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Ph.D. dissertation

DEVELOPMENT OF LC-MS METHODS FOR THE ANALYSES OF SELENIUM SPECIES OF NATURAL AND OF SYNTHETIC ORIGIN

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List of abbreviations

(2,3-DHP)-Sec-Sec	Di-N-2,3-DHP-Selenocystine
2,3-DHP	2,3-Dihydroxi-Propionyl
2,3-DOP	Dihydroxy-1-oxopropyl
AAS	Atomic Absorption Spectrometry
ACN	Acetonitrile
AES	Atomic Emission Spectroscopy
AFS	Atomic Fluorescence Spectrometry
CRM	Certified Reference Material
DCC	*N*,*N*'-Dicyclohexylcarbodiimide
D-Met	D-Methionine
DMF	*N*,*N*'-Dimethylformamide
DMSeP	Dimethylselenonium Propionate
DTT	Dithiothreitol
EFSA	European Food Safety Authority
EIC	Extracted Ion Chromatogram
EPI	Enhanced Product Ion
ESI	Electro-Spray Ionisation
GC	Gas Chromatography
GI	Gastrointestinal
GSHPx	Glutathione Peroxidase
HFBA	Heptafluorobutyric-Acid
HG	Hydride Generation
HILIC	Hydrophilic Interaction Liquid Chromatography
HPLC	High-Performance Liquid Chromatography
ICP	Inductively Coupled Plasma
ID	Isotope Dilution
IP	Ion-Pairing
IPD	Isotope Pattern Deconvolution
IUPAC	International Union for Pure and Applied Chemistry
LC	Liquid Chromatography
LOD	Limit of Detection
MALDI	Matrix-Assisted Laser Desorption-Ionization
MS	Mass Spectrometry
MSA	Methanesulphonic Acid
MSeAcG	*Se*-Methyl-N-Acetylselenohexosamine
NIBC	*N*-Isobutyril-Cysteine
NMM	4-Methylmorpholine
NMR	Nuclear Magnetic Resonance
OPA	*O*-Phthalaldehyde
PCP	Pentachlorophenol

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PTFE	Polytetrafluoroethylene
Q	Quadrupole
QQQ	Triple Quadrupole
QTOF	Quadrupole Time of Flight
RBV	Relative Oral Bioavailability
RP	Reversed Phase
SAX	Strong Anion Exchange Chromatography
SBP2	SeCys Binding Protein 2
SCX	Strong Cation Exchange Chromatography
SDS	Sodium Dodecyl Sulphate
SEC	Size Exclusion Chromatography
Sec_2	Selenocystine
SeCys	Selenocysteine
SeMet	Selenomethionine
SePP	Selenoprotein P
SeW	Selenoprotein W
T4	Tetraiodothyronine
TCA	Trichloroacetic Acid
TIC	Total Ion Chromatogram
TRIS	Tris(hydroxymethyl)Aminomethane
UHT	Ultra High Temperature
USAED	Ultrasonic Assisted Enzymatic Digestion
UV	Ultraviolet

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1. Introduction

In our age eating healthy is “trendy”. Consumers follow advertisements, jump from one fad to the other, trying to make sense of the tirade of half-information, half-truths, half-lies and occasionally outright lies that confront them. And why should they not? Food will never go out of fashion. In no age will it become unimportant, non-essential, or, not the source of enjoyment.

We often hear the saying: we are what we eat. It could not be truer, as our bodies build the new tissues, hair and muscles using the “building material” that we acquire through digestion. As no good-quality house can be built from rejected, third-rate material, no healthy body can be maintained on bad quality food. Health is an important issue for every single one of us. And the responsibility of choosing a suitable diet does not only lie with the consumer. It is the responsibility of manufacturers to produce good quality products, the responsibility of the controlling authorities to make the right regulations and monitor whether they upheld. And last, but not least, it is the responsibility of us, researchers, to produce data and results that would assist the law-makers in choosing the right regulations and controlling methods; data that would help us understand the workings of the human body and sources of sicknesses better.

The different topic I worked on can all relate to human health and food. My thesis focuses on one of the essential micro-elements, selenium. Molecules containing this element have important roles in a wide range of areas. It takes part in regulating a healthy bone growth, maintaining healthy nails, hair and skin, mental development, it has an important role in reproduction, pulse regulation, the prevention of depression and ensuring an energetic, healthy attitude. However, the minimal necessary and already poisonous levels of this element are close, and selenium poisoning is just as real of a danger as non-sufficient supplementation. In light of this it is easy to see why it is important to perfect Se-supplementation controlling methods and find out more about selenium pathways.

The most commonly used selenium supplements are selenised yeast and seleno-methionine. As selenomethionine is an amino acid, it naturally has two enantiomers, the D-, and L-enantiomers. While the L form can be easily utilised by the human body, the D-enantiomer can only be used in insignificant quantities. The importance of discriminating between the two was long recognised and several methods have been established for it through the years. However researchers are still in disagreement about their accuracy and applicability. In the first part of my thesis I describe my experiments that were executed to compare the results obtained through different methods. I examined whether the different sample preparation techniques

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influence the enantiomer ratio of selenomethionine in the sample and if the different methods yield different results.

The second part of my thesis is connected to the other commonly used selenium supplementing material: selenised yeast. To date this is the most effective supplement approved by the European Food Safety Authority. The reason behind this is the large number of Se-containing yeast metabolites that enhance and strengthen each others' effects. To date more than seventy different such metabolites were found. On the other hand fermenting yeast is more expensive than simply synthesising racemic selenomethionine, and microbiological problems could possibly occur. It is of utmost importance that selenised yeast batches can be identified, both for unveiling forgery with selenomethionine and both for retracing problematic products. The identification can be best done with the aforementioned metabolites, as their ratios change from yeast-batch to yeast-batch. However, for the measuring standards of the metabolites are necessary, and out of the seventy less than ten percent is commercially available. Therefore in the second part of my thesis I describe how I developed a method for synthesising four of these metabolites. After an optimisation these methods could not only be used for such regulations purposes, but for tests of the health effects of each metabolite as well.

The last part of my thesis work was part of a larger project started at the university, namely, to develop a functional food for selenium supplementation. For this purpose a mushroom autochthonous in Eurasia, used in Chinese medicine since the ancient times was chosen: *Hericium erinaceus*. After a selenium-enrichment and examining the selenium-metabolites, I managed to separate and extract three novel compounds, which led me to an interesting revelation: that this mushroom has a selenium-metabolism similar to that of yeast instead of other higher mushrooms, which opens up great possibilities of further research and use.

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2. Theoretical review

2.1. About selenium in general

Selenium is a non-metal element, found in the sixth column of the periodic table of elements, in the group of chalcogens. It was discovered in 1817 by Jöns Jakob Berzelius as he was trying to produce sulphuric acid using the lead chamber process from pyrite mined in Falun, Sweden. He discovered a red precipitate in the chamber that smelled like horseradish when burned, and exhibited attributes similar to both sulphur and tellurium. Realising he came across a new compound of the chalcogen group, he named the element after the moon, as tellurium was named after earth.

Selenium can rarely be found in nature in elemental state or as pure ores. It occurs in several different forms, like selenides, selenates, and selenites, but they are rare. In the largest quantities it can be cleaned from different sulphide ores, where it substitutes the sulphur.

This element has three allotropes: red, black and silver-coloured ones. Given the correct heating and cooling is performed, these allotropes can morph into each other. When selenium precipitates during chemical reactions, it usually does so in the red, amorphous form. It has three different types, α, β, and γ forms, all of which are made of Se8 rings similar to that of S. The only difference is between these three forms is in the arrangement of the rings. The α type is the most densely packaged. When red selenium is heated, none of the forms exhibits the viscosity-change typical of sulphur [1].

If melting is executed rapidly, black, vitreous allotrope is formed. In this form selenium creates complex, irregular, polymeric rings of more than a thousand atoms, whose structure results in black, glossy but brittle crystals. The largest amount of commercially available selenium is sold in this allotrope. Black selenium has a melting point of 50 °C.

With gentle heating around 180 °C any of these two forms can be morphed into the silver-coloured allotrope, but it can also be created by the slow cooling of any molten selenium or by condensing vapours not far below the melting point. Silver is the most compact and stable form of selenium with a structure of helical polymeric chains creating a hexagonal crystal. This difference in structure is the reason that while other allotropes are insulators, this type is a semiconductor and has photo conducting abilities as well [2].

Selenium in its elemental form is rare; most often it can be found in four different oxidation states, those being -2, 2, +4, and +6.

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Selenium has five stable isotopes, which are: ^{74}Se, ^{76}Se, ^{77}Se, ^{78}Se, and ^{80}Se. It has twenty-five unstable isotopes too; the two most important ones are ^{79}Se and ^{82}Se. The former has a half-life of 327,000 years, the latter halves in about 10^{20} years, which in practical terms can be considered stable.

It can form two different oxides: SeO_2 and SeO_3. Selenium dioxide is a solid polymer, which if dissolved in water, forms selenious acid (H_2SeO_3). Selenium-trioxide however, is thermodynamically unstable unlike its sulphur analogue. Dissolved in water it forms selenic acid (H_2SeO_4), but because of the instability of the trioxide form, it is impractical. Selenic acid is usually prepared by oxidizing selenium compounds in lower oxidation states. Selenic acid shows a lot of similarities with sulphuric acid: it is highly toxic, corrosive and hygroscopic. It is a strong acid, though not as strong as sulphuric acid. However, it is a much stronger oxidizing agent, it can even dissolve pure gold [3].

It forms selenium-disulphide with sulphur. It has an interesting structure; it forms rings of eight atoms, with an approximate composition of SeS_2; but the individual rings can vary. It is not a pure compound but a mixture of different rings with composition of Se_nS_{8-n}.

Similarly to other chalcogens, selenium can also form hydrides [1]. Hydrogen-selenide is a toxic, colourless gas with a strong unpleasant smell. It is more acidic than H_2S, and it hydrolyzes in water to HSe^- or even Se^{2-}. This dianion can form several different compounds, including those minerals which commercially available selenium can be acquired from*, e.g.*, mercury selenide (HgSe), lead selenide (PbSe), zinc selenide. These minerals are all semi-conductors. Selenium reacts with alkali metal selenides resulting in polyselenides, Se^{2-}_x, which form chains.

Inorganic selenium is widely used in different areas. Selenium sulphide is an anti-dandruff agent in shampoos; the electro-wiring of electrolysis cells is made of selenium dioxide; copper indium gallium selenide is used in the production of solar cells. It is used in photocopying, photocells and light meters as it is photovoltaic and photoconductive. It is also used in toning of photographic prints. On industrial scale, most selenium (50%) is used for colouring glass red. This suppresses the green or yellow tints caused by iron impurities that are typical for most glass.

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2.2. Bioavailability and biological role of selenium

Selenium is a microelement that is essential for the human body to function properly. The main source of it is naturally the food we consume. The availability of selenium varies based on several factors such as geographical conditions, agricultural practice, type of diet (vegetarian or not), availability of fish, economy of the given country and the wealth of the consumer. However, the selenium content of the soil is one of the most important factors [4].

The selenium content of soils varies by region, and certain factors can influence or change it. Plant and animal residues usually enrich soils in selenium. While mine spoils usually contain the same concentration of selenium as found in local soils, mine tailing and floodwater washouts can dissolve this selenium content and deposit it in the topsoil. Thus, selenium concentration might rise to toxic levels as it has happened in the northern Great Plain in America and in New Mexico, and can cause selenosis signs both in humans and animals. Volcanic activity along with sulphur introduces selenium into the atmosphere too, in form of SeO_2 [5]. This molecule easily dissolves in water and is washed out from the atmosphere close to the volcanoes, resulting in a selenium-rich soil. Burning fossils rich in selenium introduces Se in the air. It has been estimated that in the USA around $3.6x10^6$ kg of selenium is released into the air. Soil additives such as ash, sewage sludge and fertilizers can also cause selenium enrichment [6].

The availability of selenium also depends on the pH, other elements and organic matter present in soils [7]. The Hawaiian topsoils for example contain an average of 2.7 mg/kg selenium, yet neither humans, animals nor plants show any sign of poisoning, while soils from Kansas, with lower than 1 mg/kg concentration can produce toxic vegetations. The reason behind is the high iron content of the Hawaiian soil that fixes the selenium and renders it unavailable [8].

2.2.1. Selenium in mushrooms

Healthy diet and nutrition is becoming a trend today, and it is important to be able to control what products, what compounds and in what form reach the consumers. While there are no specific regulations about higher mushrooms' selenium content and speciation, there are European Food Safety Authority regulations about the forms of selenium that can be used for supplementation. From 2009 the European Union released a series of decrees with a list of selenium compounds allowed to be used, which includes inorganic selenium forms (Na-selenite, Na-hydrogen-selenite, selenic acid, Na-selenate, Na-hydrogen-selenate), selenised yeast, L-seleno-methionine and its hydroxi analogue. Clearly, the importance of speciation did not

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elude the lawmaker organizations either. Therefore, any research aiming for the quantification of known selenium compounds or identifying new molecules is justified.

Enrichment of mushrooms with selenium has been documented since the 90s [9,10]. However, the observation of natural selenium accumulation in mushrooms dates back more than 30 years [11,12]. As reviewed recently [13,14], several mushroom species, including *Agaricus*, *Albatrellus*, *Boletus* and *Lentinula ssp.* can be considered as natural or artificial source of selenium. However, bioavailability studies [15,16] contradict in this statement. Selenium speciation should provide information necessary either to support or to decline the intention to use selenium containing mushroom in selenium supplementation.

Concerning the single cell fungi species, *Saccharomyces cerevisiae*, more than 100 selenium species have been identified with high species coverage (>90%) [17,18]. This is exactly the opposite of what is known about selenium speciation from higher fungi: up to now, only a few selenium species could be unambiguously identified in mushroom samples. Basically, selenomethionine, *Se*-methylselenocysteine and inorganic selenium are the forms detected with high performance liquid chromatography (HPLC)- inductively coupled plasma (ICP) – mass spectroscopy (MS) or HPLC-(UV decomposition)- hydride generation (HG) - AFS based techniques [19–23]. Also, most of the selenium usually remained either unidentified or in inorganic forms, even in cases when relatively high organic selenium concentrations were observed [19,24].

Recently, a mushroom species of *Pleurotus* genus (class *Agaricomycetes*) cultivated on selenium-rich wheat straw based compost has been found to contain 49% of accumulated selenium in the form of selenomethionine [25]. The reason for this high organic selenium ratio is uncertain as no speciation data is available for the straw, therefore, both the metabolism of inorganic selenium into selenomethionine and the uptake of selenomethionine from the compost could have occurred. Identification of other metabolites of the selenium biochemical pathways should be considered to reveal the origin of the abundant organic selenium compounds.

There is another selenium accumulating mushroom though, which attracted the interest of researchers. *Hericium erinaceus* (known as lion's mane mushroom), also belonging to the mushroom class *Agaricomycetes*, is the most widespread edible species of the genus *Hericium*. It is autochthonous in Eurasia, and regarded as a parasitic mushroom of deciduous forests. In Figure 1 pictures of different types of this mushroom can be seen. *Hericium erinaceus* has been used as medicine in China since the ancient times, and it is becoming popular worldwide due to the nutritional values attributed to its special polysaccharide composition [26–28]. Another feature of this mushroom species is its relatively high protein content (~23.8 g/100 g d.w.)

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[29,30]. In 2014 Wang *et al.* published a review [31] comparison about edible mushroom composition. Their results can be seen in Table 1.

Figure 1: *Hericium erinaceus*

Table 1: Proximate composition of some edible wild-grown mushrooms of China [31]

Proximate composition of some edible wild-grown mushrooms of China (mean values; % of dry matter)						
Species	Number of samples (n)	Carbohydrates	Crude fibre	Crude protein	Crude fat	Ash
Boletus aereus	1	34	17	26.9	2.1	8.5
B. edulis	1	30.6	15.3	28.7	4.1	9.2
B. speciosus	1	28.6	21	28.1	2.9	7.6
Lactarius deliciosus	1	25	36.3	20.2	2.5	7.5
Lactarius hatsudake	1	38.2	31.8	15.3	1	7.3
Lactarius volemus	1	15	40	17.6	6.7	13.3
L. crocipodium	1	12.8	37.9	29.3	1	5.8
Lentinula edodes	1	30.2	39.4	17.1	1.9	4.3
Russula virescens	1	13.4	32.8	28.3	1.5	11.9
Sarcodon aspratus	1	64.6	5.1	12	2.8	10.4
Tricholoma matsutake	3	36.7	29.1	14.3	5	8.9

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2.2.2 Selenium in plants

While except for certain algae selenium is not essential for plants, certain bacteria do need it for the synthesis of selenoproteins, but that strongly depends on the linage. Up to now, only Gram-positive bacteria have been found specifically needing Se for their life circle. Such proteins are, for example, formate dehydrogenase in *Moorella thermoacetica*, glycine reductase $P_{A\ in}$ *Clostridium sticklandii*, glycine reductase P_B in *Eubacterium acidaminophilum.* Unlike bacteria, fungi have lost selenoproteins during evolution [32].

Certain algae require Se for the production of selenoproteins, while higher plants have no such requirements [33]. Plants take up and metabolise selenium through the sulphur pathways because of the chemical similarity between the two elements; inorganic selenium compounds are reduced and converted into organic forms this way. The first species produced this way is selenocysteine. As this amino acid might be non-specifically incorporated into proteins rendering them dysfunctional, high levels of this compound can be toxic. To prevent selenosys, selenocysteine can be methylised into *Se*-methylselenocysteine, a non-toxic molecule as it cannot be accidentally incorporated into proteins. Selenocysteine may also enter the methionine synthesis pathway, resulting in selenomethionine, which like selenocysteine can be incorporated to various proteins, but the side effects are less harmful. Another pathway is to convert selenomethionine into dimethylselenide then dimethyldiselenide, which are volatile compounds used for Se-excretion. Plants with a tendency to accumulate S, like the *Brassica* species (cabbages and mustard) will likely accumulate Se too. These plants have no specific Se-pathways, they merely accumulate Se as a side effect. On the other hand, there is a group of plants that are Se-hyperaccumulators. They are found only in seleniferous areas, and they preferably take up Se over S, accumulating it up to 1% of dry weight, but to date no evidence has been found that these plants need Se for their metabolism [34].

2.2.3 Selenium in humans

In short, the amount of selenium available for humans depends on what is available from food: meat and plants (mostly crops). The source of selenium for animals is the feed they consume, so everything boils down to the plants grown in the region. According to literature, the average daily selenium intake all over the world varies between 10 and 200 µg, but if the areas with extremely high or low soil selenium content are included, these values range from 3-6500 µg/day. The richest sources are Brazil nuts, fish (salmon, halibut, tuna), sunflower seeds, shellfish, meat, eggs and certain mushrooms. Fifty percent of the selenium intake comes from five groups of foods: crops (bread), meat, poultry, fish and eggs.

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The daily intake falling outside of the suggested limits (50-200 μg) for an extended amount of time has grave consequences [35]. The Keshan disease was first described more than a hundred years ago, but it took several decades before the reason behind it was found. This disease had a limited geographic distribution, covering the area from northwest to southwest China, and it was caused by the soils being acidic with a high organic matter and iron oxide content that resulted in the fixation of selenium in forms which are poorly absorbed by crops. This caused a selenium deficiency in the inhabitants. Typical manifestations are loss of appetite, fatigue after even mild exercise, cardiac arrhythmia and palpitations, cardiac insufficiency and heart failure. The illness may appear after only three months exposure to selenium deficient food; but once established, selenium is of little or no therapeutic value. On the other hand, oral administration of selenium three months before the periods of exposure is highly effective. The symptoms and the number of patients showed fluctuation depending on seasons and weather (among other things), something typical of virus infection and not malnourishments [36]. Therefore, further experiments were executed and Chinese scientists found that the heart failure was the result of Coxsackie virus infections. In 2004, Melinda A. Beck *et. al.* completed a research about the connections between selenium deficiency and the symptoms caused by the infections [36]. They found that in selenium-deficient mice the non-virulent strain of the virus went through a mutation that resulted in the virulent strain. After the mutation, it caused sickness in non-selenium-depleted mice too. The Kaschin-beck disease is similar to the above-mentioned disease, and it is also described in regions of insufficient selenium-availability. This disease has been detected in children aged 5-13 years in China. Typical signs are the necrosis of joints. The limb joints are degraded, resulting in structural shortening of fingers, bone growth retardation and stanting. The selenium content of hair and blood became abnormally low. Selenium supplementation can lessen the symptoms. By 1986, the ratio of children suffering from this syndrome was reduced from 44% to 1%, which is attributed to improved food supplementation of the area [37].

Since then several other important roles of selenium have been discovered and several more sicknesses have been directly or indirectly ascribed to its deficiency, such as adult diabetes, grey cataract, column ulcer or cystic fibrosis.

Since replacement of S by Se in proteins and other S compounds disrupts the function of these molecules, selenium is toxic at elevated levels to most organisms. The difference between the amount of Se required as a nutrient and the amount that is toxic is small; as a consequence both Se deficiency and toxicity are common problems worldwide.

