reference material was available but the peak was identified as γ-glutamyl-Se-methylselenocysteine with the help of the findings of Kotrebai et al. (1999).

Although a great effort and advances have been done in the area, the information is mostly based on selenized food or high selenium food supplements, where the total selenium concentration is high enough for ESI-MS. In the early 2000's, the sensitivity of ESI-MS for most selenium compounds was often 2 orders of magnitude lower than for ICP-MS and the identification of low molecular selenium species with low concentration levels in complex matrices was demanding (Pedrero and Madrid 2009). Recent developments in sensitivity have offered new techniques and modern ESI time-of-flight (TOF), Orbitrap and Fourier transform ion cyclotron resonance (FT-ICR-MS) instruments are capable of 1 pmol/ml detection limits, high resolution and high mass accuracy (Cardoso et al. 2019).

The difficulties in molecular MS analysis arise from poor ionization of sample analytes which leads to higher detection limits along with ion suppression from sample matrix. The extracts need to be extremely well purified which means multidimensional chromatographic systems. Matrix assisted laser desorption ionization (MALDI) is less vulnerable to matrix effects than ESI and produces mostly single charged ions which makes it a good option for structural studies (Rogers et al. 2010). One of the newest instrumental developments are dual-source mass spectrometers which combine an ICP and ESI source with TOF-MS system as the LC detector for simultaneous obtainment of isotopic and quantitative together with structural information.

2.3 Hyphenated separation methods

2.3.1 Liquid chromatography (LC)

Liquid chromatographic methods are the most used separation techniques in selenium speciation. LC can be operated in different modes depending on the molecular size, charge of the compound or ionization (Pyrzynska and Sentkowska 2019). LC methods can be applied for the compounds which are non-volatile and thermally labile. There are huge variability of different stationary and mobile phases which can be used, depending on the analyte. Chromatographic resolution of high-performance liquid chromatography (HPLC) can be further improved by using ultra-high-performance liquid chromatography (UHPLC).

2.3.1.1 Size-exclusion chromatography (SEC)

Size-exclusion chromatography is mainly used for separation of high-molecular mass compounds such as proteins from selenoamino acids and it is based on separation of different molecule sizes of the analytes (Tie et al. 2014). It is usually employed for biomolecules with a molecular weight between 10 and 1000 kDa. In SEC the analyte is dissolved in a solvent which is injected into a column packed with porous network. The analyte molecules larger than the pores in the stationary phase are excluded and therefore eluted first. Smaller molecules interact more with the stationary phase and they elute later based on their molecule sizes. This technique favors high-molecular compounds, such as proteins and peptides, because low-molecular weight species (especially ions with a high charge-to-mass ratio) can

be affected by adsorption or ionic exchange process as a secondary separation mechanism (Montes-Bayón et al. 2003; Połatajko et al. 2006; Pedrero and Madrid 2009).

SEC is often used as a purification step followed by another liquid chromatography in multidimensional schemes. Other LC techniques are often needed because relatively small number of theoretical plates in SEC can generate poor resolution and therefore each separated fraction may still contain a lot of different compounds (Pedrero and Madrid 2009). As a first purification step, the selenocompounds are first fractionated by SEC and the collected fractions are further processed by other type of liquid chromatography such as ion exchange or reverse phase (Montes-Bayón et al. 2003).

The mobile phases in SEC are usually buffered solutions, such as Tris-HCl buffer, which enables lowest possible competition with the stationary phase (Pyrzynska and Sentkowska 2019). These recommended solutions are usually directly compatible with the biomolecule in focus and do not hinder the further detection for example by ICP-MS. However, some high saline content mobile phases (e.g. NaCl) can cause clogging of the cones in the ICP–MS. One of the benefits that can be achieved using SEC with ICP-MS is the correlation between retention time and molecular mass, which enables the detection of the metal bound to macromolecular ligands present in the sample (Montes-Bayón et al. 2003). SEC can also be calibrated by molecular weight standards which permits an approximate estimation of molecular size. Selenoprotein detection, however, can be limited mostly because of the large dilution factor which reduces detection sensitivity (Połatajko et al. 2006).

In the study by McSheehy and others (2000) two-dimensional liquid chromatography method with ICP-MS was used in garlic speciation. They used SEC as a preparative chromatography with 1% (v/v) acetic acid eluent at pH 2.97 and offline ICP-MS detection. Fractions were collected from SEC, lyophilized, dissolved in water and further analyzed in a reversed phase column. Casiot and others (1999) stated in their report that SEC was beneficial method in the speciation of selenium compounds extracted from the yeast. Kannamkumarath and others (2002) used SEC-ICP-MS in the absence of hydrolyzing agents to study selenium distribution in Brazil nuts. They concluded that approximately 12% of total selenium was weakly bound to proteins and the rest was firmly bound to proteins.

More recently, Preud'homme et al. (2012) used a two-dimensional chromatographic separation, SEC-RP-UPLC, to improve chromatographic resolution and online ESI-linear

ion trap (LTQ)-Orbitrap MS to qualitatively identify selenium species in yeast. A smaller 4.6 mm SEC column (10 mm is commonly used) was employed to reduce the volume of diluted fractions by 20-fold, thereby eliminating concentration steps prior to the second-dimension chromatography. 8 unknown selenocompounds were identified based on the exact molecular mass and together with previously reported, resulting in a total of 49 metabolites characterised in yeast samples. Additionally, Gilbert-López et al. (2017) spotted 103 selenocompounds in yeast using an ultrasound assisted water extraction followed by SEC-ICP-MS prior to RP-LC-TOF-MS.

2.3.1.2 Ion-exchange chromatography (IEC)

Ion exchange is based on the interactions of anion or cation analytes with the positively or negatively charged functional groups of the stationary phase. The relative affinities of the ions rule the retention time in the stationary phase. When positively charged analytes react with negatively charged functional groups of the stationary phase in the column, it is regarded as cation-exchange, and when negatively charged analytes interact with positively charged groups, it is considered as anion-exchange. Both systems are influenced by the pH of the mobile phases because it affects the separation of weakly acidic or basic compounds. Anion and cation exchange have both been successfully used in selenium speciation (Montes-Bayón et al. 2003; Połatajko et al. 2006) however, cation exchange separation has been used more as an alternative system for confirmation the results from anion exchange HPLC (Pyrzynska and Sentkowska 2019).

The mobile phases usually consist of an aqueous salt buffer solution, often mixed with organic modifier, such as such as methanol or acetonitrile. Gradients are used in IEC to increase ionic strength. Gradients are beneficial in IEC because they enable separation of complex mixtures but when used in HPLC-ICP-MS they are also time consuming as the column needs to equilibrate with the new mobile phase after the chromatographic run. The changes in the mobile phase during the run can also initiate changes in the background or sensitivity. Furthermore, high saline concentrations of the mobile phase can cause clogging of the cones in the ICP–MS, similar to SEC (Montes-Bayón et al. 2003).

Larsen et al. (2001) applied anion and cation-exchange chromatography to the study of selenium enriched yeast and they found out that a cation-exchange column with a pyridinium formate elution gradient was capable to separate 8 selenoamino acids in 30 minutes. IEC has

also been employed for multidimensional chromatography (Casiot et al. 1999; Kotrebai et al. 2000a), where SEC is used to fractionate yeast extracts into 3-6 selenium containing fractions which are further fractionated by anion-exchange chromatography according to the charge or polarity. Cationic and uncharged compounds must be further purified by cation-exchange chromatography. The obtained fractions from 2D and 3D can be analyzed by quadrupole ESI-MS or TOF mass analyzer.

2.3.1.3 Reversed-phase chromatography (RP)

Reversed-phase chromatography is widely used in analysis of ionic and neutral selenium species. It consists of a non-polar stationary phase (most often C_{18} or C_8) bound to a solid support which is usually microparticulate non-polar silica gel (Pyrzynska and Sentkowska 2019). Mobile phases used in reversed phase are polar and the analytes are divided between stationary and mobile phases. Polarity of the mobile phase is often reduced during elution to increase separation of different species by using gradient where an organic solvent, such as isopropanol, methanol or acetonitrile is added. The pH of the mobile phase also affects solute retention which influences the separation level of the analyte and its division between the stationary and mobile phases (Montes-Bayón et al. 2003; Połatajko et al. 2006).

RP is often used for separation of species prior to ICP-MS because the technique is simple, and the stationary phases are well-known and widely used. However, there are few limitations with RP-HPLC–ICP–MS. The main problem is that most organic solvent are not fully compatible with ICP–MS and they can cause additional spectral interferences from hydrocarbons leading to increase of background and therefore can hinder the limit of detection. There can also be problems with the gradient elution which can produce a severe matrix induced change of sensitivity in ICP-MS causing complications in quantification (Wind et al. 2002). Only low percentages of MeOH or EtOH can be run in ICP without severe deterioration of the sensitivity using long-established introduction devices, such as concentric nebulizer (Montes-Bayón et al. 2003).

Replacing the concentric nebulizer by micro and nano-nebulizer has allowed the use of other organic solvents, for instance acetonitrile in conjunction with microbore columns (Acon et al. 2001; Wind et al. 2002; Połatajko et al. 2006). As an alternative, microconcentric nebulizers in association with desolvating systems have also been used to reduce signal changes.

2.3.1.4 Reversed-phase ion-pairing chromatography (RPIP)

RPIP is used for separation of ionic or ionizable compounds in conventional reversed phase system with the difference of a counter ion addition of to the mobile phase. An ion-pair is formed between the solute ion and an appropriate ion of the opposite charge (the counter ion). The method is effective because it enables simultaneous separation of anionic, cationic and neutral molecules (Połatajko et al. 2006). Therefore, it is a good method for selenoamino acids because they are amphoteric, meaning that they can act as either acid or a base. The non-ionic form occurs only in slight amounts in aqueous solutions and the zwitterion dominates at the isoelectric point. The pH of the mobile phase therefore concludes the charge and consequently the retentive performance. At low pH values the most abundant form of amino acids is the protonated form (Vonderheide et al. 2002).

Mobile phases in RPIP are similar to ones used in RP (water-methanol, water acetonitrile) with the supplementary addition of an ion-pairing reagent. Ion-pairing reagent is a compound with a polar head and a non-polar tail. RPIP enables the analysis of charged and uncharged compounds in a single chromatographic run with great reproducibility and short analysis time. Perfluorinated ion-pairing reagents, such as trifluoracetic acid (TFA), heptafluorobutanoic acid (HFBA) and pentafluoropropanoic acid (PFPA) have been widely used and proved effective to enhance resolution and separate many organoselenium compounds (Kotrebai et al. 2000b; Połatajko et al. 2006).

Bird et al. (1997) studied different chromatographic systems and they found out that reversed phase column and ion-pairing agent produced the best separation of the selenocompounds in yeast. The optimal mobile phase composition in their work was 98:2 water-methanol with 0.1% TFA. Kotrebai et al. (2000a) used 0.1% HFBA or 0.1% TFA as an ion-pairing agent in 1+99 v/v methanol-water mobile phase among reversed-phase column with a polar modifier between the C_8 and silica base. This method made over 70 selenium species to be available for separation under isocratic conditions. The efficiency, resolution and speed of separation was increased in the study by Bendahl et al. (2005) where four aqueous selenium standards were separated with reversed phase column in an ion-pair chromatographic system using UHPLC instead of HPLC.

2.3.2 Gas chromatography (GC)

Gas chromatography is mostly utilized in detection of volatile selenocompounds by using different detectors, such as inductively coupled plasma atomic emission spectrometry, –mass spectrometry (ICP-AES, ICP-MS) and microwave induced plasma atomic emission spectrometry (MIP-AES). It has also been employed for the detection of non-volatile selenium species after derivatization, however the method is time-consuming and may be unsuccessful for oligopeptides. For these reasons, liquid chromatography is considered as a better separation method for non-volatile selenoamino acids and selenopeptides (Pedrero and Madrid 2009).

2.3.3 Electrophoretic techniques

Electrophoretic techniques (capillary and gel electrophoresis) are based on differences in the electrophoretic mobilities of ions which are highly influenced by its mass-to-charge ratio, physical dimensions and interactions with buffer components (Pedrero and Madrid 2009).
Gel electrophoresis has been mostly applied to selenoprotein separation due to its better resolution than HPLC. Capillary electrophoresis (CE) has been frequently used for selenoamino acid speciation and it provides accomplished features for separation and verification of the chromatographic purity of target compound. It offers high resolution, need for low amount of sample providing good detection limits and also analysis of relatively labile species. In addition, there is no stationary phase which could affect the sample integrity (Połatajko et al. 2006).

CE was first connected to ICP-MS by Olesik et al. (1995). The challenge in coupling these two methods was the modification of flow rates for both methods (nl/min and ml/min). Several approaches have been developed and a commercial interface is available which is able to optimize electrophoretic and nebulizer flows (Uden 2002). Even though CE was considered as an applicable tool for speciation analysis for a while, it has been stated that it actually has only a limited use (Gammelgaard et al. 2011).

Mounicou et al. (2002) conducted a study by using CE-ICP-MS for selenoamino acid speciation in water soluble fraction of selenized yeast. It was, however, shown to be time consuming as 30% of the selenium compounds did not elute within 30min. Bendahl et al.

(2001) managed to separate more than 20 selenocompounds from aqueous yeast extracts from nutritional supplement tablets by a capillary coated with polyvinylsuplhonate. CE-ICP-MS was also used in the study by Kannamkumarath et al. (2005) in which selenomethionine, selenite, selenate and selenocysteine were detected from proteolytic nut extracts.

2.3.4 Isotope dilution analysis (IDA)

Isotope analysis is an important tool in selenium speciation. It can be classified into species-unspecific or species-specific mode depending on when the isotope is added to the sample or the chemical form of the isotope tracer. Isotope dilution analysis also requires an accurate determination of the element isotope ratio.

In the species-unspecific spiking mode (post-column isotope dilution) the spike is added to the sample between species separation and ionization. In this mode the tracer can be in a different chemical form than the species in question. Therefore, it can be used to precisely quantify compounds having an unknown structure or compounds that do not have commercially available standards (Pedrero and Madrid 2009). In a study by Díaz Huerta et al. (2005) soluble selenium species from three mushroom varieties were determined by the species-unspecific method using the isotope ratio $^{78}Se/^{77}Se$ after hot water extraction and SEC-ICP-MS. Many unknown species of low molecular weight were detected and quantified, however, not identified.

The species-specific mode can be applied to precise quantitative elemental speciation. In this mode the isotopically labelled tracer is in a same chemical form as the species to be determined. The main advantage is that it enables correction of species transformation or degradation because the natural species will be degraded in the same extent (Huo et al. 2000). The application of species-specific double isotope dilution analysis by LC-ICP-MS was introduced by Goenaga Infante et al. (2008) to the precise quantification (ng/g level) of γ-glutamyl-selenomethylselenocysteine in garlic sample extracts. The ^{77}Se-enriched species were obtained from the corresponding LC fraction from ^{77}Se selenized yeast extracts.

3 EXPERIMENTAL RESEARCH

3.1 Aims

The aim of this study was to develop a simple and rapid mass spectrometric method which could be used to detect and specify low molecular weight selenoamino acids from different food materials in order to find and quantify probable cancer protective species.

Determination of selenoamino acids have been the interest of several studies investigating potential methods to reveal low molecular weight forms of selenium. There is evidence of these forms, but it is mostly based on retention time matching with standards (Vacchina et al. 2018). Structural evidence is still needed via fragmentation of the molecular ion with MS data. There are several hyphenated LC-MS methods studied in selenium speciation but most of them are complex, time consuming or expensive.

3.2 Materials

3.2.1 Reagents and chemicals

The reagents used in the derivatization and UHPLC-ESI-MS analysis were mainly from AccQ·Tag™ Ultra derivatization Kit. AccQ·Tag™ Ultra Eluent A concentrate, AccQ·Tag™ Ultra Eluent B, AccQ·Tag™ Ultra Borate Buffer, AccQ·Tag™ Ultra Reagent Diluent and AccQ·Tag™ Ultra Reagent Powder were obtained from Waters Corporation (Manchester, UK). 0.5M hydrochlorid acid and HPLC grade acetonitrile (CH_3CN) were supplied by Sigma Aldrich (Steinheim, Germany). The Milli-Q water used throughout the experiments was purified by the Milli-Q system (Millipore Corp., Bedford, MA, USA). Proteinase K used in the enzymatic extraction was from Promega (Madison, Wisconsin, USA). The 5 mmol/l ammonium citrate buffer (pH 5.2) for HPLC-ICP-MS analysis was obtained from the laboratory of Finnish Food Safety Authority, prepared by Kirsti Risunen.

3.2.2 Samples

Garlic (*Allium sativum)* and Brazil nut (*Bertholletia excelsa)* were selected as samples because they are known to contain relatively high amounts of natural selenium (Kannamkumarath et al. 2002; Rayman et al. 2008). They are also both quite easily found from supermarkets and easy to use in normal diet. These plants are able to accumulate selenium and transform inorganic selenium into monomethylated forms. Concentrations of selenium in the nuts and the garlic depend on how effectively the element is taken up by the roots as well as on the concentration and the chemical form of the selenium on the soil (Block 1998; Reilly 1999).

Brazil nuts (origin unknown) were obtained from a local shop "Punnitse ja säästä", where the nuts are sold unpacked. Only shelled nuts were found. Spanish garlic cloves were purchased from a local supermarket.

3.2.3 Standards

Amino acid standards

Sigma® amino acid standards (Sigma-Aldrich, St. Louis, MO, USA) were obtained from the chemical storage of the University of Helsinki. The amino acids were selected to the study to correspond the Waters AccQ·Tag™ Ultra Derivatization Kit's Amino Acid Hydrolysate Standard. These amino acids, however, were not from the original kit by Waters.

The aim was to have an amino acid standard which would have a concentration of 100 pmol/µl. The correct quantity of each amino acid was calculated and then weighed with Milliscale in an aluminium vial cap. All the weighed amino acid standards were transferred into a 200 ml volumetric flask and the weighing dish was rinsed with Milli-Q water to ensure all the material was obtained. After all the amino acids were transferred to the flask, Milli-Q water was added to dilute the mixture to 200 ml, corresponding to the concentration of 100 pmol/µl. New standards were prepared regularly to ensure the repeatability. The amino acid standard was stored in a refrigerator for further analysis.

Following amino acids (Table 3) were selected to the study corresponding to the Waters AccQ·Tag Ultra Derivatization Kit.