Studies focusing on Keshan disease and selenosis have shifted focus onto the importance of selenium in human metabolism. It was found that this microelement has a role in protecting

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body tissues from oxidative stress, in regulation of growth and development and in the defence against infections.

2.2.4 Selenoproteins and selenoenzymes

To understand the effects of selenium in human health, we have to take a quick look into the human selenium metabolism. These pathways can be separated into two main groups: regulated selenium metabolism, and non-specific incorporation. The reason selenium is a microelement essential for the human body is the presence of the selenocystine-containing enzymes [8]. These enzymes lose three orders of magnitude of their activity if Se replaced by S. Selenium deficiency symptoms are consequences of the lack of properly working Se-enzymes. The human body has only a limited capacity to store selenium safely [38]. Once the storage options are exhausted, which happens rather fast, Se is nonspecifically incorporated into body tissues in place of S. These substituted molecules lose their ability to function, causing selenosis. Selenocysteine, inorganic selenate and selenite enters directly the regulated selenium metabolism. Given that selenocysteine poses a high risk if it is incorporated nonspecifically, all of these forms are first transformed into H_2Se where the pathways diverge: one leads to selenium excretion through *Se*-methyl-N-acetylselenohexosamine (MSeAcG) and $(CH_3)_3Se^+$ in urine or through $(CH_3)_2Se$ in breath; the other leads to selenoprotein synthesis through Se-phosphate and selenocysteine tRNS. Selenomethionine, on the other hand, enters the amino acid pool and goes to the non-specific incorporation pathway unless no other, more easily available selenium source is present.

Up to now four main groups of selenoenzymes have been established. The first one contains peroxidases and thioredoxin reductase, responsible for controlling reactive metabolites carrying oxygen, which are necessary for the cell's defence against infections but dangerous if overproduced. The mechanism of cytosolic enzyme glutathione peroxidase (GSHPx) family was first described in 1973. In cases of infection, stress or tissue injury, they protect against oxygen-rich free radicals, destroying hydrogen-peroxide and lipid-hydrogen peroxides [39].

The second group of selenoenzymes has a crucial role in converting thyroxin or tetraiodothyronine (T4) into their active form, triiodothyronine. Thus, selenium deficiency can result in iodine shortage, causing complex physiologic problems, like in the case of Kaschin-beck disease [37].

About 60-80% of selenoprotein in human plasma belongs to the group of selenoprotein P. Selenium plays an important role in bone physiology, also demonstrated by Kashin-Beck disease. The already described symptoms, such as disfigured growth and stunting are the result of delayed bone development caused by mutations in selenocysteine binding protein 2 (SBP2).

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This binding protein is a central factor for selenoprotein biosynthesis. While the function of circulating selenoprotein P (SePP) for bone homeostasis is not yet known, it is positively associated with bone turnover in humans. Bone Se is found exclusively in the organic matrix. In 2014 Nicole Pietschmann *et al.* analyzed murine models of altered Se metabolism. They found that most of the known selenoprotein genes and factors are expressed in bones, and they are needed for the selenoprotein biosynthesis. Their data highlighted the importance of selenoprotein P in the Se transport to bones. The results also implied that there is a hitherto unknown feedback mechanism for preferential uptake of Se in Se-deprived bones [40]. Several other selenoproteins exist. One is the component of the mitochondrial capsule of sperm cells, the lack of which causes deformed and dysfunctional sperm cell production, decreased progenitivity. Michaelis *et al*. found that selenoprotein P is also needed for sperm production [41]. They examined selenoprotein P knockout mice, and found them to be infertile. They also examined the seminal plasma from different donors and found that selenoprotein P concentrations correlated positively to sperm density and fraction of vital sperm.

Enyzmes similar to the ones described above can be found in all mammals as well.

Another one of the most abundant selenoproteins is selenoprotein-W (SeW) This protein can be found in various animals and humans, including rats, mice, monkeys, sheep pig, fish and chickens. It shows the highest expression in skeletal muscle and heart (with the exception of rodents). The sequences of selenoprotein-W are identical in rats and mice, as well as in monkeys and in humans [42]. Up to date the rodent selenoprotein-W is the only one reported of containing four cysteines, others contain only two cysteines. In all eight species of animals, cysteine is present at residue number 9 and selenocysteine at residue number 13. The biological function of selenoprotein-W has not yet been indisputably described. It has been reported that it can serve as an antioxidant, responds to stress, it is involved in cell immunity, it is the specific target for methylmercury, and has thioredoxin-like function [43].

2.3. Selenium speciation analysis

It did not take a long time for researchers to realise the importance of selenium in human health; and soon focus shifted to it. At the dawn of selenium analysis only the total amount of selenium was determined. However, researches in the fields of other elements have highlighted the huge differences in biological effect between different forms. For example, while inorganic arsenic compounds are highly toxic, arsenobetaine passes through the human body without being metabolised. While inorganic tin is considered non-dangerous for human health, the organic

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forms are highly toxic; therefore, they have to be measured separately. With that realisation the science of analytical speciation was born [8].

Finally, the International Union for Pure and Applied Chemistry (IUPAC) has published guidelines [44] or recommendations for the definition of speciation analysis:

"Speciation analysis is the analytical activity of identifying and/or measuring the quantities of one or more individual chemical species in a sample. The chemical species are specific forms of an element defined as to isotopic composition, electronic or oxidation state, and/or complex or molecular structure. The speciation of an element is the distribution of an element amongst defined chemical species in a system."

2.3.1. The importance of selenium speciation

In case of selenium, there are no such huge differences between the lethal doses of the different oxidation states as in that of arsenic and tin. All forms can be metabolised and used in a selenium-deficient diet [35]. Once the intake reaches the absolute minimal necessary level, what happens with the "surplus" depends on the speciation of the given component. Biological availability, accessibility and possible accumulation are all speciation-dependent; given the small margin between the necessary and lethal selenium doses this is a reason for concern and further research.

It was Clark *et al*. who first recognised a connection between an increased Se-intake and cancer prevention in 1996 [45]. They completed a multicenter, double-blind, randomized, placebo-controlled cancer prevention trial in seven dermatology clinics in the eastern United States. They examined all-cause mortality and total cancer mortality, total cancer incidence, and the incidences of lung, prostate and colorectal cancers. Their results supported the hypothesis that supplemental selenium may reduce the incidence and mortality of carcinomas. To confirm or contradict this statement the so-called SELECT project was started in 2005 [46]. However, before they could complete the program it had to be terminated early – many of the patients developed diabetes as a result from the administration of high enough levels of selenium and vitamin E [47]. These two studies might seem antinomic. However, the latter study neglected a number of important factors. Clark has chosen his test subject from a population base of with a history of basal cell or squamous cell carcinomas of the skin, while the latter study examined average, healthy individuals. In the first study, patients received the selenium supplementation in form of selenised yeast. This contained 80% of selenomethionine, and a huge number of still undiscovered organic selenium-compounds. Later in 2005, patients were simply fed selenomethionine. The first study worked in areas that had a varying degree of selenium-

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deficiency, the latter one picked their subjects from well-supplemented areas. Therefore the results cannot be compared, it can only be concluded that selenomethionine by itself has no anti-carcinogen effects.

Once anti-cancer effects have been attributed to selenium, several selenium-enriched products appeared on the shelves. They do not only differ in prices but also the speciation of the Se-containing compounds.

From those products that contain selenates only about 25% of all selenium-content is biologically available; the rest leaves the body through urine without alteration [48]. While inorganic selenites are biologically available, during uptake reactive oxygen radicals are formed, resulting in possible carcinogen effects [48]. Some products are claimed to contain organic selenium. However, many of them contain selenomethionine in largest quantities, the amino acid that is mostly used for non-specific selenium incorporation. While it can be used for selenium supplementation to prevent selenium-deficiency, in extra quantities it merely enters the protein synthesis pathways displaying no anti-carcinogen effects, and causing tissue selenium enrichment, something undesirable, potentially leading to selenosis. Whanger [49] and Block [50] both came to the conclusion that *Se*-methylselenocysteine and γ-glutamyl-*Se*-methylselenocysteine are the most effective anti-cancerous agents. Whanger used Se-enriched ramps to feed rats, and found that there was a ~43% reduction in chemically induced mammary tumours. Block used Se-enriched garlic and yeast to feed rats. He found that daily supplementation with selenised yeast (Se-yeast) led to a decrease in the overall cancer morbidity and mortality by nearly 50%; past research has also demonstrated that selenised garlic (Se-garlic) is very effective in mammary cancer chemoprevention in the rat model.

This is only true for mammals, but it was found that for birds and fishes it is the inorganic selenium that shows lower toxicity.

2.3.2. The importance of chirality

The issue of chirality is also of great importance. Almost exclusively the L-selenoaminoacids can be found in nature, but when they are produced chemically, a 50%-50% racemic mixture is the result. However, racemization can occur around 100 ^{o}C, and the L-form is transformed into the D-enantiomer. Given that most food preparations include heat treatment of some kind the presence of the D-enantiomer in human metabolism cannot be ignored.

If significant percentages of the protein-bound amino acids are in the D-configuration the digestibility of the proteins decreases because of the stereospecificity of proteinases and peptidases [51]. The absorption can discriminate against D-amino acids [52] and the bioavailability of all amino acids can be diminished due to the lower D-amino acid oxidase

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activity [53,54]. The efficiency of D-amino acid oxidases largely varies with species, age, organ, tissue and the substrate. It was found [53] that mammals can utilize only small ratios of the D-amino acids, and in some cases the D-stereoisomers of the essential amino acids caused growth inhibition and were mainly excreted through urine.

Table 2: Reports on the enantiomer ratio of selenomethionine in different matrices. LOD denotes for limit of detection

Reference	Sample preparation	Instrumentation	Sample	D/L ratio
Mendez *et al*. [55]	Protease	GC-ICP-MS	Selenized yeast	15:85
Mendez *et* al. [56]	Protease	HPLC-ICP-MS	Selenized yeast	18:82
Sutton *et al.* [57]	Gastric digestion	HPLC-ICP-MS	Selenized yeast tablets	D < LOD
Mendez *et al.* [58]	Protease	HPLC-ICP-MS	Selenized yeast	17:83
Montes-Bayón *et al.* [59]	Proteinase K	HPLC-ICP-MS	Selenized yeast	D < LOD
	Proteinase K and aminopeptidase M		Selenized yeast tablets	D < LOD
Day *et al.* [60]	Proteinase K	CE-UV-ICP-MS	Selenized yeast	D < LOD
Devos *et al.* [61]	Acidic digestion then extraction with 0.1 M HCl and chloroform	GC-ICP-MS	Selenized yeast formulation	~1:99
Bergmann *et al.* [62]	Water extraction	HPLC-ICP-MS	Antarctic krill	D < LOD
Huang *et al.* [63]	Water extraction	HPLC-UV-VIS	Selenized yeast	7:93
			Garlic	D < LOD
Gómez-Ariza *et al*. [64]	Fat and protein elimination	HPLC-MAD-HG-AFS	Breast milk	D < LOD
			Formula milk	26:74

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For D-methionine the value of relative oral bioavailability (RBV) is only 30% in humans [65]. From a nutritional point of view, racemization could result in the loss of protein, which is one of the most important components of food [66]. In general, D-amino acids are non-bioavailable for humans and most living organisms. The reason behind this is that "life itself is chiral". Our own proteins are all formed from the L-variety amino acids, and our metabolism is only equipped to deal with this formation. Proteins that contain the D-enantiomers do not fit into our enzymes and cannot be digested. The undigested part lowers the value of the protein rendering more essential amino acids unavailable. Such sources are heat-treated protein products, especially if the treatment is combined with a higher pH, like in case of ultra high temperature treated milk.

If D-amino acids reside in toxins, their toxicity is elevated by an order of magnitude. Studies indicate the different uptake of the two selenomethionine enantiomers [67] but similar bioavailability [68,69] in mice and rats. In humans, the bioavailability of D-methionine is considered almost equal to that of L-methionine [70]. However, utilization of D-selenomethionine in humans seems to be poorer compared to that of L-selenomethionine [71,72].

Accordingly, the ratio of D- and L-selenomethionine content of food and food related products can be an important quality parameter. This is of special significance in case of selenised feed and food supplements where the replacement of organic selenium source: selenised yeast. This is the most widespread, European Food Safety Authority -approved food and feed additive that can used for Se-supplementation. Therefore, it is used by thousands of tons each year. In addition, through the animals, it enters the human food chain. The European Food Safety Authority order also decrees that at least 60% of selenium must be in the form of selenomethionine and the concentration of inorganic forms cannot exceed 1% of total Se-content.

When one single amino acid enters the human food sources in such high quantities, even a small scale of racemization can be a reason for concern; not to mention cheaper, synthetic and racemic selenomethionine that is sometimes added to the feeds, and inherently influences product quality. This is something to be monitored closely. A reliable and robust method, that avoids racemisation, is needed not only for the detection but also for the sample preparation. The importance of amino acid chirality is highlighted by the fact that the European Food Safety Authority (EFSA) has also published its opinion on the use of the natural, L-enantiomer of selenomethionine [73]. However, so far no law, order or decree has been released with an official sample preparation method.

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Former studies have determined the enantiomer ratio of selenomethionine in different matrices.Table 2 shows the obtained direct quantification result [55–64]. These attempts applied various sample preparation and detection methods and resulted in several controversial achievements. The D,L-enantiomer ratios reported by different research groups do not only show remarkable differences, but so far also no relation has been established between the sample preparation and the reported D,L-ratio.

Considering these examples, it is obvious that the determination of total selenium content is far not informative enough to base decisions on, especially when human health is concerned.

2.3.3. The history of selenium speciation

In early times the full speciation of selenium required months of work. Without mass spectrometry detection available the structure had to be determined through a number of different chemical reactions and measurements, drawing conclusions from them. That is why relatively large amounts of the target component had to be purified from the samples, to provide sufficient mass for the reactions. One good example is the work of Barak [74], who completed the determination of selenomethionine from rat liver using paper chromatography coupled with neutron activation analysis. The samples were extracted with a special mixture of organic solvents, filtered, centrifuged, lyophilized, dissolved and placed on filtrate papers. They were irrigated for twelve hours, activated with ninhidrin, and the selenomethionine-containing peak area had to be cut out from the paper, irradiated and analyzed with a γ-ray spectrometer. The method was not only tedious and long, it was hard to reproduce; the chromatograms were destroyed during the process and the irradiation had strong interference with the O_2 present.

Even a decade later, there was not much progress with the speed of speciation. In certain cases, it was not even attempted to identify the organic forms. However, it has already been recognised that the different oxidation state of the elements can have different levels of toxicity, especially in samples where the number of organic compounds is limited. This allowed for quicker methods to be developed and be used for different control purposes. Numerous articles have been published about the oxidation state speciations from different samples: drugs, foods, river and drinking water. They were usually performed with energy-dispersive X-ray fluorescence after different preconcentration steps [75]. Depending on the method applied, the oxidation state could change during preconcentration and all data regarding molecular weight or structure were lost in the process. A few years later HG AAS also became popular and gave more accurate and faster results. At the time, however, with most trace elements both fractionation methods and analytical procedures had to be combined and speciation had to be carried out by determining the elemental content in the separated fractions [76].

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The appearance of both HPLC and ICP-AES meant a huge step forward in selenium speciation. While the importance of speciation had been understood for many years, it was only around this time that technical requirements became available complete such studies. While the detection of selenium with ICPremained non-specific, and lost all data relevant to molecular structure, the possibility to couple it directly to a separation technique allowed a relatively easy and fast determination compared to previous techniques. The new chromatographic methods shortened the elution time from twelve hours to about twenty minutes, and sped up not only the determination itself but the method development too. The relatively high detection limits of AES required both sample pre-concentration and the use of former methods, *i.e.*, electrothermal AAS and neutron activation analysis. The drawback of the application of these more sensitive methods was that they worked with discrete sample volumes and had a discontinuous monitoring of the chromatographic effluent [77].

Atomic absorption spectrometry (AAS) and atomic fluorescence spectrometry (AFS) were also very popular as they could be used in conjunction with hydride-generating (HG) sample introduction to achieve suitable signal-to-noise ratios. Dozens of articles applying these instruments have been published. For example, in 1994 Pitts published his work on an on-line system for the determination of inorganic selenium species [78]. In 2013 six further articles used AAS for selenium speciation including the work of Sun *et al.* on the speciation of organic and inorganic selenium in selenium-enriched rice by graphite furnace AAS after cloud point extraction [79]. The method is rather sensitive, and the detection limits can be lowered by the application of HG, though it has certain drawbacks. HG process requires the selenium to be in the selenium (IV) oxidation state, the reduction of any selenium(VI) atoms present in the sample is necessary. This step is conventionally accomplished by maintaining the sample at an elevated temperature in the presence of hydrochloric acid. Although this approach suffers from a number of disadvantages including the loss of information on the original selenium species present in the sample [78]. Furthermore all information on molecular structure is lost.

Nuclear magnetic resonance (NMR) was the only available method for molecular-level detection or identification at the time. However, it was mostly used for examining already known components, since for a successful experiment several milligrams of pure compound are needed. Such large amounts require immense amounts of sample and sample preparation. It was used in 1988 by Isab [80] for the characterization of selenomethionine. It was much later, only in 1998 that Fan *et al.* could use the technique for structure characterization of selenium metabolites [81].

At the beginning of the 1990s the advent of ICP-MS suddenly lowered the detection limits by several orders of magnitude and enabled isotopic separations and the measurement of different isotopes.

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Due to the destructive nature of ICP-MS, no information of molecule composition can be acquired, let alone about molecule structure. It may seem to be an unfit method for speciation. However, with the appearance of this hyphenated method the number of speciation articles published sky-rocketed, using ICP-MS with indirect speciation methods.

Block *et al.* compared different Se-enriched organic products and used liquid chromatography (LC) and gas chromatography (GC) - ICP-MS for the identification of organic Se-species [82]. Also in 1997, Bird could separate twenty different organic selenium compounds from Se-enriched yeast with IP-RP-HPLC, identifying selenocystine, selenomethionine and methylselenocysteine with the use of standards [83]. Unfortunately, he had no proposition for the rest of the components. In case of measuring volatile compounds, GC-ICP-MS became popular, not only because of the low detection limits, but also because of the ability of the GC system to transfer the entire injected sample to the detector. No loss of sensitivity caused by low efficiency nebulizers applied with HPLC had to suffer from. Species detection by ICP-MS brought a previously unseen, large linearity range rise covering several orders of magnitudes. On the other hand, selenium detection with ICP-MS suffers from problems including low ionisation efficiency and isobaric interferences. The former can be resolved by using ionization-promoting solutions, the latter by the application of collision cells.

Detection limits can further be lowered by ultrasonic nebulisers or post column HG. The greatest disadvantage of the ICP-MS method is still its standard-dependence, as the identification is usually based on retention time signal and spiking [84]. Since this new method still did not allow complete speciation, other systems were often used in parallel, such as X-ray chromatography, HG AAS, chatodic stripping voltammetry, *etc*. It was only later observed by Dernovics et al., that certain conditions may cause artefact formation, for example, acidic pH with methanol present can cause methylation. If such conditions were present ICP-MS results were easy to misinterpret [85].

The real significant change in Se speciation came with the application of molecular MS that enabled entire molecules to be measured and examined, though ICP-MS retained its leading role in identification until this technology, the so-called soft ionisation, become more widely available. The most user-friendly and efficient ion-source was the ESI-MS. Selenium has a typical, easy-to-recognise isotopic pattern, which makes MS the ideal tool for discovery, verification and quantification in Se-speciation. Identification and exclusion of “false positive” Se-containing compounds is further facilitated by the relatively large mass defect of selenium. The Se-isotopic pattern can be seen in Figure 2a. The figure also contains a mass spectrum of a Se-containing compound, *Se*-dimethyl-5-selenonium-adenosine, (2b) where the phenomenon of the mass defect can be observed. The isotopologues 359 and 361 are the results of the molecule

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containing ^{13}C isotope. That is why there is no corresponding line in Figure 2a. In Figure 2c the structure of this molecule can be observed.

In comparison Figure 2d presents the mass spectrum of sulphur, and 2e shows the isotopic pattern of the S-analogue of the above mentioned compound, *S*-dimethyl-5-adenosine. The differences between the mass defects and isotope-pattern complexity of the S and Se analogues can be observed.