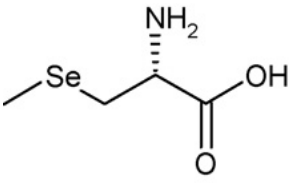
Table 3 Amino acid standards

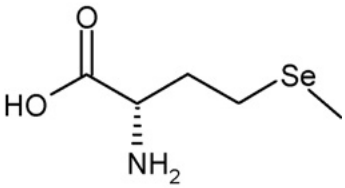
Amino acids	
L-Alanine M 89,09 g/mol	L-Lysine M 182,6 g/mol
L-Arginine M 210,7 g/mol	L-Methionine M 149,2 g/mol
L-Asparagine M 132,1 g/mol	L-Norvaline M 117,1 g/mol
L-Cysteine M 121,2 g/mol	L-Phenylalanine M 165,2 g/mol
L-Glutamine M 146,1 g/mol	L-Proline M 115,1 g/mol
Glysine M 75,07 g/mol	L-Serine M 105,1 g/mol
L-Histidine M 209,6 g/mol	L-Threonine M 119,1 g/mol
L-Isoleusine M 131,2 g/mol	L-Tyrosine M 181,2 g/mol
L-Leusine M 131.2 g/mol	L-Valine M 117,1 g/mol

Selenoamino acid standards

Commercial selenoamino acid standards (Sigma-Aldrich, St. Louis, MO, USA), Se-methyl-seleno-L-cysteine (MeSeCys M 182.08 g/mol) and seleno-DL-methionine (SeMet M 196,1 g/mol) were obtained from a previous thesis project (Liu 2011). MeSeCys and SeMet have both been found in garlic (Dumont et al. 2006b) among other selenocompounds. In Brazil nuts, SeMet is the most frequently found selenoamino acid.

Selenomethylselenocysteine (MeSeCys)





Selenomethionine (SeMet)

Standards were carefully weighed with Milliscale and separately diluted to 100 ml volumetric flasks with Milli-Q water to the concentration of 100 pmol/µl. One standard was also made containing both Se-amino acids. New standards were prepared regularly to ensure the repeatability. Selenoamino acid standards were stored in a refrigerator.

3.3 Methods

3.3.1 The outline of the methods

Different methods and steps conducted in this study are illustrated in the Figure 2. These methods are further discussed in the following chapters.

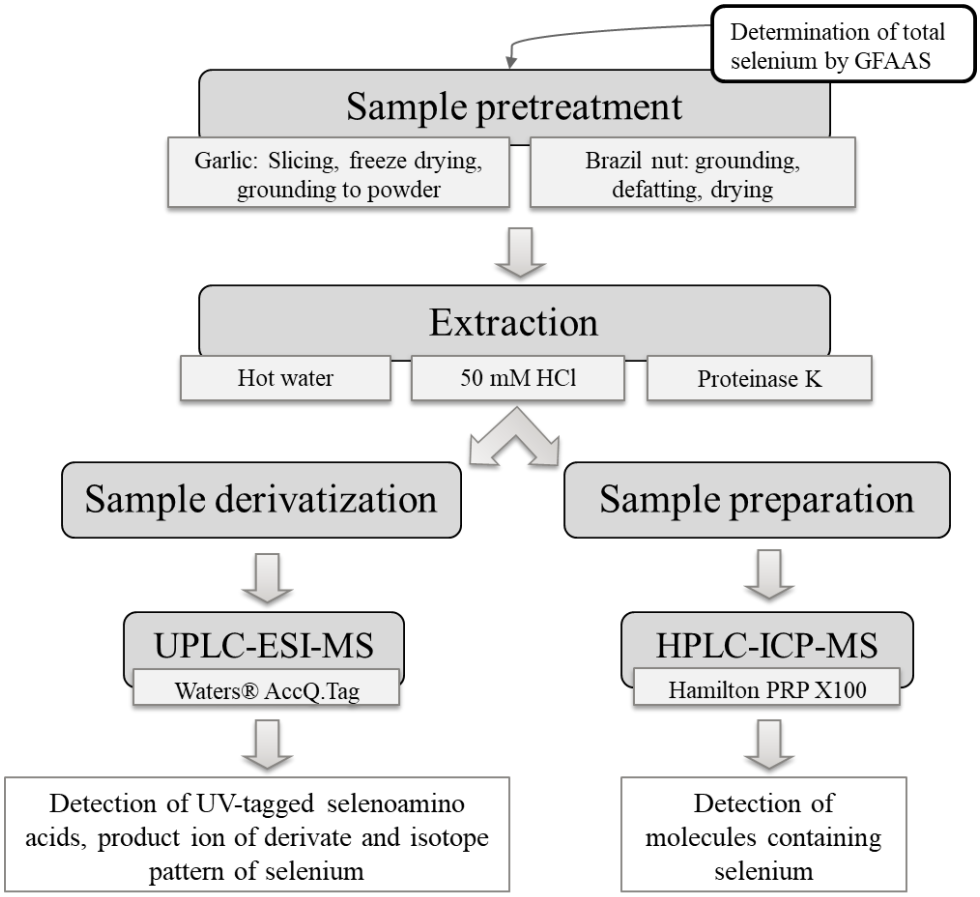


Figure 2 The outline of the methods used in the study

3.3.2 Pre-treatment of samples

Garlic

Cloves were peeled and sliced manually into ~2 mm slices. Slices were frozen in a freezer at 70℃ for 1 hour and lyophilized in a freeze dryer（Christ Alpha 2-4 LD plus, Osterode,

Germany) for 24 hours. Finally, the samples were ground to fine powder by an electric mixer (Grindomix, Retsch GmbH, Haan, Germany).

Brazil nut

Shelled Brazil nuts were crushed to paste by electric mixer (Grindomix, Retsch GmbH, Haan, Germany) and the paste was defatted with n-heptane and filtered by a vacuum filtration (x 3). Residue was dried in a fume cupboard.

3.3.3 Determination of total selenium concentration

Total selenium concentration was determined with the electrothermal atomic absorption spectrometer (AAS) (Zeeman spectrometer, model 5100, Perkin Elmer Inc., Waltham, MA, USA) connected to a graphite furnace (GF) (model HGA 600; Perkin Elmer Inc., Waltham, MA, USA) equipped with an AS-60 autosampler (Perkin Elmer Inc., Waltham, MA, USA). Graphite furnace atomic absorption spectrometry (GFAAS) is a widely used technique for the total selenium determination at trace levels due to its selectivity, simplicity and high sensitivity (Manjusha et al. 2007). Furthermore, it is relatively cheap to purchase and operate.

Samples were dried overnight at 70 °C following an acid mixture digestion (HNO_3, $HClO_4$ and H_2SO_4) and chelation-extraction by ammonium pyrrolidine dithiocarbamate (APDC) and methyl isobutyl ketone (MIBK). Inhouse reference samples (jauho 1, 2 and 3) and NIST standard reference sample 1567 (wheat flour) were included in the sample set to confirm the accuracy of the measurement.

The same procedure was later conducted to find out the efficiency of the extraction methods. Samples were prepared after extraction by weighing the supernatants and the precipitants of the three samples (H_20, HCl and proteinase K extraction).

3.3.4 Extraction

Extraction of low molecular weight selenium compounds from plant materials has mostly been done by hot water, buffer solutions or hydrochlorid acid (HCl) (McSheehy et al. 2001;

Bañuelos et al. 2011; Milovanovic et al. 2019; Ward et al. 2019; Yin et al. 2019). These extraction methods extract only Se species present in free, non-protein bounded forms. For this experiment, hot water and HCl were selected in order to find out the best option to extract free low molecular weight selenoamino acids. In her thesis, Liu(2011) performed hot water and diluted HCl extractions and suggested that the best option would be the hot water extraction. However, she encountered problems with matrix effect, so these methods were applied with better sample filtration.

Enzymatic digestion is the most frequently used extraction method for speciation analysis of selenium, which transforms Se species from protein-bound to soluble forms (Kannamkumarath et al. 2002; Vonderheide et al. 2002; Bañuelos et al. 2011; Tie et al. 2018). The efficiency of enzymatic hydrolysis is relatively high but variable (40%–90%) and depends on the choice of enzyme, pH, and time of extraction. Proteinase K enzymatic extraction was later employed in order to extract also the protein bound selenoamino acids for bigger yield.

After the pre-treatment of Brazil nuts and garlic cloves the samples went through the following three extraction methods as described below.

Hot water extraction

Powdered garlic/Brazil nut (2.5 g) was weighed into a plastic tube as triplicates and 20 ml of Milli-Q water (Millipore system, Bedford, MA, USA) was added. Samples were homogenized with Ultra-Turrax (T25, Janke & Kunkel, IKA Labortechnik, Staufen, Germany) for 2 minutes, the tubes were capped tightly, and the samples were incubated in 80 °C hot water bath (Grant GLS 400, Linear shaking thermostatic bath, Cambridgeshire, England) for 3h with agitation (140rpm). After cooling, the samples were centrifuged (Hermle Z323 centrifuge with fixed-angle rotor, Wehinghen, Germany) at 6000 rpm for 30 minutes; the supernatants were collected and bulk filtered through 0.45 µm syringe filter (GHP Acrodisc 13, Pall Corporation, Michigan, USA). The filtration residue was stored in a refrigerator for further analyzes.

Diluted hydrochloride acid (HCl) extraction

Powdered garlic/Brazil nut (2.5 g) was weighed into a plastic tube as triplicates and 20 ml of diluted HCl (50 mM) was added to the tubes. Samples were homogenized, incubated,

centrifuged and filtered the same way as in hot water extraction. The residue was also stored in a refrigerator for further analyses.

Proteinase K enzymatic extraction

Small amount of powdered garlic/Brazil nut (0.25g) was weighed into a plastic tube as duplicates (due to a limited amount of Proteinase K) and 0.025g of Proteinase K was added. Tris-HCl buffer (pH 7.33) was prepared from Trizma base and diluted HCl, and 5 ml was added to the tube. Samples were homogenized with Ultra-turrax for two minutes, the tubes were capped tightly, and the samples were incubated in 37 °C water bath for 20h with agitation (140rpm) and vortexed few times during the incubation. After cooling a while, the samples were centrifuged at 2500 rpm for 15 min to separate the particulates, and the supernatants were filtered through 0.45 µm filter. The filtration residue was stored in a refrigerator for further analyses.

3.3.5 Derivatization

Derivatization process was introduced to increase the sensitivity of the amino acid analysis and to speed up the process. Derivatization agent selected was 6-aminoquinolyl-N hydroxysuccinimidyl-carbamate (AQC), commercially known as AccQ·Tag Ultra™. It increases the hydrophobicity of the selenoamino acids, enables better retention by improving reversed-phase chromatographic properties and enhances ionisation efficiency to increase MS-detection. AccQ·Tag™ derivatization technology is easy and straightforward method as it is possible to derivatize samples in less than 10 minutes (Boogers et al. 2008). It utilizes AQC to transform primary and secondary amines into highly stable fluorescent derivatives. Therefore, this technique could be used in UHPLC with PDA detector as it enables separation with reversed phase UHPLC, and quantitation based on UV absorbance. In addition, it has also been used in MS detection and enables the detection of the combination of parent ion and the common fragment ion (m/z 171) resulting from the loss of the aminoquinoline moiety (Gray et al. 2019).

The derivatization reaction is shown in Figure 3. First the primary and secondary amino acids undergo reaction with the 6-aminoquinolyl-N-hydroxsuccinimidyl carbamate (AQC) less than a second. The second step is slower where the excess AQC reacts with water in around 15 seconds and forms the by-products 6-aminoquinoline (AMQ), N-hydroxy

succinimide (NHS) and carbon dioxide. The last step is a reaction between the major by-product AMQ and the excess of AQC which forms the highly stable urea. AMQ is the major product that gives a significant peak in the chromatogram. Bis-aminoquinoline urea creates the derivatization peak which is the smaller peak in the chromatogram. The derivatives are stable for days.

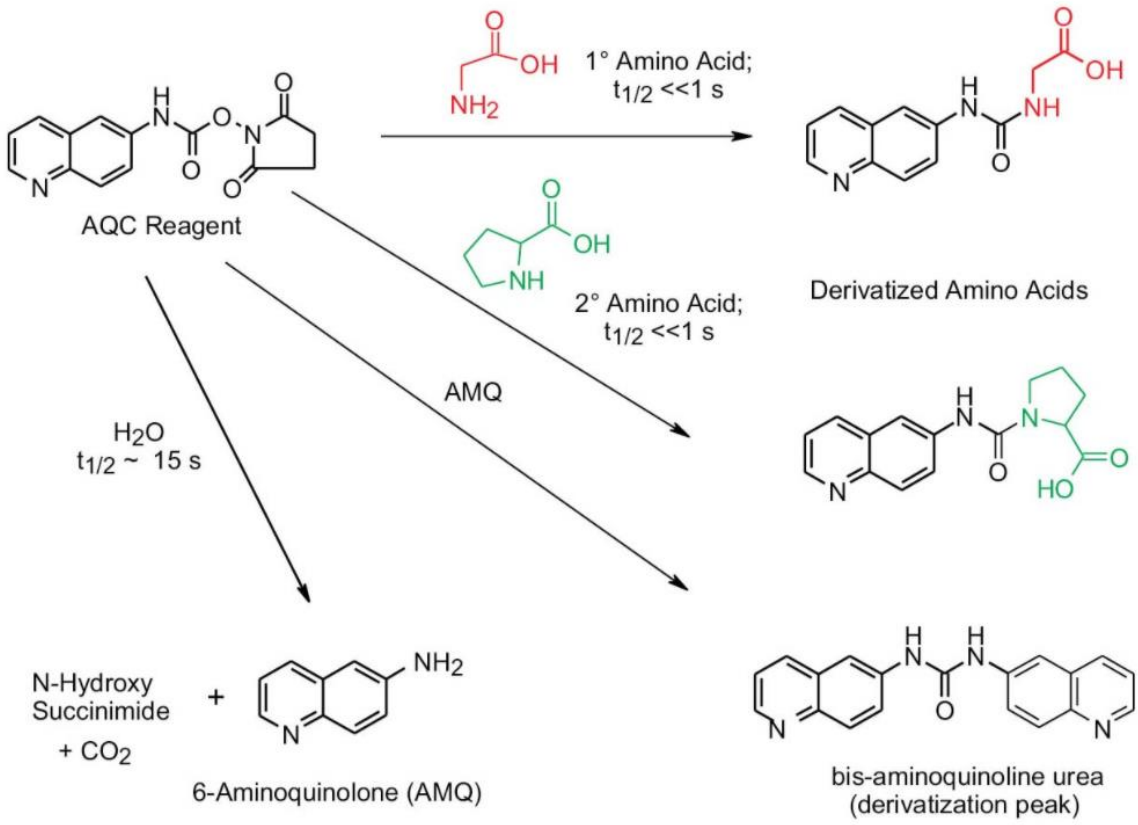


Figure 3 AccQ·Tag Ultra reaction (Waters Chromatography Europe 2020)

Derivatization process was performed for amino acid standards, selenoamino acid standards and samples (differently extracted garlic and Brazil nut samples) with the Waters AccQ·Tag™ Ultra Derivatization Kit (Waters Corporation, Manchester, UK). In contrast to the original instructions, three times bigger volumes were applied in order to produce more samples.

- First, the reagent powder was reconstituted by adding 1 ml of reagent diluent and heating the vial on the heating block at 55 °C (Pierce Reacti-Therm III Heating/Stirring, Thermo Fisher Scientific Inc., Rockford, IL, USA) until the powder was dissolved.
- 210 µl of borate buffer was delivered to a Total Recovery vial (Total Recovery Vial Kit, Screw Cap with bonded preslit PTFE/Silicone septa, Waters Corporation, Manchester, UK)

- 30 µl of the dissolved standard amino acids/selenoamino acids /samples was added to the vial
- 60 µl of reconstituted reagent was added to the vial
- The vial was capped and vortexed for several seconds and heated in the heating block for 10 minutes at 55°C
- The vial was removed from the heating block. The resulting standard had a concentration of 10 pmol/µl

3.3.6 UHPLC-ESI-MS

In order to detect the UV tagged selenoamino acids, production of derivate and isotope pattern of selenium, UHPLC-ESI-MS method was employed. ESI-MS was used to identify any co-eluted or unknown peaks of the chromatographic separations. Furthermore, ESI-MS provides more specific structural information, which can be useful for the identification and characterization of the Se compounds. Analysis was carried out on a Waters ACQUITY UltraPerformance LC® (UPLC®) system with PDA detector (Waters Corporation, Manchester, UK) coupled on-line to a Esquire LC ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) by means of an electrospray ionization (ESI) probe.

Setting up the ACQUITY UPLC system

It was assured that the UPLC system was clean before installing the solutions. Only HPLC grade solvents and water were used. It was also confirmed that PDA detector was plumbed, and Ethernet and solvent supply were connected according to the Waters UPLC Amino Acid Analysis Solution system guide.

The glassware used in this study was rinsed with the same HPLC grade solvents that were later used. Eluent A was prepared by mixing 475 ml AccQ·Tag™ Ultra Eluent A concentrate (acetonitrile+formic acid HCOOH) and 25 ml Milli-Q water thoroughly. Eluent B was supplied as a working solution, AccQ·Tag™ Ultra Eluent B, and no additional dilution was required. The presumed amount of Eluent B was transferred to mobile phase reservoir. Eluent A and B were consumed within 2-3 days and prepared again if needed. Weak needle wash solvent (5% acetonitrile in water), strong needle wash (95% acetonitrile) and seal wash solvent (50% acetonitrile in water) were prepared from HPLC grade acetonitrile and Milli-Q water and mixed thoroughly. Solvents were prepared again when needed.

Chromatographic separation

Derivatized amino acid standards and selenoamino acid standards were separated on a Waters AccQ•Tag Ultra column (1.7 µm particles, Waters Corporation, Manchester, UK) with parameters described in Table 4. Used gradients are shown in the Table 5.

Table 4. The most important parameters of UHPLC system

UHPLC	Waters® Acquity UPLC system
Column	AccQ·Tag Ultra
Column dimensions	2.1 mm x 100 mm
Column particle size	1.7 µm
Column packing material	Silica base bonded with C_{18}
Column heater	55 °C
Sample temperature	20 °C
Mobile phase flow rate	0.7 ml/min
Injection volume	2 µl
Needle placement (from bottom)	4.0 mm
PDA detector	260 nm
Eluent A	Acetonitrile + formic acid + Milli-Q
Eluent B	Acetonitrile

Table 5 Gradient table parameter values.

	Time(min)	Flow (mL/min)	%A	%B	Curve
1	0.00	0.7	99.9	0.1	-
2	0.54	0.7	99.9	0.1	6
3	5.74	0.7	90.9	9.1	7
4	7.74	0.7	78.8	21.2	6
5	8.04	0.7	40.4	59.6	6
6	8.05	0.7	10	90	6
7	8.64	0.7	10	90	6
8	8.73	0.7	99.9	0.1	6
9	9.50	0.7	99.9	0.1	6

The UHPLC run for 18 aminoacid standards (Figure 4) was accomplished according to the AccQ·Tag sample chromatogram. Figure 5 shows the chromatogram for the UHPLC analysis of the AccQ·Tag derivatized selenoamino acid standards. The first peak observed

at 6.34 corresponds to the main hydrolysis product, 6-aminoquinoline (AMQ) of unreacted AQC reagent. There are excess of the reagent to ensure that the reaction goes to completion. Selenoamino acid standards eluted clearly and the elution times were 6.495 for MeSeCys and 7.156 for SeMet.