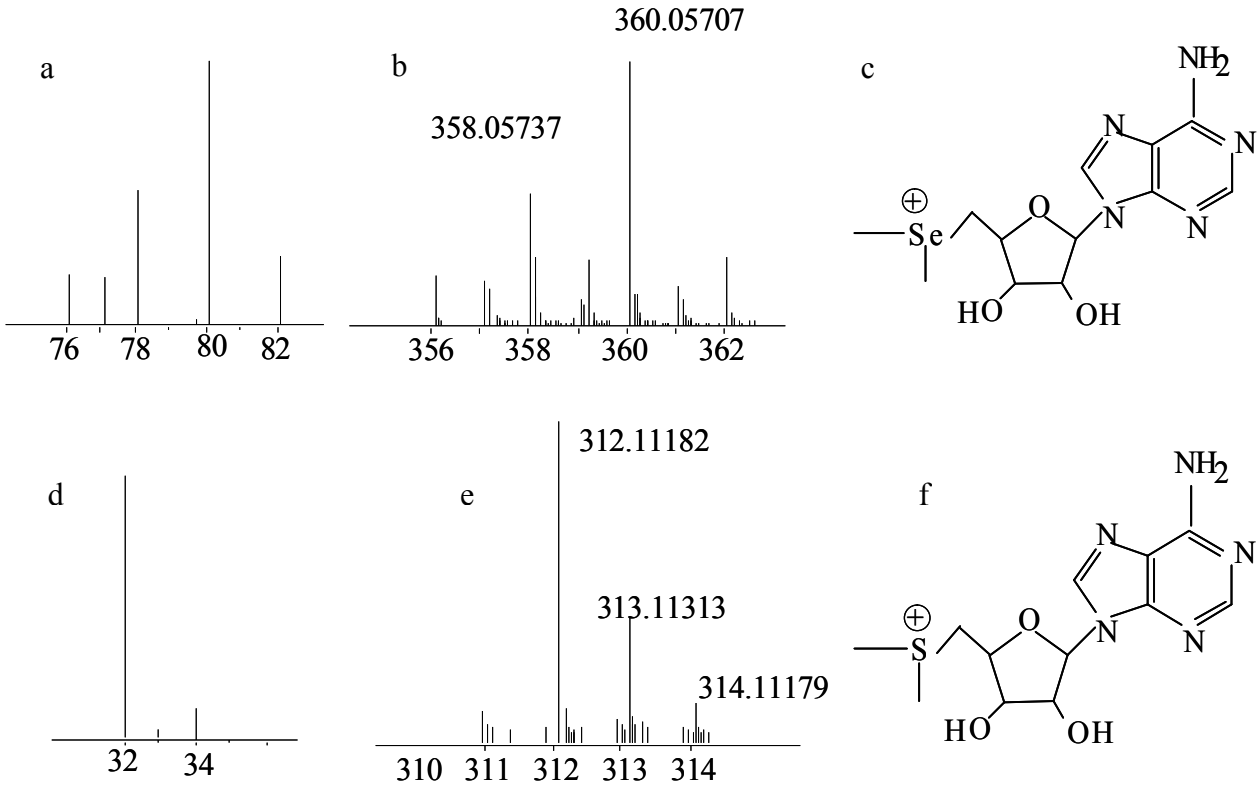

Figure 2: 2a: The isotope distribution of Se. 2b: the isotopologue distribution of a Se-containing molecule, *Se*-dimethyl-5-selenonium-adenosine. 2c: molecule structure of *Se*-dimethyl-5-selenonium-adenosine. 2d: The isotope distribution of S. 2e: the isotope distribution of a S-containing molecule, *S*-dimethyl-5-adenosine. 2c: molecule structure of *S*-dimethyl-5 – adenosine

In 1996 Crews was trying to identify unknown Se-compounds from a cod extract. In the process she injected selenomethionine, selenocystine, sodium selenite and sodium selenate standards into an ESI-MS system. While the standards were recognisable, she could not complete the identification of new components [86].

First it was used in 1999 by Casiot *et al.* who published an article on the identification of *Se*-adenosylselenohomocysteine, the major selenium species in an extract of a selenised yeast

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sample. Even though the mass accuracy still left a lot to be desired, another identifying attribute has been discovered: the fragmentation pattern [87].

The same year Kotrebai *et al*. used ICP-MS and ESI-MS in parallel experiments to identify major selenium compounds from Se-enriched yeast and garlic. It was found that selenomethionine and *Se*-adenosyl-selenohomocysteine made up 85% of the selenium content of garlic, and γ-glutamyl-*Se*-methyl-selenocysteine and γ-glutamyl-selenomethionine contributed to 90% of selenium in garlic [88]. Later that year, with the help of different colleagues, Kotrebai completed the optimization of an HPLC-ICP-MS system for the separation of Se-containing components extracted from organic sources. Once baseline separation was achieved, they applied the HPLC method for the characterization of amino acids with the use of ESI-MS [89]. A year later, also Kotrebai *et al*. has examined several ion-pairing agents for the separation of Se-containing compounds extracted from natural sources. With the optimized method and the γ-glutamyl-*Se*-methylselenocysteine and *Se*-methylselenocysteine standards oxidized with H_2O_2, several new oxidized products were identified from the extracts, using ESI-MS [90].

Even to date the leading indirect identification and speciation method is based on fragmentation-pattern, which by 2000 allowed McSheehy *et al*. to identify γ-glutamyl-*Se*-methylselenocysteine from garlic harvested in naturally seleniferous soil without the need for an authentic standard [91]. They also discovered a number of Se-containing compounds that they were unable to identify. In 2002 McSheehy *et al.* reported a large number of new Se-containing compounds found in selenised yeast but she could not yet identify their structure [92]. The same year Ogra *et al.* used ESI-MS detection for the identification of a new Se-containing compound, *Se*-methyl-N-acetylselenohexosamine from rat urine [93]. The method was also used for the characterization of non-protein selenium compounds from selenium accumulating plants by Montes-Bayón [94]. In 2002 Vonderheide *et al.* has identified several metabolites from Brazil nuts, including selenomethionine [95]. In 2007, coupling ESI with tandem MS enabled Ogra et *al*. to identify selenohomolanthionine from pungent radish, detecting this component from natural sources for the first time [96]. ESI-MS is a widely used method even today and a multitude of different samples have been examined with it. The drawback of the method is that molecule composition cannot be determined solely based on molecular mass, as the mass accuracy of the instrument might not be high enough. While ICP-MS is a robust method, and samples could be analyzed after minimal sample treatment, ESI-MS requires further purification, and the linear range of detection is much smaller.

The problem of the mass accuracy could be solved when the previous Q-MS and QQQ-MS systems were upgraded to time of flight (TOF) system. Molecular weight data acquired with a quadrupole time of flight (QTOF) detector is accurate enough to use for the

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determination of molecule or fragment composition. This higher mass accuracy coupled with the fragmentation databases may provide enough information for the accurate determination of most compounds. In 2008, with the help of higher mass accuracy of QTOF Dernovics *et al*. [97] was able to tentatively assign structure to the Se-compounds McSheehy *et al.* had discovered in 2002 [92].

Another of the soft ionization techniques is matrix-assisted laser-desorption/ionization (MALDI). Owing to a lower detection limit and superior matrix tolerance to electrospray MS, MALDI allowed a successful detection of selenocompounds in samples where ESI-MS had failed. In 2003 Encinar *et al.* [98] found several previously unidentified Se-containing components in selenised yeast, even though they did not have a proposed structure for most of them yet. The higher mass accuracy and better separation techniques also allowed previous works to be re-observed, finding new compounds, unveiling artefacts and indentifying so far unknown components. During the following years, several articles were published identifying selenoproteins with this method. The advantage of the technique is the high mass accuracy; while its drawback is that in numerous occasions the presence of Se cannot be confirmed. That is why in many cases ICP-MS is used in parallel, to gain quick and accurate information about the retention time, the concentration of the Se-containing compound, its location in gel electrophoretic media, artefact formations and enzymolysis. That was the case in 2007 when Ballihaut *et al*. reported his findings on the detection of bovine glutathione peroxidase selenoprotein using several hyphenated techniques [99].

One of the numerous selenoproteomic studies must be highlighted. In 2005 Giusti *et al.* has examined the efficiency of enzymatic digestion with trypsine [100]. The digest was separated with HPLC, and the effluent was split into two branches, one going to ICP-MS for quantification with isotope dilution, the other entering ESI-MS for identification. Since the tryptic peptides, miscleaved and/or oxidized peptides, incompletely digested protein and undigested protein could be determined in one run, the method allowed the precise evaluation of the efficiency and quality of tryptic digestion using several nanolitres of sample only.

In 2006 a new detector, the hybrid linear ion trap/orbital ion trap Ms^n has appeared on the market, raising mass accuracy into unseen heights, which facilitated molecular structure identification. The coupling of a normal bore (4.6 mm) hydrophilic interaction liquid chromatography (HILIC) column with a hybrid linear ion trap/orbital ion trap mass spectrometer allowed the detection of the selenium-isotopic pattern in mass spectra down to the intrascan abundance of 0.001 with the low-and sub-part per million (ppm) mass accuracy regardless of the concentration [101]. In 2010 a method was developed for low-molecular weight component measurements, such as cysteine, homocysteine, selenocysteine, glutathione, selenomethionine

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and cysteinyl-glycine. With the Orbitrap instrument their detection limits fall into the fmol range [102]. In 2011 a novel Se-metabolite was discovered from human urine despite of the concentration level barely reaching ppb magnitude [103]. A year later Arnaudguilhem *et al.* [17] identified numerous Se-containing components discovered by McSheehy in 2000 [91]. The method developed enabled the detection of 64 metabolites including 30 SeSe or SeS conjugates (3 triple S/Se/S ones) and 14 selenoethers. Aureli *et al.* [104] discovered nine selenosugars from staple crops grown on soils naturally rich in selenium. Among the identified compounds, Se-containing monosaccharides (hexose moiety, *m/z* 317 and *m/z* 358) or Se-containing disaccharides (hexose-pentose moiety, *m/z* 407 and *m/z* 408) were the first selenosugars reported in edible plants. In 2013 Ouerdane *et al.* [105] could detect and characterize over 30 Se species, also with the help of the Orbitrap, from black mustard seeds (*Brassica nigra*) grown on Se-rich soil.

To date hybrid linear ion trap/orbital ion trap Ms^n has remained the most effective and most accurate method for detection. However, it must not be forgotten that simpler techniques are often needed for effective sample cleanup. In certain cases, with high enough target compound concentrations and well-known fragmentation patterns simpler and cheaper detections may also be sufficient.

2.4. Sample preparation for selenium-speciation

Speciation analysis, naturally, requires sample preparation. The more sensitive and accurate the analytical system the cleaner the samples have to be to enable measuring. There are a number of different methods widely used in Se-speciation; which one is chosen for a particular sample depends on different factors, like complexity, fat content and selenium concentration of the sample, whether the sample is liquid or solid, what kind of instrumentation is intended to be used, what type of components are expected to be found, *etc*.

2.4.1 Complete digestion

It might seem redundant to talk about complete sample digestion in speciation, as the method is supposed to bring all different species into the same form, losing all information even on the oxidation state of selenium let alone the molecule structure. Yet, it is a method rarely missing from speciation articles, because it gives a frame for the entire analytical process. With the determination of total selenium content the efficiency of extraction can be monitored, the

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recovery of Se-compounds can be calculated, and it enables making a decision if speciation analysis is needed at all.

In case of Se-containing compounds only closed digestion systems can be considered regardless of the method used, as Se is prone to hydride formation, resulting in volatile compounds. The chosen method has to be efficient enough to transform all species into the same form, as different oxidation states not only have different stability, but usually can be measured with different sensitivity too, yielding misleading results. In certain cases when HG is required before detection the sample needs to be reduced so Se would be present in Se(IV) form, as the result of most digestion methods, and Se(VI) is incapable of hydride formation [106].

By today, nearly exclusively strong, often oxidising acids are used for complete digestions, such as the mixtures of HNO_3, HCl, many times with the addition of H_2O_2. The dominant method for digestion became the high-pressure microwave treatment, and with reason. The available sample holders are produced from PTFE that resist high temperature and pressure, low and high pH, and with the digester system the procedure can be highly automated, closely monitored and each sample's pressure and temperature change diagram is easy to acquire. There are several publications appearing each year. In 2013 Funes-Collado *et al.* [107] used complete acidic, microwave digestion for the determination of total Se-content during their study of selenocompounds from selenium-enriched culture of edible sprouts. In 2010 Vale *et al.* used microwave pressurized acid digestion for total selenium and selenium species control in Se-enriched food supplements [108].

2.4.2. Extraction with different solvents

The primary aim of sample preparation in speciation analysis is to be able to extract target components without causing a change in their speciation. Methods complying with the unaltered speciation requirements are usually gentle, at the cost of much lower extraction efficiency [109]. This might not be a problem as in certain cases 100% extraction is not the goal, merely the preservation of certain compounds. On the other hand, some components are so strongly bound that 100% extraction is impossible without analyte decomposition. The expected efficiency depends more strongly on the sample type than the method itself. The sample preparation techniques have been developed in parallel to the analytical methods and certain well-working procedures are used as standard methods in comparison with the newly developed preparations. As a result, each sample type has been subjected to different treatments [104]. With certain samples a preferred, more effective procedure can be chosen but in many cases the

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differences are negligible. In certain cases [110] if the removal or enrichment of several different components is desired, sequential extraction is used.

In Se-speciation there are some limiting factors regarding the methods that can be used. First, at high temperatures (70-80 °C) Se(IV) can be oxidised into Se(VI). Second, the chemical properties of most Se-containing compounds are unknown, therefore simpler and gentler methods are preferred.

The gentlest preparation is considered to be aqueous extraction with deionised water or a buffer [111]. The solutions usually are in the biological pH-range (5.9-9), usually shaken for a few hours from room temperature to 80 °C [112]. This method can extract unbound inorganic Se salts, free amino acids and their derivatives, and in case of neutral pH, 10-30% of proteins. This extraction can be executed not only to acquire the above mentioned components, but also to remove them to prevent their effects in following sample treatment, for example the enzyme inhibition of certain inorganic metal ions. Sometimes other additives are used in the extracting solution for different purposes, for example NaN_3 as a bactericide and enzyme inhibitor, β-mercaptoethanol as antioxidant and reducing agent, sodium dodecyl sulphate (SDS) as a denaturalizing agent, *etc*.

In 2010 Qin *et al.* used phosphate buffer to extract Se-species from soil [110]. The same year Cuderman *et al.* used different buffer concentrations for the extraction of Se species in buckwheat sprouts grown from seeds soaked in various Se solutions [112]. In 2009 Wang *et al.* used buffer solutions for the extraction of selenium species in pharmaceutical tablets using enzymatic and chemical methods [113].

Another group of solvents used for Se-speciation is the one of hydrophilic organic solvents, such as methanol and ethanol. To date, not many hydrophobic Se-species have been found so far. In 2013 Németh *et al.* [114] used extraction with organic solvents during their work of examining the relationship of selenium tolerance and speciation in *Lecythidaceae* species. The same year Ouerdane *et al*. [105] discovered a group of Se-species with limited lipophilic character. They examined black mustard seeds *(Brassica nigra)* grown on selenium-rich soil. Careful system optimisation enabled them to detect and identify more than 30 Se species using ESI MS, including a large number of minor (<0.5%) metabolites. Selenoglucosinolates species made up at least 15% of the total Se present and over 50% of all the metabolites. Compounds belonging to this group were found to be particularly unstable during aqueous extraction. However, they could be efficiently recovered by extraction with 70% methanol. As the number of even partially lipophilic Se-species is very limited the primary use of organic solvents in sample preparation for Se-speciation is not for extraction but for cleanup. High concentrations of the organic solvent can be used for the removal of the fat content of the sample.

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Kannamkumarath *et al*. [115] used chloroform-methanol mixture for defatting nut samples and Gómez-Ariza used chloroform for the cleanup of shellfish samples [116]. In an aqueous mixture containing 40% alcohol nearly all proteins precipitate enabling a cleanup step. The goal can be the removal of unwanted proteins, such as enzymes used for digestions or the separation of target proteins even with the use of several fractionation steps. There were a few experiments applying water-methanol mixtures for the extraction of Se-compounds, but the efficiency stayed below 50%.

Next the high concentration (> 1mol/L) acid solution extractions have to be mentioned. The pH range of in this case is far below the biological pH range. If complete digestion is not the goal, HNO_3, H_2SO_4, $HClO_4$ cannot be used. However, when it comes to HCl, the opinions are diverse. While the solution changes the inorganic (SeVI) and (SeVI) ratios, it can be used for Se-protein hydrolysis. With the use of microwave digestion the hydrolysis time can be as short as 30 minutes. According to Behne [117] 90% Se-extraction was achieved from the different organs of laboratory animals, successfully preventing the disintegration of the sensitive Se-amino-acids. However, not all authors favour the method. Several articles mention that even the addition of anti-oxidizing agent phenol was unable to prevent the destruction of Se-amino-acids [118,119].

Low concentration (< 1 M) acid solutions are also used for sample preparations. One of the most often used reagents is TCA (trichloroacetic acid). It is used in a 12 m/m % ice-cold solution for the precipitation of large protein molecules enabling the examination of small molecular weight Se-compounds, like inorganic salts, free amino acids, methylated derivatives. The acid is volatile therefore, it can be removed through lyophilisation insuring it would not influence future fraction collection. The method can be used for a wide range of samples, for example in 2001 Larsen [120] used it for the preparation of different fish samples; and in 2002 Vonderheide [121] applied the method for the cleanup of lactobacillus samples. In most cases it is used as an alternative method if size exclusion chromatography (SEC) and ultrafiltration are unavailable. On the other hand, the line between small and large proteins is blurred as precipitation is not only determined by size but also the hydrophobic character of the protein.

2.4.3. Enzymatic digestion

For a long time digestion was nearly exclusively executed with 6 mol/L HCl solution, but when the widely different effects of different Se species started to become known, the importance of the biological availability of Se was also revealed. It became soon obvious that human digestion cannot be compared to simple acidic digestion, not to mention the fact that the Se-amino acids suffered degradation during the process. It was Yoshida [122] in 1984 who first

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applied the enzymatic digestion and since then it became dominant due to its many advantages. Most enzymes do not require an elevated temperature, merely 35-50 °C. Except for experiments modelling the human stomach they need no extremely high or low pH, as most enzymes operate in the biological pH range. All these factors contribute to the preservation of the original Se-species. No special equipment is needed for the digestion. However, compared to acidic digestion it can take a very long time, the enzymes are sometimes very expensive and not so robust when it comes to the particle size of the sample.

First, the most popular question was: how much of the Se is bio-available for humans from a given sample; therefore enzymes from the human digestion system were used. Usually the aim was not to extract all Se present, but to model human digestion disregarding extraction efficiency. For the success of this method, the applied enzymes need to be able to access the proteins, and the samples should contain sufficient amount of them. In most cases pepsin, pancreatin and pronaze enzymes are used at an appropriate pH over a period of 36-72 hours. The experiments also concluded that to achieve complete digestion to amino acid level non-specific enzymes are indispensable. In 1996 Crews *et al.* [86] used an enzymatic digestion on a tuna sample, trying to mimic human digestion. They used pepsin and pancreatin, but the short incubation time and lack of crude enzymes did not allow for the determination of organic Se compounds. Thomas *et al.* [123] examined the digestibility of roasted meat and sea bread and dietary supplements. They found the availability of Se in the food to be between 40-50%, and 70-100% in the supplements.

While the previously mentioned methods yielded good results, they took a very long time to be completed, risking microbiological problems that might result in misleading data. About a hundred years ago it was Van Slyke and Meyer who started to research the question of protein digestion; and based on extensive experiments, they formulated several hypotheses about the metabolic fate of dietary proteins [124]. The researches following their work emphasized that in certain cases intact proteins, even as large as egg ovalbumin, can be adsorbed in large enough quantities to be detected in urine, unhydrolyzed, though this behaviour is abnormal. Great majority of proteins are extensively hydrolyzed along the stages of gastrointestinal (GI) digestion up to free amino acids (AAs) or short oligopeptides. According to large amounts of recent literature, peptides released from food proteins in the gastrointestinal tract can elude digestion and can be absorbed into the intestinal lymphatic system [125]. There are a few proteins though, that have a structure specifically preventing our enzymes to access them, such as prion proteins or proteins denatured to a great extent by, *e.g.*, heat treatments.

A new period started in enzymatic digestion sample preparations in 1996, when Gilon [126] proposed a new method: a one-step, simple, complete enzymatic digestion, with high

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Se-recovery. The method Gilon published introduced a non-substrate specific, crude enzyme mixture called pronase-E to the homogenised sample powder in large quantities, to compensate the lack of pre-digestion and the denatured state caused by sample preparation. Since then several similar crude enzymes have been introduced, such as subtilisine, proteinase-K, flavourzyme. For the optimal working of these mixtures buffer solutions are necessary as the appearing amino-acids would shift the pH of the solution outside the optimal pH-range. Ever since enzymatic preparation have been widely used. In 2009 Amoako *et al.* used it during their work of examining the speciation of selenium dietary supplements [127]. In 2010 Lipiec *et al*. [128] optimized their sample preparation to achieve complete protein digestion and to avoid selenocysteine losses during their examination of selenomethionine, selenocysteine, and inorganic selenium in eggs. The same year Jitaru *et al*. [129] executed an enzymatic sample preparation as part of their work on a systematic approach to the accurate quantification of selenium in serum selenoalbumin by HPLC - ICP-MS. A year later Bañuelos *et al.* [130] applied enzymatic digestion as an extraction method during their work researching the selenium accumulation, distribution, and speciation in spineless prickly pear cactus; a drought- and salt-tolerant, selenium-enriched nutraceutical fruit crop for biofortified foods. [107]In 2014 Shao *et al.* have used proteolitic digestion for the Se-specitation of Se-enriched sting bean (*Phaseolus vulgaris* vulgaris) [131].