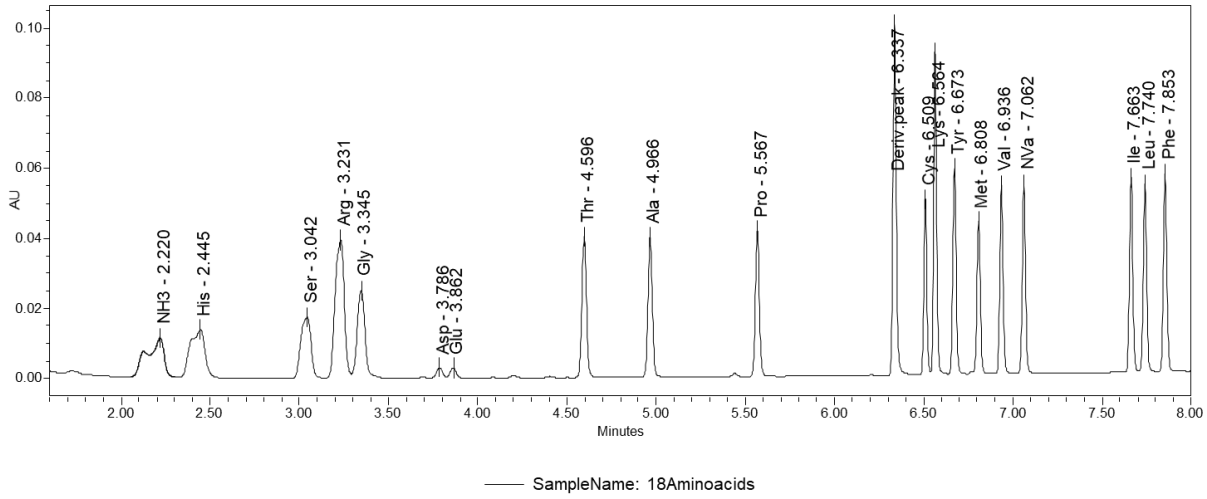


Figure 4 18 aminoacid peaks and their elution times in UHPLC, 10pmol/µl, 2 ul injection.

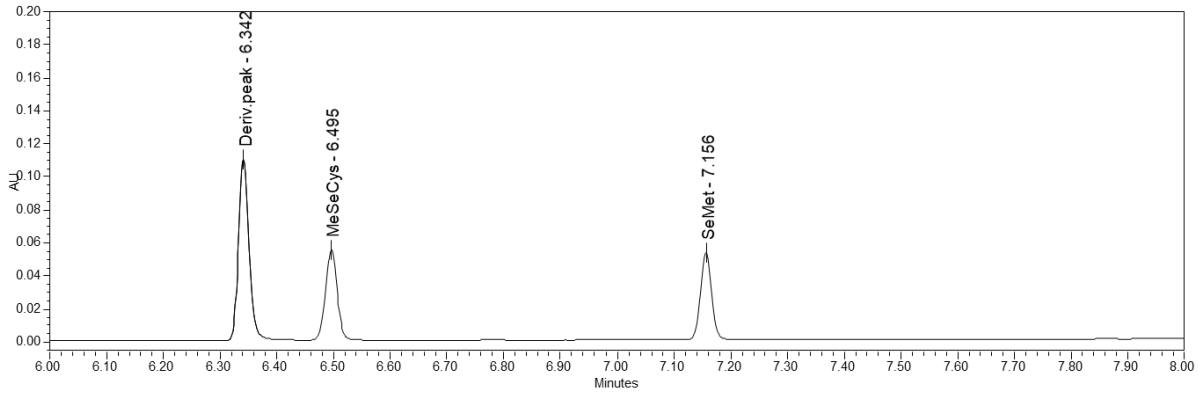


Figure 5 Chromatogram of selenoamino acid standards MeSeCys and SeMet from UHPLC analysis, 10pmol/µl, 2 µl injection.

ESI-MS

The mass spectrometer was employed to detect the standards and the possible seleno compounds from the samples. The main search was done to detect AQC derivatized SeMet (m/z 368) and MeSeCys (m/z 354). The fragmentation of derivatized MeSeCys is presented in the Figure 6. Because both seleno compounds were derivatized with AccQ·Tag reagents, MS/MS fragmentation would generate the same fragment ion, m/z 171.

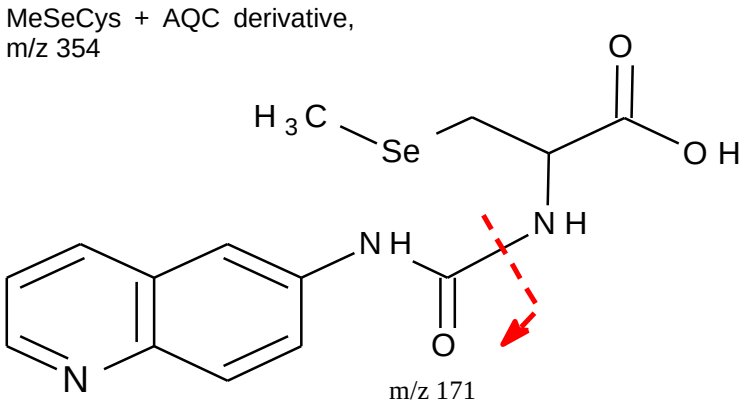


Figure 6 Derivatized MeSeCys m/z 354 and its fragment ion m/z 171.

After UHPLC, the column eluate 0.7 ml/min was split 1:10 with a splitter. The detection of derivatized samples and standards was conducted in the mass spectrometer operated with electrospray ionisation (ESI) in positive ion mode, with both full scan MS and MS/MS modes. Nitrogen served as the nebulizing and collision gas. The source conditions were as follows; positive ion, standard mass range, capillary exit 90 V, scan range 150-550 m/z, trap drive 39.5, capillary 4000V, skimmer 1 25 V, end plate offset 800V. The mass spectra were processed with Bruker Daltonics DataAnalysis 3.0 software.

In order to detect selenoamino acids from the samples, following methods were tested:

- Spiking the samples with selenoamino acid standards. The volume of standards used for spiking was 10% of the sample volume.
- Full scan mode was employed to search for other possible selenium compounds. Ions with isotopic variation were selected and new windows were made for them for MS/MS
- Search for oxidation products. It has been reported that SeMet can go through oxidation in enzymatic extraction turning into SeMetO (Pedrero et al. 2007). In this compound O is connected to selenium by two bonds. The m/z of AQC derivate connected to SeMetO would be m/z 384, AQC derivate connected to SeMeSeCysO it would be m/z 370. It is also possible they could turn into SeMetO2 or SeMeCysO2, m/z 400 and 286 respectively. These compounds were separately screened.
- Gradient alteration was investigated if longer gradient would influence elution and detection of species. Only the time was changed (Table 6 Gradient alterationTable 6), other parameters (flow, %A, %B and curve) remained the same.

Table 6 Gradient alteration.

	Time(min)	Flow (mL/min)	%A	%B	Curve
1	0.00	0.7	99.9	0.1	-
2	0.54	0.7	99.9	0.1	6
3	6.74	0.7	90.9	9.1	7
4	9.5	0.7	78.8	21.2	6
5	10.0	0.7	40.4	59.6	6
6	10.01	0.7	10	90	6
7	10.65	0.7	10	90	6
8	10.8	0.7	99.9	0.1	6
9	12.0	0.7	99.9	0.1	6

Matrix effect studies

Matrix effects were tested because they are a typical reason for loss in response (ion suppression) or increase in response (ion enhancement) in ESI-MS. They are caused by the alteration of ionization efficiency of target analytes in the presence of co-eluting compounds in the same matrix. Matrix components or mobile-phase additives that act as ion-pairing reagents can reduce ionization efficiency and result in low response (Panuwet et al. 2016).

Matrix effect was tested for all the samples with continuous 0.3 ml/h post column infusion of standard into the ionization source by a syringe pump (EW-74900-00 single-syringe infusion pump, Cole-Parmer®, Illinois, USA) connected to the T-union. After simultaneously injecting a sample into the system, a drop or rise along the baseline indicates suppression or enhancement in ionization of the analyte due to the presence of co-eluting components. The run was done for both ions m/z 368 and m/z 354 in extracted-ion chromatogram (EIC) mode (fragmented and isolated m/z 171).

AQC UHPLC-ESI-MS/MS Method Evaluation and Applicability

The performance of the UHPLC-ESI-MS/MS method for the analysis of AQC-derivatized selenoamino acids was evaluated by measuring the repeatability, linearity, and sensitivity of the analysis. The repeatability of the method was determined by examination of the retention time and peak area ratios after injections of standard solutions of derivatized selenoamino acids. An ANOVA test was run to find out if the results from the two days were significant. Standard curve was made by injecting selenoamino acid standards at concentrations of 2, 10,

20, 50 and 100 pmol/µl. Two replicate injections were made for each concentration level. Limits of detection were calculated from signal to noise ratio, LOD=3 x Signal/Noise.

3.3.7 HPLC-ICP-MS

HPLC-ICP-MS was employed to find out whether it was possible to detect any seleno compounds in the garlic and Brazil nut samples. It was important to try this method, even briefly, because it was possible that there were also some unfamiliar selenocompounds in the samples or that the selenocompounds had transformed to something else. The Chemistry and Toxicology Research Unit of the Finnish Food Safety Authority, Evira (currently Ruokavirasto), kindly granted a possibility to test their ICP-MS for a few days.

In order to ionize and detect elemental selenium, chromatographic separation was carried out using the Waters HPLC (2690 Separations Module, Waters, USA) connected to the Thermo Fisher Scientific X Series II quadrupole inductively coupled plasma mass spectrometer (Waltham, Massachusetts, USA). The ICP-MS was equipped with HPLC–ICP-MS Coupling Kit, Integrated PlasmaLab software (Thermo Fisher Scientific, Waltham Massachusetts, USA). The analytical column was an anion exchange column Hamilton PRP-X100 (Bonaduz, Switzerland), 10 µm particle size, 250 x 4.1 mm. 5 mmol/l ammonium citrate buffer (pH 5.2) served as the mobile phase. Selenoamino acid standards and extracted samples were not derivatized as before ESI-MS, but they were diluted 1/10 with weak nitric acid (1%). Injection volume was 100 µl and the mobile phase flow rate was set to 1 ml/min isocratically. The resolution was in the standard mode. The machine did not include reaction cell system, so Argon interference was possible. The data was collected on-line for selenium isotope Se^{78} in order to avoid the main interference with Se^{80} and argon ($^{40}Ar^{40}Ar+$).

3.4 Results

3.4.1 Total selenium content in the samples and sample extracts

Total selenium content was determined by graphite oven AAS from freeze dried garlic sample and defatted Brazil nut sample to find out the total selenium content in the same samples which were further used in extraction and sample preparation. Table 7 shows that Brazil nut contained 44 times more selenium than garlic.

Table 7 Total selenium content in garlic and Brazil nuts (dry weight).

Sample	Total selenium (ng/g)	Total selenium (µg/g)
Garlic	100	0.1
Brazil nut	4400	4.4

Total selenium content was also determined from the sample extract supernatants but also from the precipitates after different extractions. This was done to find out if the extraction method was efficient enough to extract any selenium from the sample material. It was discovered that most of the total selenium remained in the precipitate regardless of the sample or the extraction method (Table 8). The total selenium content in the supernatants was around 10% compared to the precipitates. This itself, however, did not prove that there were not enough selenoamino acids present in the supernatants (used in the sample preparation) for ICP-MS and ESI-MS analysis.

Table 8 Total selenium content in extraction precipitate and supernatant.

Sample and extraction method	Precipitate (ng/g)	Supernatant (ng/g)
Garlic H_20	180	20
Garlic HCl	180	20
Garlic prot K	230	15
Brazil nut H_20	3400	300
Brazil nut HCl	3300	297
Brazil nut prot K	2100	196

H_2O=hot water extraction, HCl=diluted hydrochloride extraction, prot K= proteinase K extraction

3.4.2 Attempt to specify selenoaminoacids by UHPLC-ESI-MS

Selenoaminoacid standards (10 pmol/µl, 2 µl injection) were clearly detected after method optimization by both PDA and MS detectors as illustrated in Figure 7. It also shows the fragment ion m/z 171 for both standards from tandem MS.

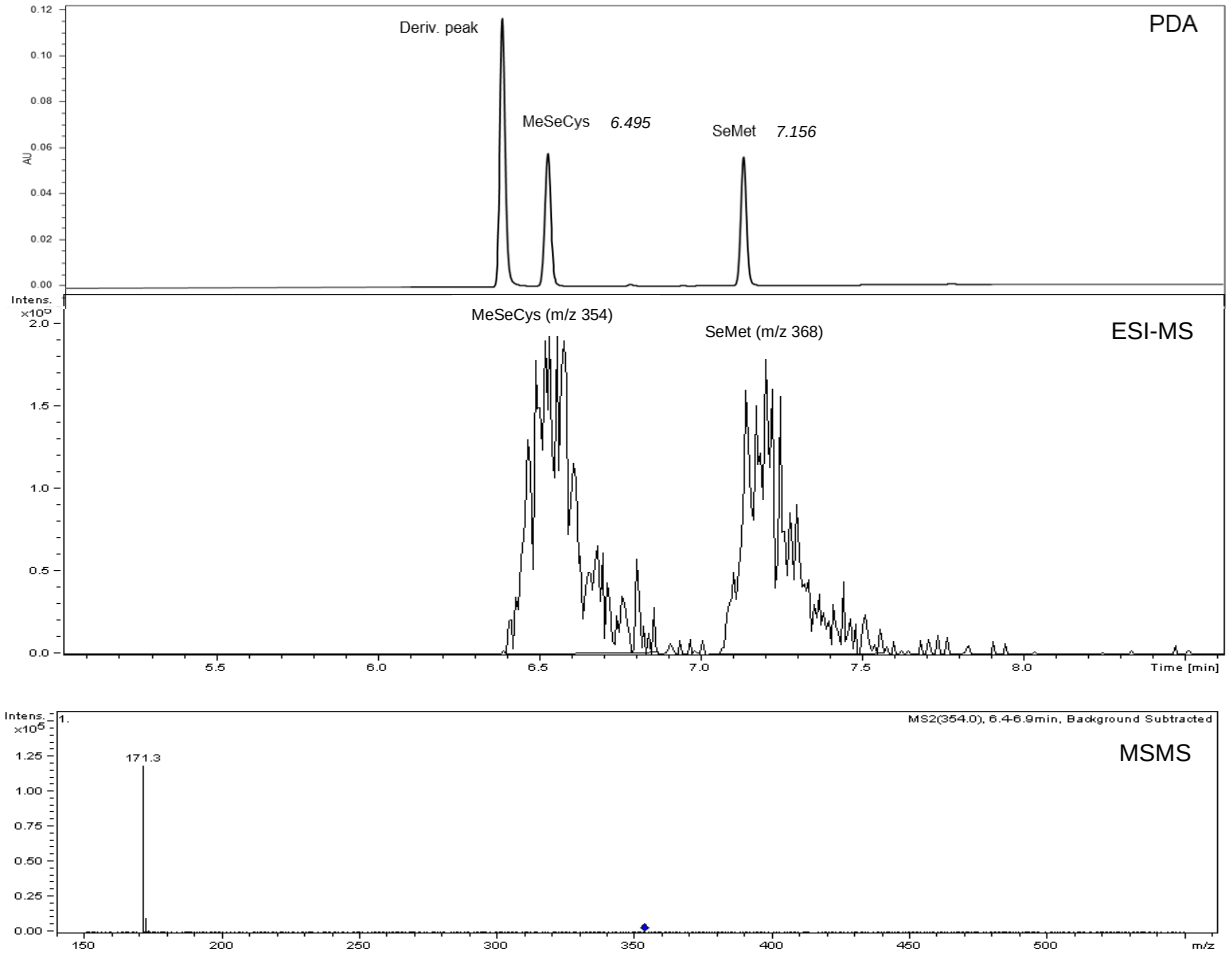


Figure 7 Retention times of derivatized MeSeCys and SeMet and the fragment ion from tandem MS.

Matrix effect was studied for all the extraction methods of both garlic and Brazil nut samples with continuous post-column infusion system in ESI-MS. No matrix effects were observed for any sample. Signals were not suppressed, and the baseline was continuous in all the samples. Figure 8 shows the extracted ion chromatogram of matrix effect test for SeMet with no drop or rise in the baseline during SeMet elution time.

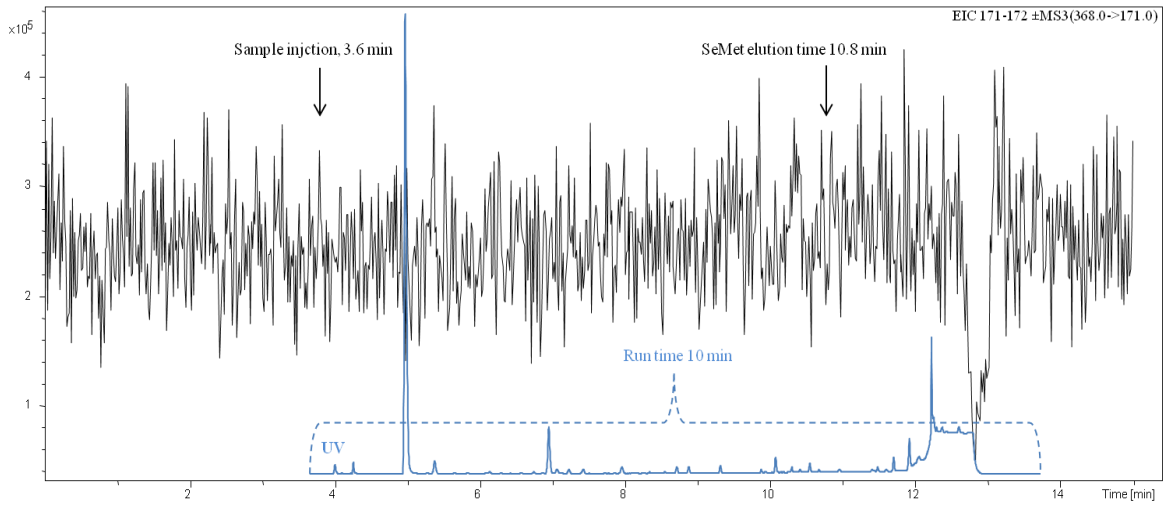


Figure 8 Matrix effect test of diluted HCl extraction of Brazil nut for SeMet (m/z 368→m/z171).

Identification of selenoamino acids from garlic and Brazil nut

MeSeCys and SeMet were optimized and searched with full scan and isolation but nothing was seen from the mass spectra of the samples. Spiking and gradient alterations were tested with no observable results. Oxidation products were searched from proteinase K digested samples and finally full scan mode was applied to search any seleno compounds with isotopic pattern. Despite all the efforts no results were obtained. The sensitivity was not sufficient enough to detect very low concentrations of selenoamino acids in garlic and Brazil nuts.

3.4.3 Evaluation of the AQC UHPLC-ESI-MS method

Derivatization of selenoamino acid standards with AccQ·Tag kit and detection with UHPLC and ESI-MS proved to be fast and easy as both standards (MeSeCys and SeMet) were analysed in less than eight minutes.