On the other hand, it must be noted that foods contain other components than proteins as well, and the human body has an array of enzymes for their digestion. Therefore, the application of non-protease or non-human enzymes is justified. With plant, bacteria and mushroom samples the cell wall, in plants and animal tissues the lipid content might prevent the protease enzymes from accessing the Se-proteins, and there might be Se-species bound to non-protein molecules. Consequently, not long after the appearance of pronase E, different lipase, amylase, cellulase and pectinase enzymes were introduced depending on the sample type. It was Casio*t et al.* [132] in 1999 who first used a lipase enzyme during sample preparation. The lipide and protein digestion happened simultaneously; the protease freeing the lipids for the digestion. It was also this publication that introduced driselase, a crude enzyme mixture for the digestion of cell walls. The idea of using different enzymes has been used by different authors since then. In 2002 Gómez-Ariza *et al.* [116] needed the lipid digestion as part of sample preparation during his research of Se-species in oysters and crabs. In 2002 Michalke *et al*. [133] used lysozyme for the cell wall degradation of lactobacillus samples; the enzymes were applied both simultaneously and separately from the protease. The Se extraction was efficient, but the number of identified species was low, presumably due to the low number of standards available and the harsh treatment of the sample. Larsen *et al*. [120,134] executed the optimisation of a two-step

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enzymatic digestion using β-glucosidase for cell wall digestion and a wide substrate-spectrum crude enzyme for proteolysis.

2.4.4. Chromatographic cleanup

As mentioned before, with the appearance of more selective and less robust equipments, cleaner samples were demanded. Foods are complex matrices, and even the simplest extraction can dissolve thousands of components. Often, most of them need to be removed selectively from the sample before analysis. For this purpose different chromatographic methods are often used, based mostly on different principles. Combining more of these methods can lead to greater selectivity and cleaner samples. The large number of available combinations enables chromatography to be used for nearly any type of sample or target compound. However, this could never be achieved without ICP-MS in case of selenium speciation. The destructive nature and the atom-specific selectivity of this detector make it possible to define the retention time of the target molecules through their seleno-atoms.

In most cases chromatographic cleanup is executed in two or even more steps. The first step is the most robust one, for the removal of complex matrix and fractionation of target compound based on molecule mass. The first step is usually SEC (SEC), because samples with high concentration of matrix compounds can be injected without causing overload. It has a lower resolution, but it is also the least invasive technique, nearly all components survive SEC separation [135]. The inorganic Se(VI) and Se(IV) retain their forms and the large proteins are not precipitated either. The drawback is that the separation is not really “sharp”, thus, the collected fractions need to be inspected one by one before they can be pooled, usually with ICP-MS flow injection.

SEC separation has two different types. The most commonly used method is the classic SEC separation, where large concentrations (0.1 M) of buffers are used. The second type, low buffered SEC separation is a relatively new technique. The ion concentrations are kept low in the eluent in order to allow for the secondary interactions between the column and target compound to enhance the separation. Encinar *et al.* has used 10 mmol/L ammonium acetate solution for the fractionation of selenised yeast extract [136].

Even though each fraction still contains hundreds of different compounds, the method allows the separation of low and high molecular weight compounds without using extreme pH ranges or oxidative agents. Before the sample can be deemed “clean” at least one more, but usually several additional cleanup steps are needed.

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In an orthogonal chromatographic cleanup method the second step is often ion exchange chromatography. It can be either anion (SAX) or strong cation exchange chromatography (SCX). They offer a better resolution than SEC separation, but they are also less robust, more invasive and the number of components that can be cleaned with them without suffering degradation is also smaller. They are very commonly used in Se-speciation for a variety of purposes such as for the cleanup of selenised garlic extract human serum [137] from broiler chicken muscle tissue [138] or broccoli [139].

On the other hand, sometimes even a two-dimensional cleanup is not enough; or, the complexity of the matrix does not require SEC separation fractionation. If more steps are necessary some of the highest resolutions can be achieved with reversed phase chromatography (RP), but only covalent bonds survive separation. As inorganic ions elute in the void, it can also be used for desalting the sample from previous buffer or extracting solution. The apolar phase used is most often acetonitrile: rarely methanol, as the low pH and the presence of methanol could cause methylation. In certain cases when retention on the column is not sufficient by itself to separate the target compounds ion-pairing agents can be used. Only volatile compounds, like HFBA (heptafluorobutyric acid) that can be removed using lyophilisation are suitable for this purpose. Depending on the target molecule and the properties of the agent, it may decrease the reproducibility of the measurement and may add a time limit to how long the sample stays stable after the introduction of the agent.

Gilar *et al.* found in 2005 that as a general approach, usually a two-dimensional (2D) chromatographic cleanup is both necessary and adequate. SCX - RP, HILIC - RP, and RP - RP 2D systems were found to provide suitable orthogonality. The RP - RP system (employing significantly different pH in both RP separation dimensions) had the highest practical peak capacity of 2D-LC systems investigated [140]. SEC separation followed by anion-exchange, cation-exchange, RP-HPLC, has been extensively applied to the speciation of selenium speciation in human milk [141], garlic [91] and yeast [142].

The greatest change in the last decade of chromatographic separation has been the increasing use of HILIC technique. With the appearance of HILIC new separation options became available. This method enables the simultaneous use of separation based on different chromatographic principles, including normal phase LC, ion chromatography and RP-LC. It applies hydrophilic stationary phases and reversed-phase type eluents.HILIC separation in Se-speciation is now commonly used during yeast metabolite examinations [101] but it has also been used for the identification of selenosugars in high selenium cereal crops [104] and Se-metabolites from human urine and blood [103].

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2.5. Qualitative and quantitative aspects of selenium speciation

As for the speciation, there are two main questions that have to be answered: what is there in the sample, and how much is present. Naturally, the latter one presumes previous knowledge of the possible content. What methods can be chosen for the analysis depends heavily whether a standard for the target component is available for purchase or not.

2.5.1 Quantification

2.5.1.1 Standard addition – external calibration

While external calibration is not the most precise method, in certain cases it can be used with great success. For example, during determination of all selenium content, after a complete acidic digestion all organic material is destroyed and there is no matrix left. If there is no standard available (isotopically enriched or not) for the target compound and the sample can be diluted enough external calibration is the only possible solution for quantification. It was the case in 2007 when Dernovics *et al.* completed the standardless identification of selenocystathionine and its γ-glutamyl derivatives in monkeypot nuts [143].

The availability of standards facilitates the quantification of the target molecule. The most commonly used method in this case is standard addition. This method is usually applied when the sample matrix influences the analytical signal thus introducing signal enhancement or suppression. The general process for standard additions is to execute measurements after adding increasing amounts of analyte to aliquots of the sample. The idea of this procedure is that the total concentration of the analyte is the combination of the unknown and the standard, and that the total concentration varies linearly. There are two different ways of doing this: one in which the samples are all made up to the same volume after the standard has been added; and another one in which the volumes of the sample solutions are different. The signal is plotted on the y-axis; in this case the x-axis is graduated in terms of the amounts of analyte added either as an absolute weight or as a concentration. The regression line is calculated and extrapolated to the point on the x-axis at which $y = 0$. This negative intercept on the x-axis corresponds to the amount of the analyte in the test sample.

Sometimes however, the dilution of the sample is not desired, as it could lead to sensitivity decrease. In such cases different amounts of standard solutions are added to the sample aliquots and measured without making them up to the same volume. However, the results obtained from the measurement of such series first undergo a correction calculation that would

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neutralize the margin caused by the volumetric indifferences. After that point the result calculation works the same in the other method. Selenocysteine, selenomethionine, *Se*-methyl-selenocysteine are some of the compounds most often identified with standard addition.

In selenium speciation standard addition is used very commonly with HPLC-HG-AFS, in most cases (but not exclusively) for the determination of total Se content. Stibilj *et al.* developed a method for the verification of the declared value of selenium in food supplements. In their work they optimized a method using HG-AFS. In their work, for the quantification of Se standard addition was used as the high and varied content of Cu, Mg and Zn in the samples had to be taken into consideration [144]. In 2005 Dumont *et al.* have developed a method for the identification of the major selenium compound, selenomethionine, in three yeast (*Saccharomyces cerevisiae*) dietary supplements. During their work they developed a HPLC-ESI-MS-MS method for the monitoring of six selenium containing compounds, but the total selenium content of the samples was measured using HG-AFS after digestion [145]. In 2006 Mester *et al.* reported that during the certification of a new selenised yeast reference material (SELM-1) several laboratories opted to use a HG-AFS technique with standard addition for the quantification of the SeMet content [146]. In 2008 during the determination of total selenium in geological samples Fan *et al.* compared different digestion methods, and also used HG-AFS detection with the help of standard addition to determine the total selenium content of their samples [147].

Next to HPLC-HG-AFS, HPLC-ICP-MS is the other leading method used together with standard addition. Even to date several articles are published using this combination, and the samples cover a wide range. In 2006 Juresa *et al.* [148] conducted experiments about the stability of selenosugars in human urine, namely methyl-2-acetamido-2-deoxy-1-seleno-β-d-galactopyranoside. They conducted the same experiments at three different times and have detected a growing number of decomposition products with ICP-MS. Total selenium content was determined using standard addition and revealed that Se was lost from the solutions during storage/handling, presumably as volatile species. In 2008 Bierla *et al.* [149] have developed a method for the determination of selenocysteine and selenomethionine from animal blood using simultaneous derivatisation. The fraction containing both derivatized selenoamino acids was isolated by SEC and submitted IP-HPLC-ICP-MS analysis. The quantification of selenocysteine and selenomethionine was carried out by the method of standard additions. An internal standard of ^{77}Se-labelled selenomethionine was used to control the derivatization yield and chromatographic recovery.

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2.5.1.2 Isotope dilution

Another option of quantitation when standard is available is isotope dilution. The method was first introduced in 1913 by György Hevesy during the determination of the solubility of lead sulphide and lead chromate, and he received a Nobel–prize for his work in 1943. The technique falls into the internal standard category, as a standard (though isotopically-enriched) is added to the sample. Unlike traditional analysis methods and standard addition, all of which rely on the intensity of the obtained signal, this method is based on measuring ratios between different isotopes. This in itself can prevent problems arising from sample loss, inefficient nebulisation or ionisation. These effects influence the isotopically labelled and unlabelled molecules equally, thus leaving their ratios unchanged. The subsequent calculations follow the logic for the mark and recapture method often used in biology for the determination of population size. Isotope dilution is also one of the very few methods traceable directly to the international System of Units, a primary measurement technique.

Numerous articles have been since published using isotope dilution, and it became indispensable for solving problems that are otherwise impossible or extremely tedious to deal with. It provides correction for matrix effects and signal drifts, but it can only be used for multi-isotopic elements.

There are three types of isotope-dilution: direct (or single), inverse and double dilution methods. Direct isotope dilution is usually used for quantifying an unlabelled, non-radioactive target compound that otherwise cannot be separated from other molecules, compounds that belong to the same family usually. The target compound may be both organic and inorganic. If the direct method is used, the isotopically enriched compound is added to the sample prior to sample preparation, and homogenized well. Thus, the labelled molecule suffers from the same losses as the target compounds, their ratios remain unchanged. This enables the use of relatively low-efficiency sample preparation steps that would normally be impractical instead of tedious techniques. It has excellent precision and accuracy, without the need of using methodological calibration graphs of high number of calibration points. The ratio of the isotopes is measured and concentration is calculated from the prepared sample. It can be used in case of different samples, such as human blood serum [150]. However, like all other techniques it has its drawbacks. The number of available isotopically labelled compounds is limited which strongly restricts the use of the method. The measured isotope abundances must be accurate (spectral interferences, mass bias, detector non-linearity, *etc*.) The isotope composition of the natural abundance and isotopically labelled element or compound must be known in advance; the concentration of the

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spike must be determined by reverse isotope dilution analysis. None of these attributes is easy to establish as isotopically-enriched compounds are usually available in small quantities of questionable purity. Therefore, prior to isotope dilution is performed on the sample, the amount of the enriched analyte must be ascertained beforehand through isotope dilution. This preparatory step is called reverse isotope dilution and it involves a standard of natural isotopic-composition analyte. The entire method together is called "double isotope dilution". For selenium speciation it was first used in 1983 by Ramer *et al.* for the determination of selenium species from laboratory animal organs with the help of GC - MS.

Isotope pattern deconvolution (IPD) is a new, alternative, simpler way of calculation. This method uses multiple linear regressions, and offers simplified alternative data processing process to double spike isotope dilution computations. The greatest advantage of this mathematical tool lies in the possibility of deconvoluting the isotope pattern in a spiked sample without knowledge either about the quantities of enriched isotope tracer incorporated into the natural sample matrix or the degree of impurities and species-interconversion (*e.g.,* from sample preparation).

When no standards are available other methods have to be used, such as non-species-specific isotope dilution. Also, as discussed previously, ICP-MS suffers from matrix related effects upon the nebulizer and the signal intensity (quenching). In addition, even slight deposition on the sampler cone will cause drifting. Due in part to drifting, many times calibration curve technique with internal standardization is chosen over the technique of standard additions. In this case, instead of the addition of an isotopically enriched target compound, a different, usually inorganic molecule is added to the mixture, or a solution of the isotopically altered component can be continuously mixed to the sample solution prior to analysis. Another advantage of non-species-specific isotope dilution is that a large number of different compounds can be analysed with it in the same chromatographic run. This technique has been widely used over the last decades due to the high accuracy and its applicability to simple and complex analytes. While it can be used as a simple internal standard to prevent drifting, it is also suitable for the quantification of large organic molecules, such as selenoproteins, blood serum components or molecules found in urine. As early as 1965 by Chau and Riley [151] already used it for the determination of selenium in seawater and sea organisms. While the first applications go half a century back, until the importance of selenium speciation was realised researchers only wished to quantify total selenium, like Reamer and Veillon [152] in 1981, who published an article about the determination of total selenium content from various biological matrices. By 1991 Tanzer and Heumann [153] performed the speciation of Se in environmental waters, such as ocean, river and lake waters. Among other things they quantified the selenates and selenites,

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and a number of organic compounds such as trimethylselenonium ion but others [154] used it this method for plant, soil and sludge selenium quantification. As the technology advanced further, so did the complexity of target analytes. By 2003 Huerta *et al.* [155] used it for the quantification of selenites in yeast and wheat flour.

In 2008 Xu *et al.* conducted research with Se-tagged human serum proteins. They used species-unspecific isotope dilution ICP-DRC-MS, and could identify five selenium species including selenoprotein P, glutathione peroxidase, selenoalbumin, and two unknown selenospecies [156]. In 2009 Reyes *et al.* investigated the selenium species in petroleum refinery waste waters. Inorganic Se species selenite, selenate and selenocyanate were separated by ion chromatography (IC). Quantification of selenium in each separated species was performed using post-column isotope dilution analysis by continuous mixing of an enriched ^{77}Se spike solution [157]. In 2011 Ballihaut *et al.* conducted experiments for the identification of selenoproteins in human plasma. Using isotope dilution LC the identified compounds included nine SePP peptides, including two selenopeptides and nine GPx3 peptides; while albumin was identified with a protein coverage factor >95% [158]. The same year Li *et al.* used post-column IDA for the identification of Se-species in human plasma from patients exposed to mercury. Selenocystine, selenomethionine, selenoprotein P, selenoalbumin and glutathione peroxidase were separated and quantified [159]. Isotope pattern deconvolution, being a newer technique is not so widely spread, but equally useful. For example, in 2007 González Iglesias *et al.* used the method to differentiate and determine endogenous and supplemented selenium in lactating rats. The research group fed the rats for two weeks with formula milk containing one enriched Se isotope, ^{77}Se, as the metabolic tracer. The isotopic composition of selenium in serum and urine samples was then measured by collision cell ICP-MS after the addition of a solution containing another enriched isotope, ^{74}Se, as quantitation tracer, before analysis. Isotope pattern deconvolution allowed the transformation of measured Se isotopic abundances into concentrations of natural abundance (endogenous) selenium and enriched ^{77}Se (supplemented) present in the samples [160].

The use of species-specific isotope-dilution is limited by the lack of available standards, but when it can be used, it yields accurate data. In Se-speciation there are few compounds available, but those were used in many areas. For example in 2008, Ouerdane and Mester grew wild yeasts on Se-rich medium. Their goal was to maximise the Se-incorporation of the wild yeast, therefore the different Se-species in the medium and the metabolites were regularly monitored. Quantitation of selenomethionine and methionine was performed by species-specific isotope dilution GC - MS. In a medium containing Se(VI), the maximum replacement of Met with selenomethionine was 50%, which is considerably higher than that of obtained with the

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current commercial Se yeast formulations [161]. In 2009 Inagaki *et al.* conducted experiments analyzing the trace elements in sediments. In 2011 Matsukawa *et al.* [162] developed a method for the simultaneous determination of selenomethionine enantiomers in biological fluids by stable isotope dilution GC-MS. DL-[$^{2}H_{3,}$ ^{82}Se]selenomethionine was used as analytical internal standard to account for losses associated with the extraction, derivatization and chromatography. The same year Ohta *et al.* developed a method for the simultaneous quantization of several Se-metabolites. A mixture of the isotopically-labelled standards was spiked in a selenised garlic extract and rat urine; then the samples were subjected to speciation analysis by HPLC-ICP-MS [163].

2.5.2 Qualitative aspects

2.5.2.1. Enantiomer-selective speciation

In the field of enantiomer-selective measurements there is only one Se-components that is in the centre of interest: selenomethionine, as previously mentioned the effects of D- and L-amino acids are very different. Even today in many cases this calls for enantiomer-specific methods. For enantiomer-selective separation a chiral agent of some type is needed: a stationary phase and eluent or a derivatising/ligand forming agent.

In 2012 Duan *et al.* executed experiments also with selenised yeast, but used ligand-exchange micellar electrokinetic capillary chromatography after solid phase extraction for chiral speciation [164]. In 2013 Yang *et al.* determined the enantiomer ratios of methionine and Se-methionine in selenised yeast [165].

2.5.2.2. Identification of unknown compounds

As mentioned in the history of Se-speciation before the appearance of molecular ionization the only available method for gaining molecular information was NMR. The technique was rarely if ever used for molecule identification, as it needs large amounts of clean components to work. Se-speciation started its golden area with the appearance of ESI-MS in 1999. Even to date molecular ionization coupled with high resolution mass spectrometers remains the leading method for the identification of new compounds. Examples from the last decade of component identification can be seen in Table 3.

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Table 3: Examples from the last decade of Se-component identification

	Authors	Year	Instrumentation	Sample	No. of new components	A new/typical component
1	Block *et al.* [166]	2004	HPLC-ICP-MS, GC-AED, and GC-MS	selenised yeast		S-(methylseleno)cysteine
2	Díaz Huerta *et al.*[19]	2005	SEC-, RP- and SAX HPLC -ICP-MS, Post-column isotope dilution	edible wild mushrooms	several unidentified compounds	Selenomethionine
3	Ogra *et al.*[20]	2005	HPLC-ICP-MS equipped with ORS, ESI-MS/MS	selenized garlic and shallot	unidentified S-metabolites	γ- Glu-methylselenocysteine
4	García-Reyes *et al.* [167]	2006	LC-TOFMS	selenised yeast	2	*m/z* 513 compound
5	Dernovics *et al.* [101]	2008	ICP-MS-assisted HILIC-HPLC-hybrid linear ion trap/orbitrap MS^n	yeast-based food supplements	9	*m/z* 563 compound
6	Dernovics *et al.* [97]	2008	SEC-, SAX-HPLC ICP-MS and ESI-Q-TOFMS/MS	selenised yeast	6	*m/z* 563 compound
7	Klein *et al.* [103]	2011	RP/HILIC - ICP-MS and ESI-LTQ Orbitrap MS	human urine	1	*Se*-methylselenoneine
8	Arnaudguilhem *et al.* [17]	2012	RP-, HILIC- HPLC-ESI- hybrid quadrupole trap/Orbitrap MS	selenised yeast	21	Di-γ-glutamoylselenocysteine
9	Bianga and Szpunar [168]	2013	Laser ablation ICP-MS, 2D polyacrylamide GE and Orbitrap tandem MS	selenised yeast		Selenised glyceraldehyde-3-phosphate dehydrogenase
10	Shao *et al.* [131]	2014	SAX-LC-ICP-MS, LC-ESI-TOF-MS	Se-enriched string beans		γ-glutamyl-Se-methylselenocysteine

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2.5.2.3. Standard synthesis

As demonstrated above, molecular ionization coupled with high accuracy mass spectrometers were used for the identification of a wide range of compounds. On the other hand, the proposed structure of these molecules cannot be confirmed without the use of NMR, or, without comparing the fragmentation pattern to that of matching standards. In order to use the former method, a relatively large amount of clean compound is needed. In most cases the target molecule is a minor metabolite of some kind, present only in very small concentrations. Even for the MS examination several clean-up and concentrating steps are needed; and the resulting sample is rarely clean enough for a NMR experiment. In many cases there is not even enough of the sample to extract to clean and to concentrate enough of the target component for NMR analysis.