Repeatability and linearity of the method was evaluated by 10 inter and intraday injections of standards (Table 9). The retention times of the selenoamino acid standards by UHPLC had low coefficient of variation (CV%) but there was a significant difference between MeSeCys Day 1 and Day 2 results at the 95,0% confidence level. There was no difference between the Day 1 and Day 2 results of SeMet or with ESI-MS results of both selenoamino acid standards.

Table 9 Retention times and areas of AQC-derivatized selenoamino acid standards by UHPLC-ESI-MS. N=10 for both days.

Rt

		Day 1			Day 2		
	Compound	Mean (min)	SD (min)	CV%	Mean (min)	SD (min)	CV%
UV/UPLC	MeSeCys	6.49	0.001	0.016	6.50	0.001	0.010
	SeMet	7.16	0.001	0.012	7.16	0.001	0.012
ESI-MS	MeSeCys	6.53	0.067	1.034	6.51	0.032	0.486
	SeMet	7.22	0.063	0.876	7.19	0.057	0.789

Areas

		Day 1			Day 2		
	Compound	Mean	SD	CV%	Mean	SD	CV%
UV/UHPLC	MeSeCys	69222	4182.2	6.0	68481	1842.6	2.7
	SeMet	62580	3851.8	6.2	60366	1665.8	2.8
ESI-MS	MeSeCys	1613219	89714.8	5.6	1823366	135087.7	7.4
	SeMet	1113718	107480.7	9.7	1371583	134511.0	9.8

For the UHPLC, CV% of areas stayed under 10 and repeatability of the results were good. The ESI-MS areas had also the CV% under 10, but the P-value of the F-test was less than 0,05 for both MeSeCys and SeMet, so there was a statistically significant difference between the means of the Day1 and Day2 results at the 95,0% confidence level. However, the results were quite close to each other knowing that the results of this ESI-MS usually vary a lot.

The standard curves for SeMet and MeSeCys (in the range of 2-100 pmol/µl) gave good linearity with correlation coefficients(R^2) from 0.997 and 0.998 in ESI-MS to 0.999 and 0.999 in UHPLC. The detection limits (DL) for MeSeCys and SeMet in ESI-MS were both 0.02 pmol/µl.

3.4.4 Speciation of selenoamino acids by HPLC-ICP-MS

After the failed attempts to detect selenoamino acids in UHPLC-ESI-MS, HPLC-ICP-MS was briefly tested to find out whether there are any selenium containing compounds in the garlic and Brazil nut samples.

SeMet and MeSeCys standards (10pmol/µl, 100 µl injection) were run in the standard mode monitoring Se^{78} signal, and the peaks were clearly seen. Hot water and diluted HCl extractions of garlic and Brazil nuts showed no signs of selenium. Finally, a small peak emerged from proteinase K extracted Brazil nut sample. The peak was identified as SeMet by retention time matching with the standard (Figure 9) and quantified semi quantitatively from standard curve (Figure 10). The standard curve was prepared by injecting 50, 70 and 100 µl of 10 pmol/µl SeMet standard. The concentration was 0.16 pmol SeMet/µl of sample, and further calculated 0,06 µg SeMet /g Brazil nut.

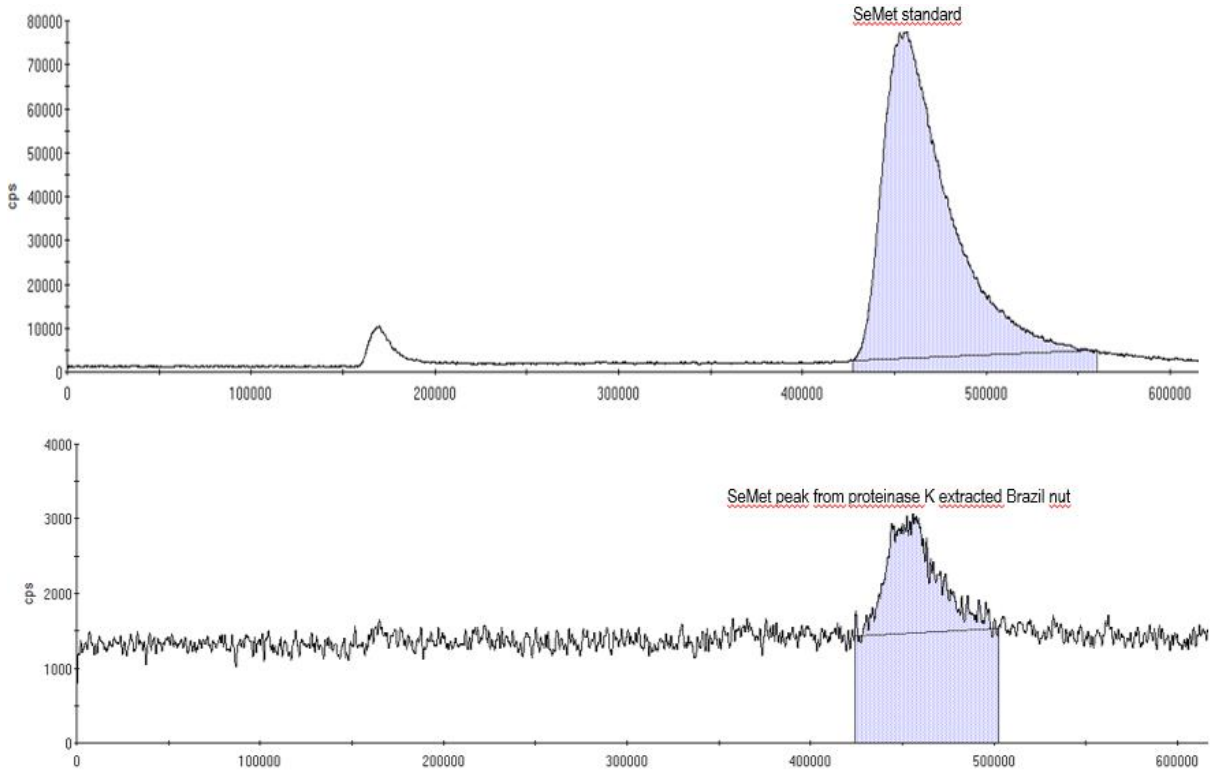


Figure 9 Chromatograms of SeMet standard (10 pmol/ µl) and proteinase K digested Brazil nut sample diluted 1/10 in weak nitric acid. 100 µl injected.

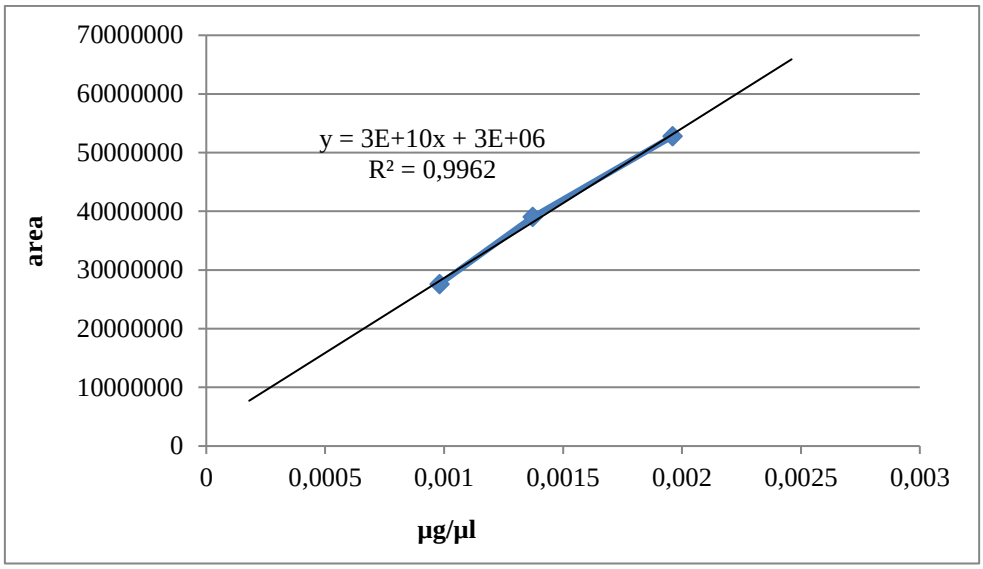


Figure 10 Standard curve of SeMet

3.5 Discussion

3.5.1 Total selenium content in the samples and extracts

GFAAS was employed to study the overall concentration of selenium in the garlic and Brazil nut samples and their extracts. Total selenium content in garlic (0,1 µg/g dry weight) were in line with other results found in the literature for natural garlic. The concentrations from 0,02 µg/g to 0,25 µg/g have been reported (Block 1998; Kotrebai et al. 2000b; Polyakov et al. 2020). Most of the selenium studies have been done with the enriched garlic or garlic supplements, as the content can be remarkably higher (from 68 µg/g to 1355 µg/g) (Cai et al. 1995; Block 1998; Reilly 2006) due to the accumulator properties, and therefore more prone to achieve better results in selenium speciation analysis.

Total selenium content in Brazil nuts (4.4 µg/g dry weight) was significantly higher than for garlic but similar to the results obtained by Vonderheide et al. (2002) and Dumont et al. (2006a) analysing the Se concentration in shelled Brazil nuts (2.54–8.30 µg/g dry weight). The variation of the total selenium content in Brazil nuts can be especially high (0.3-49.9 µg/g dry weight), depending on the soil and shelling, and because the nuts were already shelled while purchasing, the results were expected to stay in the lower range (Chang et al. 1995; Vonderheide et al. 2002; Dumont et al. 2006a; Manjusha et al. 2007; Tošić et al. 2015; Moreda-Piñeiro et al. 2018).