In the last decade the number of newly discovered and tentatively identified components has been increasing rapidly, while the number of available standards has barely grown. There are only a few articles that report synthesising standards as a part of identification. There is an increasing need for standards, therefore any method that enables the synthesis of several different compounds is not merely useful but needed very much. Identification through synthesis is not a commonly-used, but precise and useful technique. In 1993 Tamura *et al*. [169] completed the synthesis of different Se-analogues of glutathione. In 1998 Fan *et al*. [81], during their Se-metabolite research, synthesised three important precursors of volatile alkyl selenides, including dimethylselenonium propionate (DMSeP) to overcome the problem of commercially unavailable standards. In 2001 Block *et al*. [170] synthesised γ-glutamyl-Se-methylselenocysteine, selenolanthionine, Se-1-propenylselenocysteine, Se-2-methyl-2-propenyl-L-selenocysteine, and Se-2-propynyl-L-selenocysteine for the verification of *Allium* Se-species. In 2002 Kobayashi *et al*. [171] confirmed the structure of 1β3-methylseleno-N-acetyl-N-galactosamine, found in urine, using synthesis. The same year Wrobel *et al*. [172] synthesised selenoadenosylhomocysteine during their work of examining Se-enriched metabolites. This way they could verify the structure of a commercially unavailable component. In 2004 Braga *et al.* [173] published the synthesis and mass spectrometric characterization of selenotrisulfides of glutathione (GSH), cysteine (Cys) and homocysteine (HCys) by ESI-MS. The same year, Traar *et al.* [174] published the synthesis of methyl 2-acetamido-2-deoxy-1-seleno-β-D-hexopyranosides, a component found in human urine. Also in 2004 Block *et al.* [166] synthesised S-(methylseleno)cysteine for the verification of the structure of a compound found in selenised yeast. In 2009 Matich *et al*. [175] synthetised 2-(methylseleno)acetaldehyde, 2,2-bis(methylseleno)acetaldehyde, 4-(methylseleno)-(2E)-nonenal, and 4-(methylseleno)-

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(2E,6Z)-nonadienal for the identification of organoselenides from genetically modified Se-accumulationg *Nicotiana tabacum*.

Selenium speciation in the last decade has been gravely hindered by the lack of standards. The short list of successful synthesis above just underlines the fact that there is an increasing need for standards; therefore any method that enables the synthesis of several different compounds is not merely useful but very much needed.

2.5.2.4. The lack of selenised-yeast specific standards

While the number of commercially available or synthetically described Se species of plant and mammal metabolism has been slowly increasing [81,96,169–177], the availability of yeast specific Se species is extremely limited [166]. Taking into account that almost 70 Se species have been identified from selenised yeast [17,178] and the fact that Se-yeast is the only natural (that is, not a synthetic compound based) and approved source for human selenium supplementation in the EU, the list of lacking (~ 60) selenium standards is more than remarkable.

2,3-dihydroxi-propionyl (2,3-DHP) group is a specific residue detected in selenised yeast that forms numerous stable and highly abundant Se species in several different yeast strains and fermentation batches. The absolute amount and ratio of these compounds can be used for both batch identification and to prevent forging Se-yeast with the much cheaper selenomethionine. A few of these compounds can be found in every single strain and batch, some are marker components of a specific strain or fermentation method. One of the highly abundant and commercially unavailable Se-species, that can be found in every Se-yeast batch is the conjugate of glutathione and 2,3-dihydroxy-propionyl-selenocysteine (CAS No. 1006377-09-8; $C_{16}H_{27}O_{11}N_4SSe+$ [M+H]+, *m/z* 563.05568). This Se-yeast specific compound was reported first by McSheehy *et al*. [142] and Goenega-Infante *et al*. [179], while its structure was tentatively identified in 2008 based on high resolution ESI-MS data [97]. Since that time this compound has been detected and cited continuously from several producers and yeast strains [17,180,181]. While glutathione is highly concentrated in yeasts and occurs ubiquitously in eukaryotic and prokaryotic cells, the glyceric acid amine residue has only been previously reported either in antibiotics [182] or – interestingly – in selenium-containing conjugates from yeast and, recently, from black mustard (*Brassica nigra*, L) [105]. Indeed, the metabolic role and origin of the 2,3-dihydroxy-propionyl (2,3-DHP; incorrectly referred also 2,3-dihydroxy-1-oxopropyl, 2,3-DOP) group has not been elucidated yet, which is especially interesting as no sulphur analogues of any of the Se containing species (of this group) have been found.

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3. Goal of research

My work can be grouped around three main topics that are the main phases of developing a new research procedure: sample preparation improvement, new compound identification and identity verification of the target component.

The aim of the first subject was to examine the effect of sample preparation on the D,L-enantiomer ratio of extracted selenomethionine through the following steps:

1. to execute two well-known sample preparation methods on different matrices (yeast and *Lecythis minor*, Jacq. (monkeypot-nut))
2. to apply and optimize a derivatisation method for these two samples
3. to apply an analytical method for the separation and determination of L- and D-selenomethionine
4. to examine total selenium content and column recovery
5. to compare results acquired from different sample preparation methods and different samples.

The second goal was to complete the validation of the 2,3-dihydroxy-propionyl group in selenium speciation by chemical synthesis and LC - MS analyses through the following steps:

6. to perform the synthesis of four Se-compounds (namely, 2,3-dihydroxy-propionyl-selenocysteine-glutathione, 2,3-dihydroxy-propionyl-selenocysteine-selenocysteine, di-N-2,3-dihydroxy-propionyl-selenocysteine and selenocysteine-glutathione) that naturally occur in nearly all selenised yeast batches and currently are not available as standards,
7. to optimize the conditions of chemical reaction and to gather measurable amounts of target compounds,
8. to develop a clean-up method to be used between the steps of synthesis
9. to obtain elution and fragmentation data of the synthesised compounds and confirm their proposed molecule structures.

The third purpose was to examine and identify the selenium metabolites of *Hericium erinaceus* through the following steps:

10. to moderately enrich *Hericium erinaceus* with inorganic selenium
11. to develop and optimise a method for extraction and sample cleanup
12. to enrich the sample extract in selenium compounds

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13. to examine the extracts with HPLC-ESI-QTOF-MS for the identification of selenium compounds
14. to compare the selenium metabolites with those of the selenised yeast and the S-metabolic pathways of the mushroom looking for S-Se counterparts.

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4. Experimental: Materials and methods

My research can be grouped around three main theses: evaluation of sample preparation methods for the analysis of selenised yeast and their effects on target compound, method development for standard synthesis of yeast-specific compounds and identifying selenium compounds from *Hericium erinaceus*. Therefore the methods used, the results and conclusions will be discussed in three separate parts corresponding to these topics.

4.1 Instrumentation

For sample preparation purposes a Hielscher UP 100 H ultrasonic probe (Teltow, Germany) was used with full cycle time and the amplitude of 100%. For speciation studies samples were digested using a CEM Mars-5 microwave unit equipped with HP-500 vessels (CEM, Matthews, NC, USA) according the following program: 0-20 min to 17 MPa pressure; 20-40 min: kept on 17 MPa.

ICP-MS Agilent 7500cs (Agilent, Santa Clara, CA, USA) was used to monitor the isotopes of ^{77}Se, ^{78}Se, ^{80}Se, ^{82}Se, ^{88}Sr and ^{103}Rh. The instrument was coupled to an Agilent 1200 HPLC system that was equipped with an optional extended loop (+400 µL). The HPLC - ICP-MS analysis was executed with 5% O_2 as optional gas (40 ml min^{-1}; 4.6 purity) when using organic solvent based eluents and H_2 (5.0 purity) as collision gas at the flow rate of 2.2 ml min^{-1} and He as collision gas at the flow rate of 2.2 ml min^{-1}.

For the clean-up of the selenomethionine fraction a PRPX-100 (4.6 mm x 250 mm x 5 µm; Hamilton; Reno, Nevada, USA) anion exchange column was used. For enantiomeric separation an XTerra MS C_{18} (Waters; Milford, USA; 4.6 mm x 250 mm x 5 µm) RP column was applied.

Intermediate products of syntheses of 2,3-dihydroxy-propionyl-glutathione were also monitored with an HPLC-ESI-MS coupling where a QTRAP 3200 triple quadrupole (QQQ) – linear ion trap mass spectrometer (ESI -QQQ-MS; Applied Biosystems/Sciex; Foster City, CA, USA) was used either in the Enhanced Q3 mode for the full-scan experiments with an integration time of 1 s or in Enhanced Product Ion (EPI) mode for MS/MS analyses. The related instrumental parameters are described in Table 4.

For the identification of selenium species an Agilent 6530 Accurate ass ESI-QTOF-MS was used with an Agilent 6220 derived dual ion spray source. The instrument was coupled to an

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Agilent 1200 HPLC system. The operating parameters of the ESI-QTOF-MS can be seen in Table 5. In most cases the LC system was operated coupled to a mass-spectometer, thus in a LC-MS system.

Table 4: Instrumental parameters of ESI-QQQ-MS

Qtrap 3200 triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems)	
ESI source	Turbo V interface and Turbo ion Spray probe
Operation mode	Negative
Ion spray voltage (V)	-4500
Curtain gas (nitrogen) (psi)	15
Ion source gas (psi)	10
Turbo gas (psi)	10
Desolvation temperature (°C)	30
Collision activated dissociation gas (a. u.)	10
Declustering potential (eV)	55
Full scan recording range (*m/z*)	100-1100
MS/MS recording range (*m/z*)	50-1100

Table 5: The operating parameters of the ESI-QTOF-MS

6530 Accurate Mass QTOF LC-MS (Agilent)	
ESI source	Dual ESI (Agilent)
Operational mode	Positive/negative
Precursor ion isolation in MS/MS	medium (4 *m/z*)
Mass accuracy in MS mode	< 2 ppm
Mass resolution	> 10000
Detection frequency	4 GHz
Fragmentor voltage	150 V
Curtain voltage	65 V
Drying gas	13 L/min
Capillary voltage	800 V
Nebulizer pressure	40 psi
Gas temperature	325 ºC
Data analysis software	Mass Hunter Acquisition B.02.01(B211630) with SP3

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4.2 Materials

H_2O_2 (a.r., 30 m/m%) and HNO_3 (a.r. >65 m/m%) were purchased from Scharlau (Barcelona, Spain). Gradient grade methanol (99.99%), acetonitrile (ACN; far UV HPLC grade), and Dowex 50WX4 cation-exchange resin (200-400 mesh) were bought from Fischer Scientific (Loughborough, Leicestershire UK). Ammonium acetate (a.r.), cyclohexane (a.r.), acetone (a.r.), H_3BO_3 (99.5%), NaOH (99%), TRIS (a.r.), HCl (37 m/m %) and NaI (a.r.) were purchased from Reanal (Budapest, Hungary). Methanesulphonic acid (99.5%), dithiotreitol (DTT, 99%), NIBC (*N*-isobutyryl-cysteine), OPA (*O*-phthal-dialdehyde), D,L-selenomethionine (containing 52.2% L- and 47.8% D-enantiomer, as specified by the manufacturer) and protease XIV enzyme (4.8 U mg^{-1}), activated charcoal (4-14 mesh, granular, Norit® PK 3-5), Whatman Grade 1 filter paper, HCOOH (~98%, puriss), 4-methylmorpholine (NMM; 98.0%), *N,N'*-dicyclohexylcarbodiimide (DCC; 99.0%), pentachlorophenol (PCP; 98%), selenocystine (Sec_2; 97%), DL-glyceric acid hemicalcium salt hydrate (≥98%), reduced (≥98.0%) and oxidized (≥98%) glutathione, *Se*-methylselenocysteine, HCOOH (~98%, puriss), heptafluorobutyric acid (HFBA) were purchased from the Sigma-Aldrich group (Schnelldorf, Germany). SELM-1 CRM was obtained from the Institute for National Measurement Standards, National Research Council of Canada (composition certified by manufacturer: total selenium: 2059 ± 64 mg/kg; selenomethionine 3448 ± 146 mg/kg). High selenium monkeypot nut (*Lecythis minor*, Jacq.; ground and partially defatted, containing ~2300 mg Se kg^{-1}) was obtained from Dr. Winfried Behr (Germany). *N,N'*-dimethylformamide (DMF; 99%), 1.000 g L^{-1} standard solutions of Se and Rh, Pronase E enzyme (also named protease XIV; 4000 PU mg^{-1}), and H_2O_2 (a.r., 30 m/m%) were ordered from Merck (Darmstadt, Germany). Selenised wheat reference material CCQM-P86 identical with "ERM-BC210a" was obtained from LGC (Teddington, UK). Milli-Q water (18.2 MΩ*cm, Merck-Millipore, Molsheim, France) was used throughout the experiments.

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4.3 Effect of sample preparation methods on the D,L-enantiomer ratio of extracted selenomethionine

4.3.1. Clean-up of the protein fraction from the high selenium nut sample

The nut sample contains around 30% of fat and 50% of carbohydrate. As the target selenium compounds are found in the protein fraction the fat was removed with extraction and the carbohydrates with acetone precipitation.

For the removal of the fat content, 30 ml cyclohexane was added to 10 g nut sample, shaken manually for 5 minutes and centrifuged at 4100 *g* for 15 minutes at 4 °C. The solvent was decanted and the procedure was repeated two more times. The sample was dried at 37 °C for 24 hours in a drying oven then at room temperature in Petri dishes.

Afterwards 0.25 g defatted sample was mixed with 2.5 ml water and extracted with ultrasound apparatus (100 W; 30 kHz) for 1 minute, then 10 ml -18 °C acetone was added and incubated for one hour at -18 °C. The coagulated proteins were separated by centrifugation (10 min, 4 °C, 4100 *g*) The residue was washed with ice-cold acetone twice before being dried at 37 °C for 24 hours in a drying oven then at room temperature in Petri dishes for 10 hours.

4.3.2 Determination of total selenium content

The total selenium content of the SELM-1 was determined from the drags after the enzymatic digestion (see description in 4.3.3.1) using microwave digestion. 5.0 ml a.r. HNO_3 was added to 0.10-0.11 g from the drags and left to incubate overnight. 2.0 ml H_2O_2 was added to the mixture then digested with the microwave system (CEM Mars-5). After digestion the clear solutions were made up to 50.0 ml in volumetric flask. Next, 500 µl, 0.1 mg/L Rh internal standard solution was added to 100 µl of the digested sample then diluted to 10.0 ml. Calibration was executed with standard addition. 0, 20, 40, 100 ng/L concentration standard (expressed in selenium) was added to four aliquots, respectively. ICP-MS was operated in normal mode, with the monitoring of ^{77}Se, ^{82}Se and ^{103}Rh isotopes.

The same procedure was performed on the protein fraction of the monkeypot nut.

The selenium content of defatted of monkeypot nut sample after acetone precipitation was found to be 6568 mg kg^{-1}. This result is in good agreement with the original 2300 mg kg^{-1} concentration, as more than two thirds of the sample was removed, reducing the sample to the selenium-containing protein fraction, making the selenium concentration rise.

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The total selenium content was also determined for the calculation of the column recovery.

4.3.3. Sample preparation

4.3.3.1. Enzymatic preparation

Enzymatic preparation is one of the most popular methods for freeing the protein-bound selenomethionine. This is one of the two methods our research aimed to compare.

The procedure was partially based on the method of Yang *et al.* [183]. In brief, 100 mg sample (both from SELM-1 and the protein fraction of monkeypot nut) was mixed with 5 ml ammonium acetate buffer (0.1 M, pH 6.8) and vortexed. 50 mg protease XIV enzyme dissolved in 3 ml ammonium acetate buffer was added to the sample and shaken at 37 °C for 24 hours. The sample was centrifuged (4100 *g*, 4 °C, 20 min). The supernatant was made up to 10.0 ml in a volumetric flask and filtered through 0.45 µm PTFE disposable syringe filters (VWR; Radnor). The entire sample preparation was executed in five replicates separately for both the monkeypot nut and SELM-1 samples.

4.3.3.2. Acidic hydrolysis

Acidic digestion is also one of the most popular methods for freeing the protein-bound selenomethionine. This is the second method our research aimed to compare.

Acidic hydrolysis followed the method proposed by Mester et *al*. [146]. 50 mg sample (both from SELM-1 and the protein fraction of the monkeypot nut) was mixed with 10 ml 4 M methanesulphonic acid and boiled for 8 hours under reflux [184,185]. After 8 hours the samples were cooled, made up to 50.0 ml in a volumetric flask and filtered through 0.45 µm PTFE disposable syringe filters. The entire sample preparation was executed in five replicates separately for the monkeypot nut and SELM-1 samples.

4.3.3.3. Cleanup with SAX – HPLC

The digested samples contain too many matrix compounds for the direct measurement of selenomethionine. To reduce the background fraction collection was executed.

Before the D,L-enantiomer separation the selenomethionine fractions of the samples were cleaned with anion exchange chromatography (see the conditions in Table 6). The peak

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corresponding to selenomethionine was collected twelve times from each sample. The corresponding fractions were pooled and lyophilized.

Table 6: Chromatographic conditions applied during the experiments for the determination of the effect of sample preparation methods on the D,L-enantiomer ratio of extracted selenomethionine

Objective	Column	Elution program	Eluent A	Eluent B	Flow rate	Injected volume
SeMet clean-up	PRPX-100	0-5 min 100% A 5-7 min to 100% B 7-27 min 100% B 27-28 min to 0% B 28-35 min 100% A	10 mM ammonium acetate (pH=5.0)	250 mM ammonium acetate (pH=5.0)	1.8 ml min^{-1}	100 µL
D,L enantiomer separation	XTerra MS C_{18}	isocratic	52 V/V% 20 mM ammonium acetate (pH=6.0) 48 V/V% methanol	-	0.8 ml min^{-1}	See Table 9

4.3.3.4 Derivatisation and D,L-enantiomer separation

The enantiomer separation of the extracted selenomethionine was achieved through derivatisation followed by chromatographic separation.

For the derivatisation process the methods of Bergmann *et al.* and Bruckner *et al.* [185,186] were adapted and modified. The reaction mechanism of the derivatisation can be seen in Figure 3.

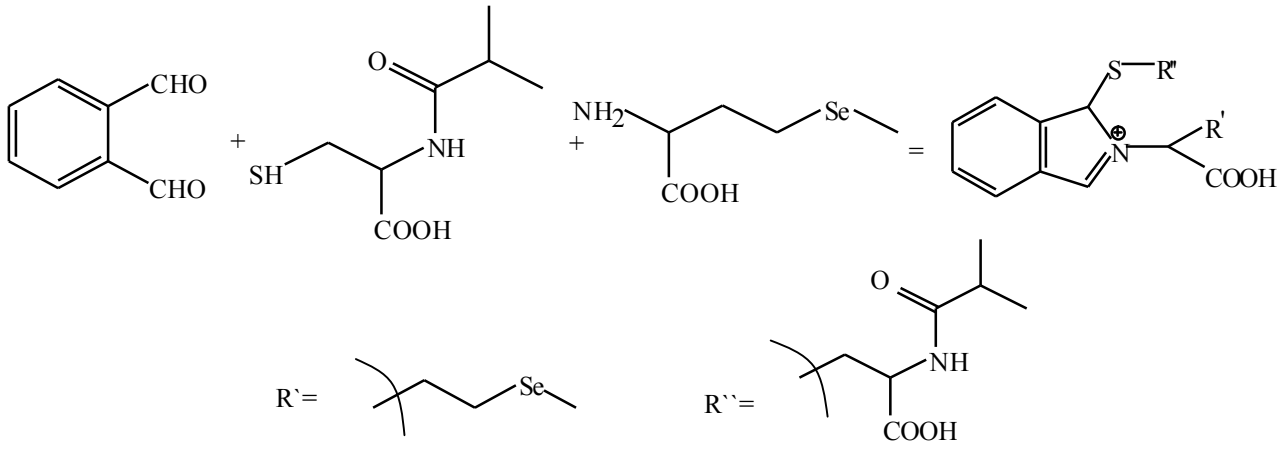

Figure 3 : Derivatisation method with NBIC and OPA by Bergman *et al.* [62]

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All samples were derivatised prior to the D,L-enantiomer separation with NIBC and OPA. In brief, 28.5 mg NIBC was dissolved in 10.0 ml methanol, while 15.2 mg OPA was dissolved in the mixture of 8.0 ml methanol and 2.0 ml boric acid buffer. For the preparation of the boric acid buffer 1.6 g boric acid was mixed with 2.5 ml 30 m/m% NaOH and made up to 50.0 ml with deionised water.

For the determination of column recovery, 20 ng of derivatised D,L-selenomethionine /calculated as Se/ was injected in triplicate onto the column. Fractions were collected directly into 25.0 ml volumetric flasks, separately to cover the elution time frames of underivatised selenoamino acids /0-15 min/ and that of the derivatised enantiomers /20-35 min/, respectively. In parallel, 20 ng of derivatised D,L-selenomethionine was directly filled in triplicate in 25.0 ml volumetric flasks. All the flasks were afterwards made up to volume with the chromatographic eluent (see Table 9), homogenized, and used for flow injection experiments. To determine column recovery, 200 µl of each flask was injected into the stream of the chromatographic eluent without fitting in the C18 column and monitored with ICP-MS on the ^{80}Se isotope with the use of 5% O_2 as optional gas He as collision gas at the flow rate of 2.2 ml min^{-1}.

4.4. Validation of the 2,3-dihydroxy-propionyl group in selenium speciation by chemical synthesis and LC - MS analyses

4.4.1. Methods

4.4.1.1. Desalting of glyceric acid

The first step to synthesise 2,3-dihydroxy-propionyl-selenocysteine-glutathione is the coupling of glyceric acid and selenocystine. To enable this, glyceric acid (commercially unavailable in its free form) has to be freed from its calcium salt.

Glyceric acid hemicalcium salt was converted to the free acid form according to Berens and Scharf [187] by dissolving 465 mg glyceric acid salt in 25 ml 50 V/V% methanol-water solution, and then 19.0 g Dowex 50WX4 cation-exchange resin was added during stirring. After 20 minutes of incubation the resin was removed by filtration, afterwards the solution was filtered first through 2.0 g activated charcoal then through a filter paper, concentrated to about 5 ml using a rotary vacuum evaporator at ambient temperature, and then strained using 0.45 µm PTFE

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filter. The leftover water content was removed at 55 °C using a vacuum rotary evaporator. 210 mg glyceric acid was acquired that was stored at -23Cº until used.

4.4.1.2. Synthesis and clean-up of pentachlorophenol–glycerate

For the coupling of glyceric acid and selenocystine glyceric acid has to be activated, which is done through coupling with pentachlorophenol. Chromatographic clean-up was executed to remove the by-products.

106 mg (1.0 mmol) glyceric acid, 237 mg (1.14 mmol) DCC, and 291 mg (1.1 mol) pentachlorophenol were dissolved in 3 ml of DMF, placed in ice water bath and stirred for 24 hours, then another 339 mg (1.64 mmol) DCC and 412 mg (1.54 mmol) pentachlorophenol in 3 ml DMF were added, and left to incubate for 24 more hours. The product was dried in a rotary vacuum evaporator and dissolved in the mixture of 4.5 ml ACN and 3 ml DMF. The solution was centrifuged at 4000 *g* for 10 min, decanted and filtered through a 0.45 μm PTFE filter. Clean-up of pentachlorophenol-glycerate was executed with fraction collection based on semi-preparative RP-HPLC-UV separation and was verified with ESI-QQQ-MS. The relevant chromatographic parameters can be seen in Table 7, while ESI-QQQ-MS instrumental parameters can be seen in Table 4. The corresponding fractions were pooled and lyophilized. 13.2 mg dry matter was acquired with a yield of 3%.

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Table 7: HPLC instrumental parameters used for the validation of the 2,3-dihydroxi-propionyl group

Method	Column	Eluents	Gradient	Flow rate	Injection	Other
preparative scale RP-HPLC-UV	Agilent Zorbax XDB-C_8; 5 µm 9.4 x 250 mm	A: H_2O B: ACN	0-5 min:50% B 5-25 min: ↑ 100% B 25-40 min: 100% B 40-41 min: ↓ 50%B 41-50 min: 50% B	2.0 ml/min	100 µl	-
preparative scale SAX-HPLC-ICP-MS	Agilent Zorbax SAX; 5 µm 9.4 x 250 mm	A: 10 mM pH=5.0 ammonium acetate B: 250 mM pH=5.0 ammonium acetate	0-3 min: 0% B 3-20 min: ↑ 100% B 20-40 min: 100% B 40-42 min: ↓ 0% B 42-50 min: 0% B	5.0 ml/min	100 µl	Flow rate divided to 4 ml/min and 1 ml/min. Only 1 ml/min entering ICP-MS
preparative scale RP-HPLC-ICP-MS	Agilent Zorbax XDB-C_8; 5 µm 9.4 x 250 mm	A: 0.1 V/V % HCOOH in H_2O B: ACN	0-10 min: 3% B	3.0 ml/min	100 µl	Flow rate divided to 2.4 ml/min and 0.6 ml/min. Only 0.6 ml/min entering ICP-MS
analytical scale RP-HPLC-ESI-QTOF-MS	Agilent Zorbax XDB-C_{18} 3.5 µm 2.1 x 50 mm	A: 0.1 V/V % HCOOH in H_2O B: 0.1 V/V % HCOOH in ACN	0-5 min:5% B 5-12 min: ↑ 95% B 12-14 min: 95% B 14-14.5 min: ↓ 5% B	350 µl/min	10 µl	-
analytical scale SAX-HPLC-ICP-MS	Hamilton PRPX-100; 10 µm 4.1 x 250 mm	A: 10 mM pH=5.0 ammonium acetate B: 250 mM pH=5.0 ammonium acetate	0-5 min: 0% B 5-15 min: ↑ 100% B 15-35 min: 100% B 35-36 min: ↓ 0% B 36-42 min: 0% B	1.5 ml/min	100 µl	-

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4.4.1.3. Coupling of pentachlorophenol-glycerate and selenocystine

Selenocystine was solubilized in DMF according to the procedure described by Dernovics et *al*. [143]. 4.0 mg pentachlorophenol-glycerate (11 µmol) was dissolved in 4 ml DMF and placed in an ice bath under Ar current and with continuous stirring, 50 µl selenocystine solution (9 µmol) and 2 µl (18 µmol) NMM were added. To keep the pH of the solution between 7 and 8, 2 µl NMM was added three more times at 15-minute intervals, and then it was incubated at room temperature for 48 hours with constant pH monitoring. The final product was lyophilized and dissolved in 4.0 ml 10 mM ammonium acetate buffer (pH=5.5). The solution was centrifuged at 4000 *g* for 10 min, decanted and filtered through 0.45 µm PTFE filters.

The formation of di-2,3-DHP-selenocysteine and 2,3-dihydroxi-propionyl-selenocysteine-selenocysteine was monitored with analytical scale SAX-HPLC-ICP-MS set-up, while their clean-up was carried out for the removal of by-products with sequential semi-preparative SAX-HPLC-ICP-MS and RP-HPLC-ICP-MS based fraction collections. The relevant parameters can be seen in Table 7. The column flow was split both cases to provide adequately low flow rate for the nebulizer of the ICP-MS. The corresponding fractions were pooled and lyophilized.

4.4.1.4. Conjugation of selenocystine with glutathione

The next step for the synthesis of 2,3-dihydroxy-propionyl-selenocysteine-glutathione is the coupling of glyceric acid-selenocystine conjugate with glutathione. As the amount of glyceric acid at our disposal was limited, the conjugation step of the glyceric acid-selenocystine conjugate was optimised using selenocystine only instead of selenocystine-glyceric acid conjugate.

For this step 0.1 M TRIS buffer (pH=8.6) was used. 3.4 mg (10 µmol) selenocystine was reduced and dissolved in 3 ml buffer containing 26.1 mg (169 µmol) dithiothreitol. 106 mg (173 µmol) oxidized glutathione dissolved in 4.0 ml buffer was added to the solution, then 3.8 mg NaI (25 µmol; as catalyst) [184] and 24 µl (160 µmol) H_2O_2 were added.. The solution was incubated for 2 hours at room temperature.

The screening of the reaction products was done with analytical scale SAX-HPLC-ICP-MS, while the clean-up of the selenocysteine-glutathione conjugate was done with semi-preparative SAX-HPLC-ICP-MS (Table 7). The reaction solution was 1+3 (V/V) diluted with 10 mM ammonium acetate buffer (pH=5.5) prior to injection. Fractions were pooled,

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lyophilized, dissolved in 300 µl 10% (V/V) ACN-H_2O solution, and injected to HPLC-ESI-QTOF-MS system for characterization. The relevant instrumental parameters can be seen in Tables 5 and 7.

4.4.1.5. Conjugation of (2,3-dihydroxi-propionyl)-selenocysteine-selenocysteine and di-N-2,3-DHP-Sec with glutathione

The pooled (2,3-dihydroxi-propionyl)-selenocysteine-selenocysteine and di-N-2,3-DHP-Sec compounds acquired from the semi-preparative anion exchange chromatography - HPLC - ICP-MS clean-up were dissolved in 2.0 ml of 0.1 M TRIS buffer (pH=8.6). First 2.5 mg (16 µmol) dithiothreitol was added, followed by 150 mg (244 µmol) oxidized glutathione, then 100 µl 16 mg/ ml NaI solution (11 µmol) and finally 24 µl (160 µmol) H_2O_2 were mixed to the solution. The solution was incubated for 2 hours at room temperature.

The clean-up and the HPLC - ESI - QTOF MS characterization of the 2,3-dihydroxi-propionyl -selenocysteine-glutathione conjugate was carried out the same manner as it was done with the selenocysteine-glutathione conjugate. Instrumental parameters can be seen in Table 5.

4.5. Metabolism of selenium in Hericium erinaceus (lion's mane mushroom)

4.5.1. Production of Se-enriched H. erinaceus

H. erinaceus was cultivated on 3-kg-sized compost bags containing sterilized sawdust – wooden chips – wheat bran in the weight ratio of 1:2:0.1. Subsequent to full colonisation the entire mushroom bag was injected with sodium selenite solution (as 300 mg Se per bag) one week before fruiting. This concentration did not trigger any visible signs of stress or fruit deformation. All harvested mushrooms were lyophilised and ground to fine powder.

4.5.2. Methods

4.5.2.1. Enzymatic sample preparation

Enzymatic preparation was executed for the determination of selenomethionine content.

250 mg of lyophilized and ground mushroom was mixed with 5.0 ml TRIS buffer (pH 6.8, 0.1 M) and 50 mg of Pronase E. The sample was shaken overnight at 37 °C, then another 50 mg of enzyme was dissolved in 3.0 ml buffer and added to the sample, and shaken for 24 hours. The supernatant was decanted and made up to 10.0 ml with deionised water in a

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volumetric flask and filtered through 0.45 µm PTFE disposable syringe filters. The entire sample preparation was executed in three replicates. The process was validated using the wheat reference material with 320 mg sample intake.

4.5.2.2. Determination of total selenium content

To determine the total selenium content in the mushroom and in the remaining drags after the enzymatic digestion the samples were digested with 5.0 ml nitric acid and 3.0 ml of H_2O_2 using the following program: 0-15 min the pressure raised to 250 psi, 15-35 min kept at 250 psi, 35-50 min cooled down to ambient pressure. The total Se concentration was determined with the Agilent 7500ce ICP-MS on the ^{77}Se and ^{82}Se isotopes by the method of standard addition using Rh as the internal standard. The process was validated using the wheat reference material with 400 mg sample intake.

4.5.2.3. Quantification of selenomethionine and Se-methylselenocysteine

The selenomethionine and *Se*-methylselenocysteine contents were measured using the method of three point standard addition with an IP-RP-HPLC-ICP-MS set-up. Instrumental parameters can be seen in Table 8.

Table 8: Chromatographic conditions used during the examination of Se-metabolism in *Hericium erinaceus*

	Column	Eluent A	Eluent B	Gradient	Injection volume	Flow rate	Column temperature
Quantification of SeMet and SeMetCys	Thermo Hypersil Keystone C_{18};250 mm x 4.6 mm x 5 µm	0.1 V/V% HFBA in deionised water	0.1 V/V% HFBA in methanol	0-5 min : 5%B 5-15 min:↑ 50%B 15-20 min: 50%B 20-21 min: ↓5%B 21-28 min: 5%B	100 µl	0.9 ml/min	30 °C
SEC cleanup	HiLoad 16/60 Superdex 30 SEC	ammonium-acetate (pH 9.5, 10 mM)	-	isocratic	1.2 ml	1.5 ml/min	not controlled
HPLC-ESI-QTOF-MS analysis	Agilent Zorbax XDB-C_{18} 2.1 mm x 50 mm x 3 µm	0.1 V/V% HCOOH in deionized water	0.1 V/V% HCOOH in ACN	0-5 min : 5%B 5-15 min:↑ 50%B 15-20 min: ↑100%B 20-25 min: 100%B 25-26 min:↓ 5%B 26-32 min: 5% B	100 µl	0.35 ml/min	30 °C

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Four aliquots of 100 µl of the enzymatic digested samples were mixed with 50 µl 0.1 m/m% dithiothreitol and standard solutions of 0, 2, 5, 10 ng selenomethionine (as Se), respectively. All four solutions were made up to 1 ml using eluent A. For *Se*-methylselenocysteine, 0, 0.2, 0.5, and 1 ng addition levels were used.

4.5.2.4. Ultrasonic extraction

Aquous ultrasonic treatment was executed for the extraction of small-molecular-weight selenium-containing compounds, which are typical of yeast metabolism.

0.5 g of lyophilized and ground mushroom powder was suspended in 10.0 ml of Milli-Q deionised water, extracted with the ultrasonic probe for two minutes, then centrifuged, decanted, and the process was repeated once more. The two aqueous extracts were pooled and lyophilized. The dry extracts were dissolved in 1.5 ml ammonium acetate buffer (pH 9.5, 10 mM) and filtered through 0.45-µm PTFE disposable syringe filters. The entire sample preparation was executed in seven replicates.

4.5.2.5. Fraction collection from SEC separation and IP-RP-HPLC clean-up

SEC was used for the removal of matrix. Instrumental parameters can be seen in Table 8. Fraction collection started 42 min after injection, and 95 fractions were collected at 1 min intervals.

The selenium content of collected fractions was monitored using flow injection - ICP-MS. Fractions corresponding to identical peaks were pooled and lyophilized.

The selected selenium containing fractions were dissolved in 500 µl deionised water, and subjected to IP - RP cleanup on the Thermo Hypersil column following the parameters described above. To 50 µl of the dissolved sample 25 µl of 0.1 m/m% dithiothreitol and 425 µl eluent A was added. 50 µl of this mixture was injected for each fraction collection that was repeated 18 times. Afterwards, the corresponding fractions were pooled and vacuum centrifuged until dryness.

4.5.2.6. HPLC-ESI-QTOF-MS analysis

HPLC-ESI-QTOF-MS analysis was executed for the identification of selenium-containing metabolites.

The dried samples were dissolved in 200 µL of 0.1 V/V% HCOOH in deionised water, and injected to the HPLC - ESI - QTOF MS system. The system was operated in positive and negative modes with the parameters described in Tables 5 and 8.

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5. Results and discussion

5.1. Effect of sample preparation methods on the D,L-enantiomer ratio of extracted selenomethionine

The most commonly applied sample preparation methods for the quantification of selenomethionine are the enzymatic and the acidic (mostly methanesulphonic acid based) digestion methods. In many cases both are used for the monitoring of selenomethionine. The scales seemed to tip towards the acidic digestion, when in 2004 Mester *et al.* [146] published their work on the comparison of extraction methods for quantitation of methionine and selenomethionine in yeast by species specific isotope dilution GC–MS. They came to the conclusion that methanesulphonic acid based extraction was the most efficient one. Later, a number of articles were published using the very same digestion methods. However, the article disregarded the possible analyte decomposition – and that high temperature and high pH are known to cause racemization. In addition, the method proposed by Mester *et al*. used elevated temperature treatment for an extended period of time. However, in 2006 the first yeast-based Se-speciation CRM, SELM-1 became available. An intercomparison exercise [146] was executed aiming to certify this reference material for the quantification of methionine, selenomethinone and total selenium. Numerous groups took part in the event, and the results were compared to data acquired from isotopic dilution measurements. The results within the accepted boundaries were all acquired through either acidic or enzymatic digestion. After this point, the popularity of acidic digestion fell again, but it never disappeared. To this day, both methods have been used for selenomethionine quantification, but it remains a question whether they cause decomposion. The aim of our work was to compare the effects these two procedures have on the detectable enantiomer ratios of selenomethionine in samples of biological origin and to determine if they have any enantiomer-specific properties that can bias the results.

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5.1.2. Sample preparation and cleaning-up of the selenomethionine fraction

In order not to influence the genuine enantiomer ratio during the enzymatic sample preparation pH ≥ 7 was avoided, so that no racemisation would occur because of the alkaline circumstances. Hence the usually applied pH ≥ 7 was changed to pH = 6.8. The certification procedure of SELM-1 was regarded as reference for enzyme selection [188]. Preliminary studies showed that anion exchange chromatography - HPLC is a robust and cost-effective method of choice for the fractionation of selenomethionine from these samples since it allows the separation of selenomethionine as the most abundant Se-containing fraction of the chromatogram. Before the injection dithiothreitol was added to the samples as a reducing agent to prevent the oxidation of selenomethionine as oxidised selenomethionine is not retained on the anion exchange chromatography - HPLC [189].

After the acidic digestion it was necessary to dilute the samples to suit the requirements of anion exchange chromatography separation. It resulted in a lower concentration of selenomethionine in these samples compared to enzymatic hydrolisates but the attempts for preconcentration by lyophilisation failed because irreversible degradation was observed: the process caused the enrichment of the acid and degradation of the target compound.

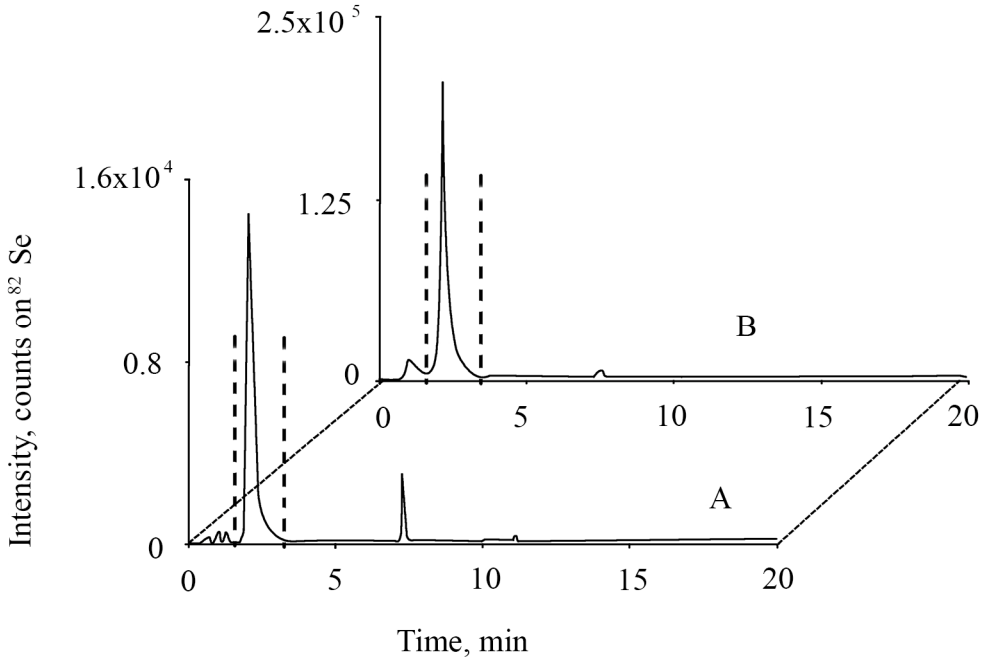

Figure 4: SAX-HPLC-ICP-MS chromatogram of the SELM-1 sample. A, prepared with acid digestion; B, prepared with enzymatic digestion. The fractions were collected between the dashed lines

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Figures 4 and 5 show the chromatograms obtained by anion exchange chromatography - HPLC - ICP-MS for the different samples and sample preparation methods. The fractions collected are highlighted in the chromatograms.

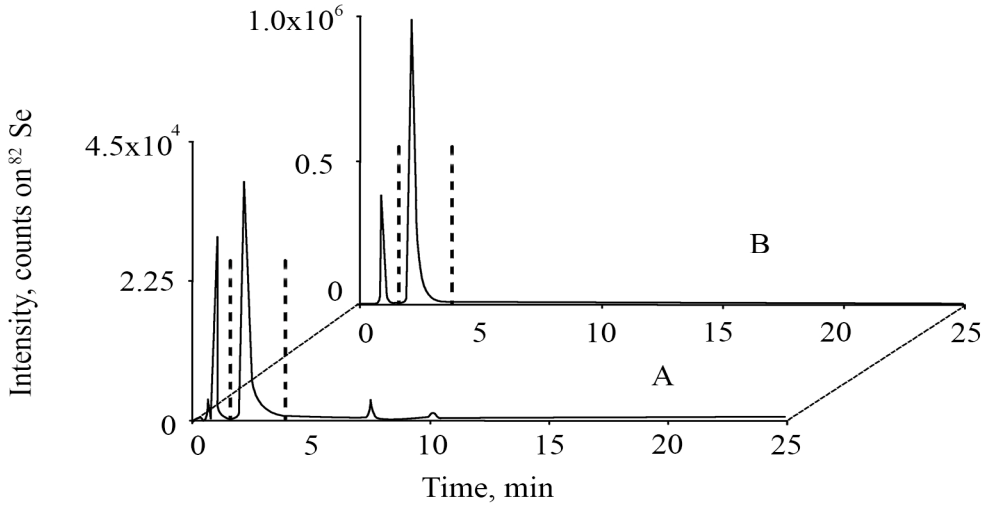

Figure 5: SAX-HPLC-ICP-MS chromatogram of the monkeypot nut sample. A, prepared with acid digestion; B, prepared with enzymatic digestion. The fractions were collected between the dashed lines. The peaks eluting in the void contain selenocystathionine [143]

5.1.3. Derivatisation and quantification

The optimization of the method was executed with the use of L-selenomethionine standard solution. After the suggested 2-minute long incubation time only partial derivatisation was achieved.