Extraction methods succeed to extract only small amounts of selenium from the samples and most of the selenium remained in the precipitate. According to the previous studies by several authors (McSheehy et al. 2001; Bañuelos et al. 2011; Milovanovic et al. 2019; Ward et al. 2019; Yin et al. 2019) it was expected that water and HCl extractions may only extract the free low molecular weight species while enzymes are needed for bigger yield to extract also the protein bound species such as SeMet. The extraction efficiency has been around 40% to 90%, depending on the food material and the extraction media. However, in this case the total selenium amount in the supernatants were about the same in hot water, diluted HCl and proteinase K extractions, as only around 10% was obtained to the supernatants.

Unfortunately, the extraction efficiency was studied too late in the experimental phase, as it should have been studied as soon it was noticed that no selenoamino acids could be seen with the UHPLC-ESI-MS method. However, similar extraction procedures have been used by others as well (Kotrebai et al. 2000; Dumont et al. 2006b; Liu 2011). Cuderman et al. (2010) studied different extraction media in order to find the optimal method to identify selenium species in selenized buckwheat sprouts. The best results were obtained by hydrolysis with HCl, breaking the cells with liquid nitrogen and then using enzymatic hydrolysis with protease XIV. As it has been successful for buckwheat sprouts, this kind of mixture of different extraction methods could have been worth trying also for garlic and Brazil nuts. Other approach could be using simulated saliva and/or gastric fluid extraction as described by Dumont et al. (2006b), which would be the closest to what happens in human body. Therefore, the information would be the most beneficial when weighing the health effects of certain selenocompounds.

3.5.2 Applicability of the AQC-UPHLC-ESI-MS Method

Application of AccQ•Tag derivatization method for selenoamino acid analysis followed by LC-MS has not been described before in the literature. Similar method has been applied by Gray et al. (2019) for the quantification of amino acids and biogenic amines in rat urine by UPLC-MS/MS, where the AQC derivatization process was found to improve retention in RP-LC and also ionisation efficiency to enhance MS-detection. The same aspects were noticed with this study. The derivatization process with AccQ•Tag derivatization kit was found to be easy and straightforward, as all the amino acid standards peaks and selenoamino

acid standard peaks eluted within 8 minutes. Optimisation of the method was successful as no matrix effects were observed in ESI-MS and AQC-derivatized selenoamino acid standards SeMet (m/z 368) and MeSeCys (m/z 354) eluted as expected. The method gave good linearity (R^2 >0.997) and repeatability (CV% <10) for selenoamino acid standards.

For the garlic and Brazil nut samples the method did not provide any results. Despite the efforts in optimizing conditions in ESI-MS, no selenoamino acids were detected. It is possible that in electrospray ionization, aminoacids in the samples were more easily ionized due to their higher amount which could have inhibited the ionisation of selenoamino acids. All the derivatized compounds also formed the same fragment ion (m/z 171) which also complicated the detection. Armstrong et al. (2007) noticed that even though the preparation of samples for LC-MS analysis using amino acid kits (such as Waters AccQ•Tag) simplifies the derivatization step, the non-volatile buffers used in these kits are not readily compatible with ESI-MS, bringing drawbacks to the LC-MS, such as signal suppression. Waters AccQ•Tag Kit includes non-volatile borate buffer which is used for optimum pH adjustment of the reaction solution in order to obtain maximum product yields. One possibility could be changing the borate buffer with the ammonium acetate buffer as tested by Salazar et al. (2012) with aminoacid analysis of *Arabidopsis* leaf extracts.

Due to the poor extraction efficiency, there was only around 10% of the possible selenium to be detected in the sample extracts (supernatants). When calculated with proteinase K extracted Brazil nut (which was the most potential to show signs of SeMet in ESI-MS), 21% of the selenium in the supernatant could be SeMet (Kannamkumarath et al. 2002). Taking into account the sample preparation, there could have been only 0,0114 pmol/µl SeMet in the proteinase K extracted Brazil nut sample. The limit of detection (LOD) in ESI-MS was 0.2 pmol/µl for both AQC-derivatized SeMet (m/z 368) and MeSeCys (m/z 354). Even if the extraction efficiency would have been 100%, SeMet would have just barely been detected, MeSeCys not at all. Therefore, it is clear that there were not enough selenoamino acids in the samples to be detected with this method. In the future, the same method could be tested with samples containing more selenium and the extraction efficiency should be verified before going further with the analyses.

3.5.3 Detection of SeMet by HPLC-ICP-MS

HPLC-ICP-MS was employed in Finnish Food Safety Authority, Evira (currently Ruokavirasto) after it was noticed there were no results from garlic and Brazil nut samples by UHPLC-ESI-MS. This option was adopted to find out if it was possible to detect any selenoamino acids from the garlic and Brazil nut samples at all. This method, however, did not include the AQC-derivatization process or the possibility to characterize possible new selenocompounds. Unfortunately, the time frame was too short, but with the help and expertise of Kirsti Risunen from Evira, some results were obtained.

Concentration of SeMet (0.06 µg SeMet/g Brazil nut) in proteinase K digested Brazil nut sample was analysed semi quantitatively from standard curve. Quite similar result was obtained by Kannanmkumarath et al. (2002), also from the shelled Brazil nuts; total selenium content was 8.3 µg/g of which 21% was SeMet, leading to the estimated result of 1.7 µg SeMet/g Brazil nut. As the total selenium concentration in the extract supernatant in this study was 0.196 µg/g, 21% of it would be around 0.04 µg/g which is close to the result (0.06 µg/g).

Other samples did not provide any results even though there were more overall selenium in hot water and diluted HCl extracted Brazil nut sample supernatants. These results show that the enzymatic digestion cleaved the protein bound SeMet from the Brazil nut which did not occur in other extraction methods, at least not enough to be detected. This was expected as described before by several authors (Kannamkumarath et al. 2002; Vonderheide et al. 2002; Bañuelos et al. 2011; Tie et al. 2018), but it was also expected to extract the most overall selenium which did not happen. In hot water and diluted HCl extractions, selenium might have remained in several selenocompounds as selenoaminoacids could not been detected in the samples. SeMet and MeSeCys has been determined to be stable in hot water and enzymatic extractions for several days by Cuderman et al. (2010), however there are suggestions that conversion of SeMet to selenomethionine oxide (SeMetO) happens in the extracts within a relatively short time after extraction (Bierla et al. 2012).

The sensitivity of ICP-MS could have been increased with the addition of methanol (e.g. 2-10%) to the mobile phase (Larsen and Stürup 1994). This was not done as the sensitivity appeared good considering the selenoamino acids. The isotopic interference of Argon was

also possible as the machine did not include reaction cell system. The data, however, was collected on-line for selenium isotope Se^{78} in order to avoid the main interference with Se^{80} and $^{40}Ar_2^+$.

Several methods for selenium specification have been tested in the literature and the newest methods tend to combine the advantages of highly sensitive ICP-MS and molecular information from ESI-MS (Preud'Homme et al. 2012). By combining LC-ICP-MS with ESI-MS, analytes of interest can be quantified with a high degree of mass accuracy and sensitivity in the MS mode, and the option of up to MS^4 fragmentation offers good aspects for successful identification of selenium. Cardoso et al. (2019) concluded in their review article that the field of selenium analysis is moving towards and the potential for a greater understand of selenium biochemistry as a result of the recent advancements in ESI-MS/MS.

4 CONCLUSIONS

The study aimed to develop a simple and rapid mass spectrometric method which could be used to detect and specify low molecular weight selenoamino acids from different food materials in order to find and quantify probable cancer protective species. The experiments showed that there are insufficient amounts of selenium and selenoamino acids in unenriched garlic and Brazil nut samples to be detected by UHPLC-ESI-MS. Despite the efforts in optimizing conditions, there were no signs of selenoamino acids in real samples. In electrospray ionization, amino acids in derivatized samples are also more easily ionized as their concentration is significantly higher, which could have hindered the ionization of Se amino acids. All the derivatized compounds also formed the same fragment ion (m/z 171) which also complicated the detection. However, the AQC-derivatisation followed by UHPLC-ESI-MS gave linear and repeatable results for selenoamino acids standards.

After different experiments with molecular ESI-MS, an element specific HPLC-ICP-MS was employed for a few days and it managed to detect SeMet in proteinase K digested Brazil nut (0.06 µg SeMet/g Brazil nut). It showed that there was protein bound SeMet available after enzymatic extraction, but no evidence was received from methylated selenoamino acids, as no results were obtained from hot water or diluted HCl extracted samples. HPLC-ICP-MS is widely used in selenium speciation and with more time and accurate optimization there could have been potential to provide better results.

The extraction methods (hot water, diluted HCl and proteinase K) were not efficient enough and need to be improved or replaced as most of the total selenium remained in the extract precipitate. There could be potential to test a mixture of different extraction methods for garlic and Brazil nuts in order to extract the highest possible amount of selenocompounds from the samples. Other approach could be using simulated saliva and/or gastric fluid extraction which would be the closest to what happens in human body. Therefore, the information would be the most beneficial when weighing the health effects of certain selenocompounds.

In future selenium speciation studies, it would beneficial to combine the advantages of highly sensitive ICP-MS and molecular information from ESI-MS. One possibility could be to use SEC-ICP-MS as a pre step, gathering the Se-containing fractions, followed by post column derivatization with AQC and detection and characterization by ESI-MS.

Finally, despite the failed experiments with the real samples, AQC-UHPLC-ESI-MS method showed adequate linearity and repeatability for selenoamino acid standards and offers a possibility for a fast and easily applicable selenoamino acid analysis.

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