It can be seen on Figure 6, that besides the derivatised format eluting at 17 minutes, another peak closer to the void also appears, that is the non-derivatised standard.

According to literature, the 3.5 µl reagent used for 40 ng amino acid (measured as Se) was supposed to be sufficient for the complete derivatisation, therefore the reaction time was lengthened to 5 minutes and used so in all following experiments. The results of the optimization can be seen in Figure 7.

While the peak of non-derivatised amino-acid disappeared, a new small peak eluted at 21 minutes. The integration results showed it was 1.2% of the entire L-selenomethionine content. According to literature [55] this phenomenon is not unprecedented. The new peak is the D-enantiomer of the component. The enantiomer-selective standards are produced from racemic

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mixtures using resolvation, therefore the presence of a small amount of D-enantiomer cannot be prevented.

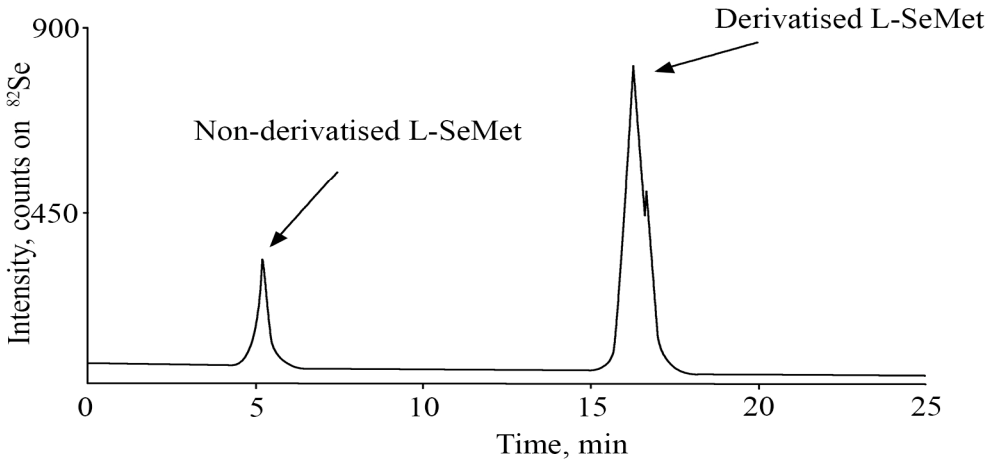

Figure 6: Incomplete derivatisation of L- selenomethionine with OPA and NIBC, monitored with RP-HPLC-ICP-MS instrumentation

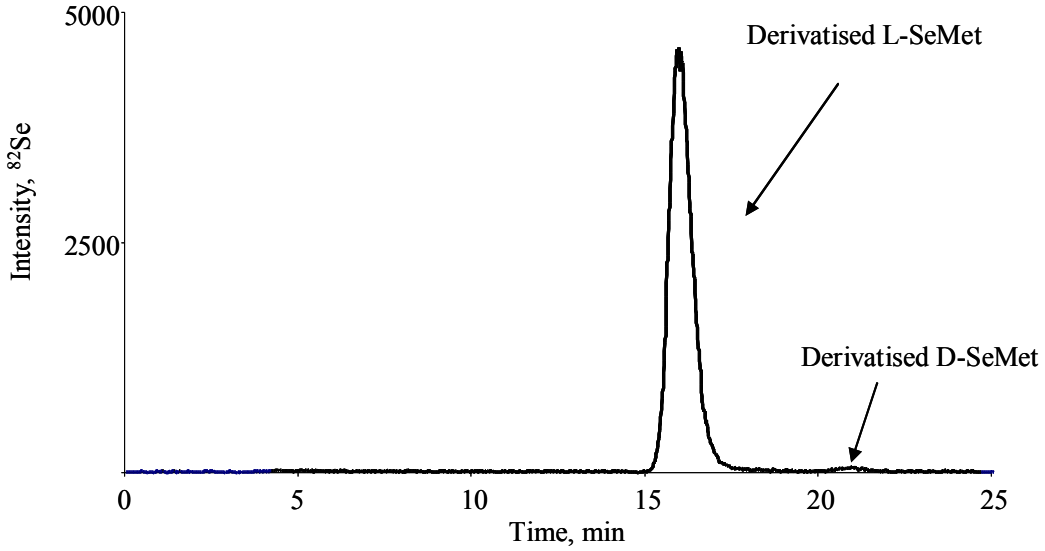

Figure 7: Result from the optimisation of the derivatisation process of the L-selenomethionine standard, monitored with RP-HPLC- ICP-MS

The verification of the derivatization for a racemic mixture was also carried out. The resulting chromatogram can be seen on Figure 8.

The ratio of the integrated areas of the L- and D-selenomethionine peaks was 55%:45%. To be able to compare this value with the original ratio, an enquiry was made to the manufacturer. The ratio was defined as containing 52.2% L- and 47.8% D-enantiomer.

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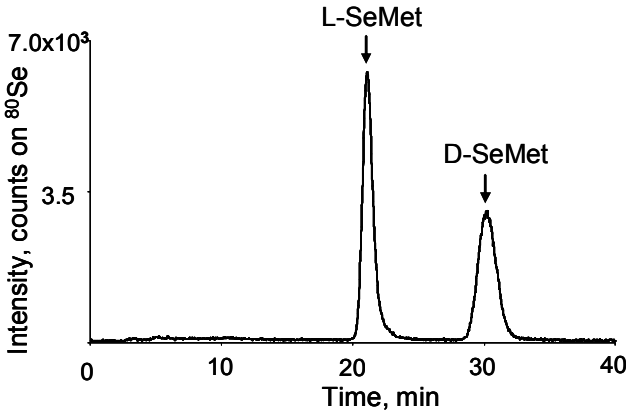

Figure 8: HPLC-ICP-MS separation of derivatised D,L- selenomethionine standard (injected amount: 20 ng as Se)

When using the optimized method for a real food sample without any kind of protein or amino acid clean up, the resulting chromatogram has a large amount of unknown components in the chromatogram. The reasons of this phenomenon could be either the relative impurity of the analysed extract or the insufficient amount of the derivatising agent applied. To eliminate these problems in our work dedicated protein clean up was used that was followed by a subsequent anion exchange chromatography - HPLC fractionation prior to the derivatisation to decrease the sample matrix. In addition, the ratio of the samples and the derivatisation agent were individually optimised (see Table 9) in order to achieve the highest possible derivatising rate of the extracted selenomethionine. This was especially important to do, as during the optimization of the derivatisation process the chromatographic peak intensities acquired from the acidic and enzymatic digested samples were considerably different, in spite of the fact that previously they were cleaned up by the same SAX - HPLC technique.

The chromatography of the enantiomers of the D,L-selenomethionine standard provided adequate separation (R=6) enabling both external calibration and standard addition (see Figure 8). However, optimization investigation showed that in the case of real samples high concentration of the sample had to be injected otherwise the D-enantiomer could not be detected. On the other hand, the large amount of matrix hampered the application of standard addition. Therefore, the added amount of D-selenomethionine could not be baseline separated from the tailing of the L-selenomethionine peak. This is why external calibration was addressed with different calibration ranges for the enantiomers. As the elution was isocratic, the ionisation efficiency provided by the ICP might be considered stable during the chromatographic run after the void section.

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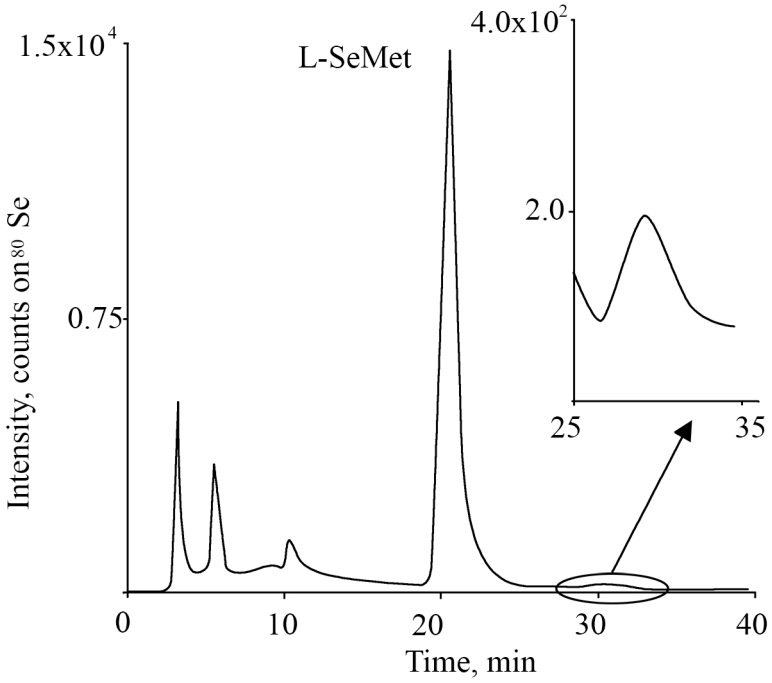

Figure 9: Enantiomer separation of selenomethionine in the SELM-1 sample following the enzymatic cleanup, monitored with RP-HPLC-ICP-MS. The inset shows the peak corresponding to D-selenomethionine

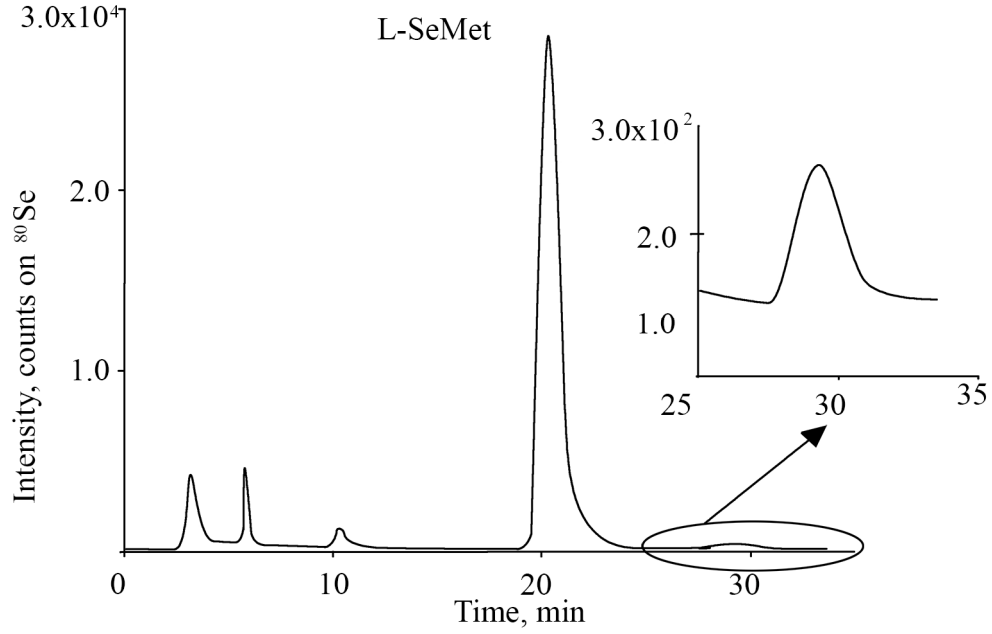

Figure 10: Enantiomer separation of selenomethionine in the monkeypot nut sample following the enzymatic cleanup, monitored with RP-HPLC-ICP-MS. The inset shows the peak corresponding to D-selenomethionine

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The concentrations of L-, and D-enantiomers differed with at least one order of magnitude. It is to note that the external calibration was carried out with the commercially available D,L-selenomethionine standard. The chromatogram acquired during the enantiomer separation of SeMet in the SELM-1 sample can be seen in Figure 9, from the monkeypot nut sample in Figure 10.

Concerning the quantification process a highly significant difference (~60%) was found between the slopes of the calibration curves of the two derivatised enantiomers. Derivatisation was carried out with a 1:1 V/V mixture of NIBC and OPA exactly 5 minutes prior to injection. The ratio of the sample, water and derivatising agents used can be seen in Table 9.

Table 9: Derivatisation setup for selenomethionine with the detailed amounts of sample, derivatising agents and water used. L denotes the quantification of L-selenomethionine, while D denotes the quantification of D-selenomethionine. L^1: the analytical range for calibration included 2, 5, 10, 20 ng D,L-selenomethionine (calculated as Se). D^2: the analytical range for calibration included 1, 2, 5, 10 ng D,L-selenomethionine (calculated as Se). The slopes of calibration were 6561 and 4127 counts/ng Se^{-1} for L and D, respectively

	Sample volume, µL		Deionized water, µL		Derivatising solution, µL		Injected volume, µL	
Sample	L	D	L	D	L	D	L^1	D^2
0.2 mg L^{-1} selenomethionine standard (expressed in Se)	100		0		100		20	10
							50	20
							100	50
							200	100
Enzymatically digested monkeypot nut	15		120		15		5	70
Acid hydrolysed monkeypot nut	80	160	0		120	240	10	100
Enzymatically digested SELM-1	15	150	30	150	15	300	5	90
Acid hydrolysed SELM-1	80	160	0		120	240	20	200

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Former studies have also revealed that ICP-MS shows different sensitivity for the various seleno-species [148,183] but such difference for enantiomer pairs was unexpected in particular in case of isocratic elution. The column recovery experiments revealed that 93% of D,L-selenomethionine was eluted from the column in derivatised form, while 1% was eluted in the void volume, *i.e.*, in underivatised form. As the OPA-NIBC based derivatisation process equally acts on both enantiomers, neither the derivatisation, nor column recovery issues are responsible for the observed difference in the instrumental response.

5.1.4. Statistical analysis

The statistical analysis presented in Table 10 showed significant differences between the enantiomer ratios of the samples treated with the different sample preparation methods. The amount of the D-enantiomer liberated by the acidic digestion (2.2% and 2.7% for the SELM-1 and the monkeypot nut, respectively) was significantly higher than the quantity freed by the enzymatic digestion (0.5% and 0.6% for the SELM-1 and the monkeypot nut, respectively), in the cases of both samples. The ratio of D-enantiomer in the monkeypot nut samples was higher numerically than in the SELM-1 samples but this difference was not significant according to the statistical analysis.

Table 10: Results of statistical analysis carried out with Student's test, at 95% significance levels. Five independent replicates were quantified for each ratio determination. For details, see text. SD denotes to standard deviation. "S" and "NS" denote to significant and not significant, respectively

Samples and preparation methods		Average D/L SeMet ratio ± 1 SD	Differences in the D/L ratio, compared to			
			Monkeypot nut		SELM-1	
			Enzymatically digested	Acid hydrolysed	Enzymatically digested	Acid hydrolyzed
Monkeypot nut	Enzymatically digested	0.6% ± 0.04%	-	S	NS	-
	Acid hydrolyzed	2.7% ± 0.6%	S	-	-	NS
SELM-1	Enzymatically digested	0.5% ± 0.03%	NS	-	-	S
	Acid hydrolyzed	2.2% ± 0.5%	-	NS difference	S	-

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As a reference, the study of Weiss *et al*. [184] can be taken, showing that Met, the sulphur analogue of selenomethionine, can undergo racemisation during MSA based hydrolysis. However, the amount of the resulting D-enantiomer extremely depends on the sample and on the way of energy transfer, *i.e.*, boiling under reflux or microwave irradiation. In that study, the racemisation of L-Met was detected in the range of 0-10% after refluxed MSA digestion. Our results on selenomethionine correspond well to this observation.

On the other hand, the enzymatic digestion should reflect the genuine D,L-enantiomer ratio of selenomethionine in the samples, as neither prolonged alkalic treatment nor highly acidic circumstances were allowed. To the best of our knowledge, no reports can be found on the ratio of D,L-Met enantiomers determined after enzymatic proteolysis. Thus the comparison with our relevant data on D,L- selenomethionine is not possible. Concerning the stereospecificity of crude pronase E, a Boehringer product showed almost perfect differentiation between racemic and enantiomerically pure synthetic L-dipeptides. This consequence is drown from the hydrolysis rate data [185]. The stereospecific action of a related crude product, protease XIV, from a different manufacturer on samples containing intact proteins cannot be directly estimated.

Clearly, the 0.5-0.6 % of D-selenomethionine after enzymatic digestion does not meet certain results of former studies (see Table 2). In some cases this can be explained by detection limit issues. However, studies reporting the ratio of D-selenomethionine that differ significantly from both the full racemic (that is, synthetic) cases and the other extremities with the amount of selenomethionine close to the detection limit, should further be investigated.

5. 2. Validation of the 2,3-dihydroxy-propionyl group in selenium speciation by chemical synthesis and LC - MS analyses

The greatest challenges of the synthesis were the commercial unavailability of any active ester form of glycerate and the tendency of polyols for condensation/polymerization. The intermediate step of the synthesis, the coupling of the active ester to selenocystine results in two yeast-specific Se-compounds, the conjugate of 2,3-dihydroxi-propionyl-selenocysteine and selenocysteine (CAS 1246200-50-9 + 3614-08-2; $C_9H_{17}O_7N_2Se_2^+$ $[M+H]^+$, *m/z* 424.93634) [17], and di-N-2,3-DHP-selenocysteine (CAS 1357479-85-6; $C_{12}H_{21}O_{10}N_2Se_2^+$ $[M+H]^+$, *m/z* 512.95211) [181]. The last step of the synthesis includes the oxidative conjugation of glutathione and a modified selenocysteine residue to form a S-Se bridge. Similarly to this step, with the conjugation of glutathione and native selenocysteine another Se-species detected in yeast (CAS No. 188609-44-1; $C_{13}H_{23}O_8N_4SSe^+$ $[M+H]^+$, *m/z* 475.03963) [181] could be also

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synthesised and purified. The clean-up procedures were monitored with HPLC -UV, - ICP-MS, and -ESI-QQQ-MS detection, while the identification was based on HPLC-ESI-QTOF-MS characterization.

5.2.1. Conjugation of selenocystine with glutathione and the characterization of selenocysteine-glutathione

From the family of selenium containing glutathione conjugates selenocysteine-glutathione is one of the least sophisticated compounds and it occurs in nearly all batches and strains in selenised yeast [189], however its concentration does not exceed that of 2,3-DHP-selenocysteine-glutathione. Apart from very low abundant selenocystine and selenocystine species [17,114], this compound is unique in terms of containing a non-modified selenocysteine residue attached through either a S-Se or a Se-Se bond. This fact might be explained by the limited availability of native selenocysteine due to its general depletion to detoxify excess Se.

The difficulty of the chemical synthesis of selenocysteine-glutathione is the effective oxidative conjugation in the presence of the huge excess of dithiothreitol required for the solubilization of selenocystine.

In literature articles can be found [190] where the author claims that reduced glutathione can be oxidized solely in the presence of H_2O_2. Based on this information, the first experiment for the conjugation included mixing the selenocystine solution with reduced glutathione and adding H_2O_2 drop by drop to the mixture in hope of being able to form Se-S bridges. After the suggested amount of H_2O_2 was used up results were evaluated with ESI - trilpe-quadrupole - MS. The acquired data showed no change in the form of glutathione. Hoping to achieve the desired outcome, more H_2O_2 was added to the solution periodically monitoring the state of oxidization. 25 times more H_2O_2 has been added than it would have been needed according to the presumed stoichiometric reaction mechanism. It was found that the method did not yield any of the conjugate nor a detectable amount of oxidized glutathione. Reduced glutathione would start decomposing long before it would start producing the oxidized form. Though the decomposition of glutathione at such high H_2O_2 concentrations was not entirely unexpected, as that agreed with the data published by Finley *et al*. [191] about the oxidization of reduced glutathione. However, even their work reported the successful formation of glutathione at 60% rate, something that in our case was not observed.

This unsuccessful experiment suggested that the authors either left information out about their procedures, or, these particular experiments were not even executed. This prompted us to look for different solutions. It was the work of Kirihara *et al.* published in 2007 that offered the

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solution: the use of catalyst was necessary. The problem was resolved by NaI catalyzed oxidation[192] and by the depletion of DTT with oxidized glutathione.

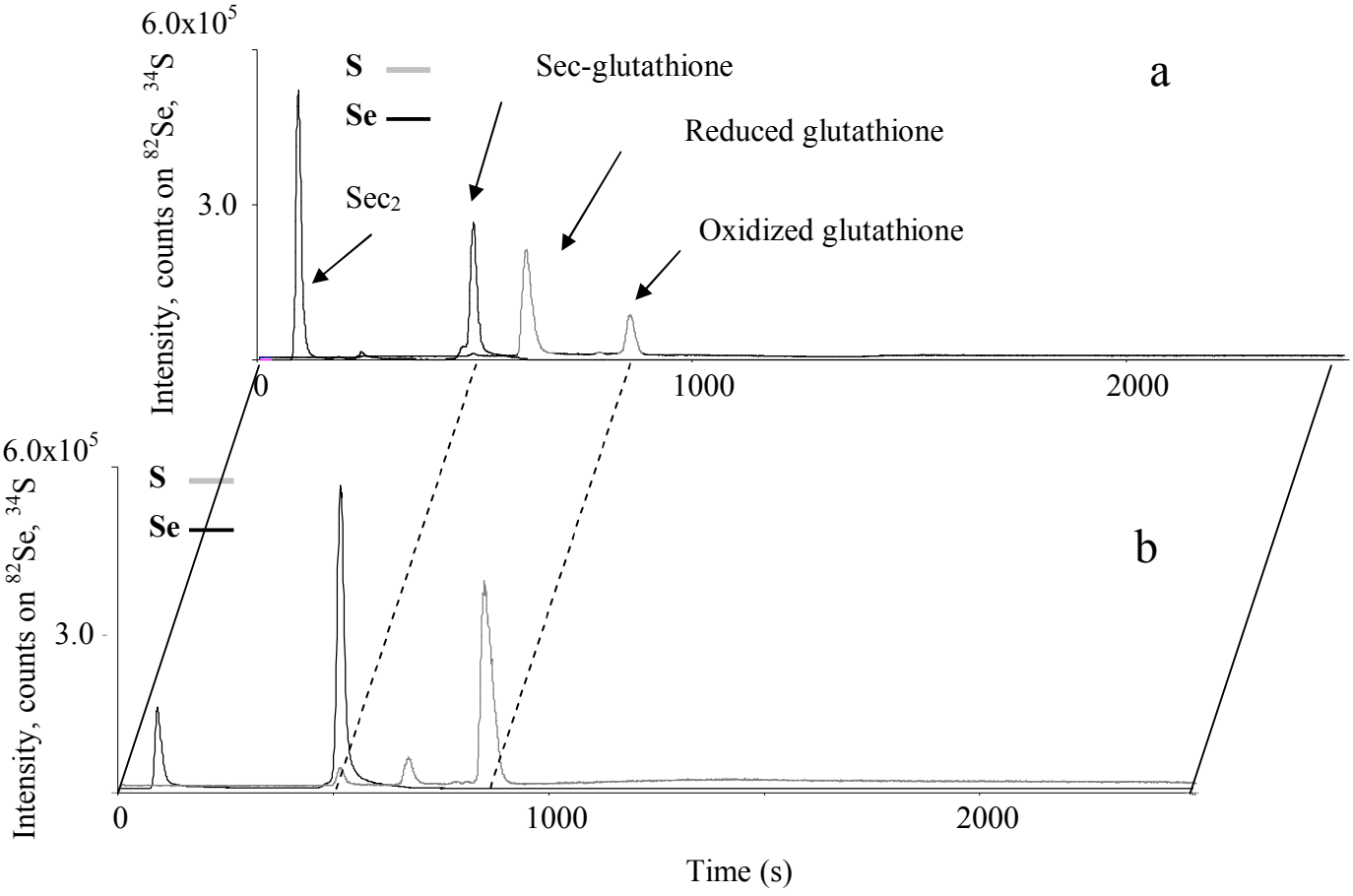

Figure 11: Results of the optimization of selenocysteine-glutathion conjugation: a, before, and b, after (buffer modified from 50 mM, pH=7.5 to 0.1 M pH=8.6 time increased to 1 h from 15 minutes)

It was found that an incubation time of 1 h, and a pH strictly in the range of 8.0-.8.5 is necessary for the successful coupling. It was also found that the reaction products would shift the pH towards the acidic range; therefore, a higher concentration buffer solution was used. The results of optimization are shown in Figure 11. The unconjugated selenocystine peak that elutes in the void has been reduced, and the amount of target compound has increased. With the changes made it is no longer the oxidized glutathione that is reduced by the dithiothreitol, the reduced glutathione peak was considerably decreased.

As presented in Figure 11 the arising selenocysteine-glutathione elutes between selenocystine and oxidized/reduced glutathione on SAX-HPLC, thus providing adequate separation for the actual chromatographic clean up.

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Table 11: mass accuracy of target components and fragments

	Measured *m/z*	Calculated *m/z*	ppm difference
$[C_{16}H_{27}N_4O_8SSe]^+$	**563.0555**	**563.0562**	**-1.31**
$[C_8H_{13}O_4N_2SeS]^+$	312.9720	312.9761	-13.2
$[C_6H_{10}NO_5Se]^+$	255.9709	255.9719	-3.83
$[C_8H_{11}O_4N_2S]^+$	231.0438	231.0429	4.03
$[C_3H_6NO_2Se]^+$	167.9548	167.9558	-6.25
$[C_5H_8NO_3]^+$	130.0490	130.0498	-6.15
$[C_{13}H_{21}N_4O_8SSe]^+$	**475.0396**	**475.0400**	**-0.91**
$[C_{11}H_{18}N_3O_6SeS]^+$	400.0027	400.0075	-11.9
$[C_8H_{16}N_3O_5SeS]^+$	345.9925	345.9970	-13.0
$[C_5H_9N_2O_3SSe]^+$	256.9455	256.9499	-17.1
$[C_8H_{11}N_2O_4S]^+$	231.0398	231.0434	-15.8
$[C_3H_6NO_2Se]^+$	167.9532	167.9558	-15.8
$[C_5H_8NO_3]^+$	130.0480	130.0498	-13.5
$[C_{12}H_{21}N_2O_{10}Se_2]^+$	**512.9534**	**512.9530**	**0.78**
$[C_7H_{10}N_2O_5Se_2]^+$	361.9084	361.9046	10.5
$[C_6H_{10}NO_5Se_2]^+$	335.8921	335.8889	9.41
$[C_6H_8NO_4Se_2]^+$	317.8837	317.8784	16.7
$[C_6H_{10}SeNO_5]^+$	255.9738	255.9719	7.42
$[C_3H_6NO_2Se]^+$	167.9573	167.9558	8.93
$[C_9H_{17}N_2O_7Se_2]^+$	**424.9361**	**424.9368**	**-1.72**
$[C_6H_{10}NO_5Se_2]^+$	335.8910	335.8889	6.13
$[C_6H_8NO_4Se_2]^+$	317.8810	317.8784	8.24
$[C_6H_{10}SeNO_5]^+$	255.9721	255.9719	0.78
$[C_3H_6NO_2Se_2]^+$	247.8729	247.8740	-4.60
$[C_3H_6NO_2Se]^+$	167.9561	167.9558	1.79

The purified selenocysteine-glutathione was characterized with HPLC-ESI-QTOF-MS and MS/MS experiments. Figure 12a shows the total ion chromatogram (TIC) and extracted ion chromatogram(EIC) of the compound, Figure 12b shows the full scan recorded at the apex of the related extracted ion chromatogram ($C_{13}H_{23}O_8N_4SSe^+$ $[M+H]^+$, *m/z* 475.03959, Δ= -0.08 ppm) and Figure 12c presents the MS/MS data (see also Table 11). Similarly to the fragmentation of the Se-containing glutathione family and selenocystine in positive ion mode [86,97], the intense fragments arrive from the loss of Gly, γ-Glu residues and neutral losses of NH_3 and HCOOH, while the S-Se bond is hardly fragmented and the intact glutathione and selenocysteine residues are only of low abundance.

It is important to mention that the synthesis of selenocysteine-glutathione was addressed both as an optimization step and as an independent method for the synthesis of a commercially unavailable compound.

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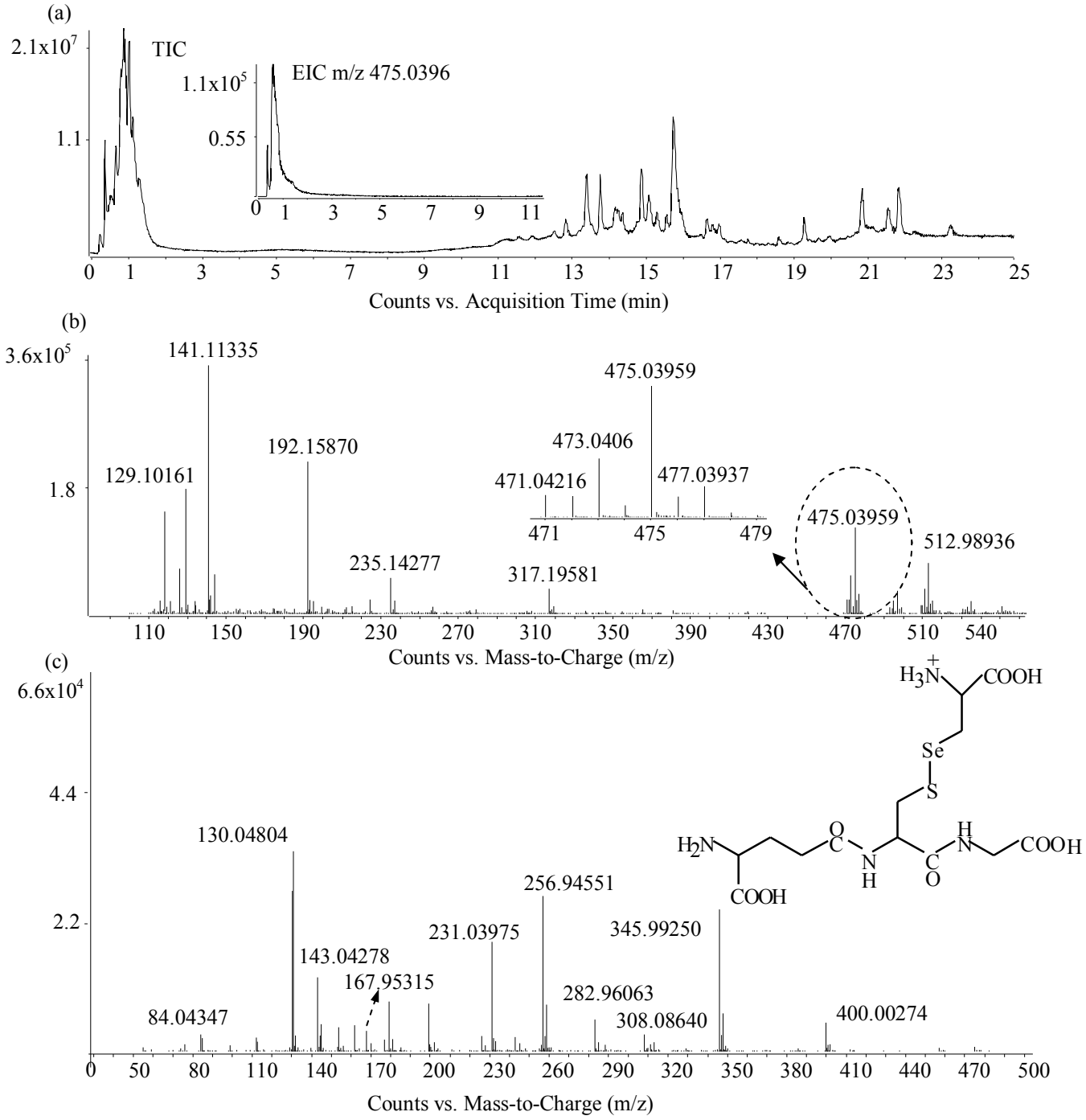

Figure 12: (a) HPLC-ESI-QTOF-MS TIC) of the compound collected from SAX-HPLC. The inset presents the EIC for *m/z* 475.0396. (b) Full scan spectrum recorded near the apex of the EIC for *m/z* 475.0396. The inset shows the selenium pattern of the target compound. (c) MS/MS spectrum and structure of the compound at *m/z* 475.0396

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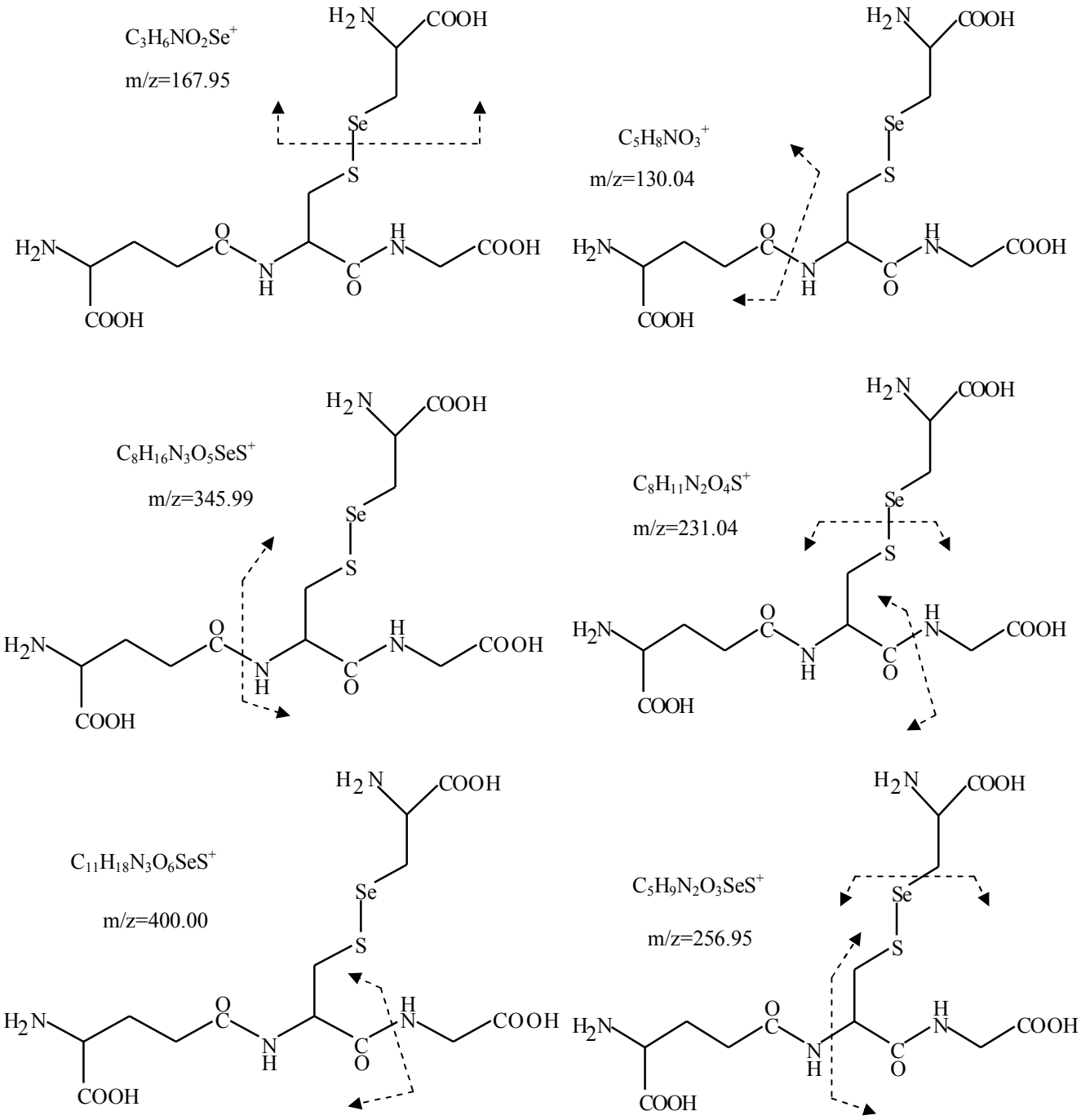

Figure13: Proposed MS/MS fragmentation mechanisms of the conjugate of selenocysteine and glutathione (*m/z* 475.03)

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5.2.2. Synthesis and clean-up of pentachlorophenol-glycerate

The use of pentafluorophenol (PFP) might be preferred over pentachlorophenol, as PFP esters react faster and the removal of pentachlorophenol may be difficult.[30] However, in our research it was found that the reaction of glyceric acid with PFP did not yield any detectable amount of ester (results not shown); therefore, the step was repeated with the use of pentachlorophenol.

During the experiment it was also found that the reaction was relatively slow; the second 24 hours of incubation yielded an order of magnitude more synthesis product.

Pentachlorophenol renders to the pentachlorophenol-glycerate hydrophobic properties, thus providing the possibility for an RP-HPLC based clean-up. Figure 14a presents the relevant HPLC-UV chromatogram where the compound eluting at 14.3 min was identified with ESI - MS/MS as pentachlorophenol-glycerate after preparative scale fraction collection. The compound could be identified due to its unique isotopic pattern containing five chlorine atoms and it could be characterized with the same fragmentation mechanism during both the ionization process in the ion source (Figure 14b) and the MS/MS fragmentation (Figure 14c), *i.e.*, the arising of pentachlorophenyl anion (*m/z* 351.0 $[C_9H_4Cl_5O_4]^- \rightarrow$ *m/z* 264.8 $[C_6Cl_5O]^-$). The low yield of synthesis can be partly attributed to the polyolic structure of glyceric acid that facilitates the formation of by-products, and partly to the need for water-containing HPLC eluent.

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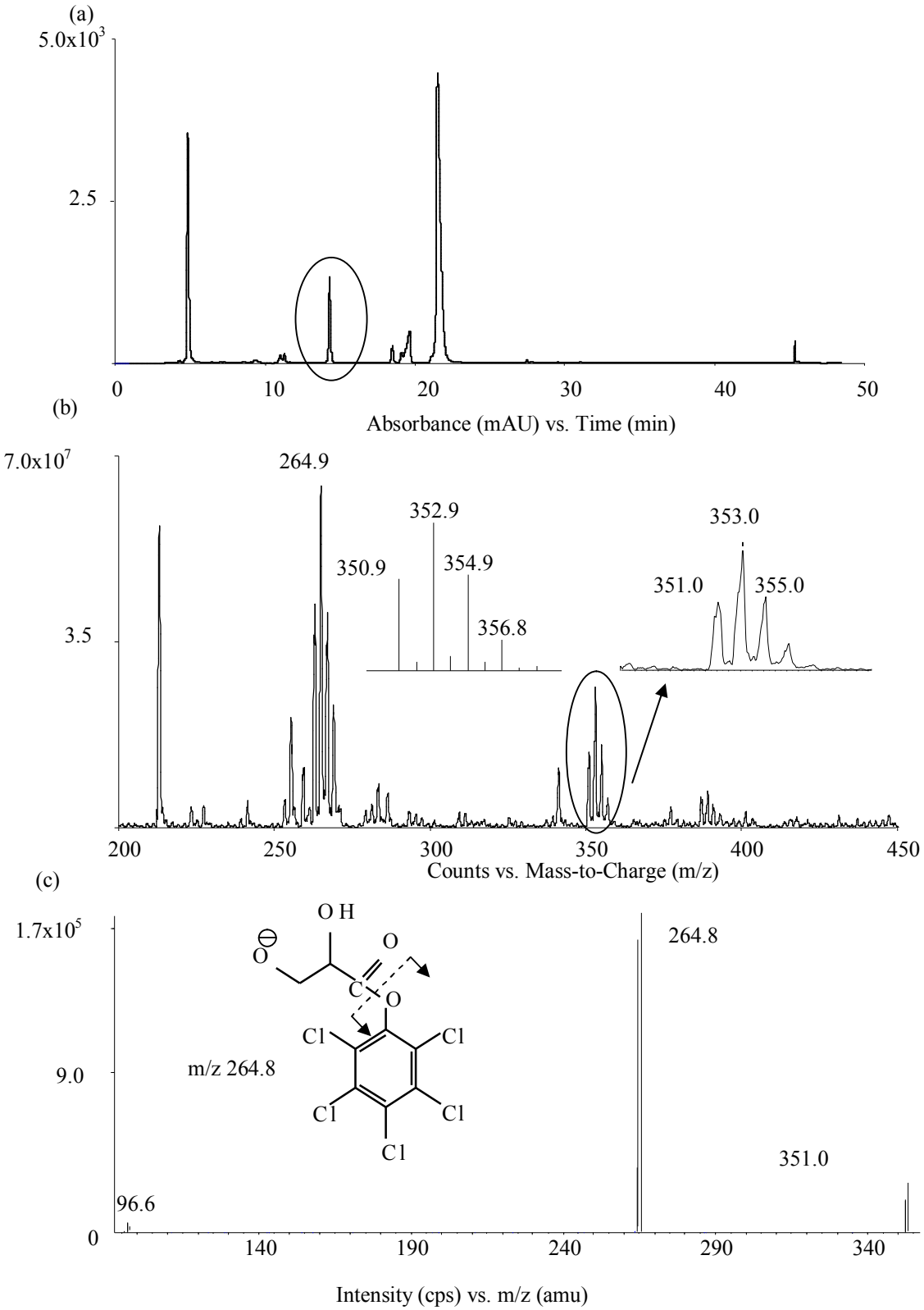

Figure 14 (a) Preparative scale RP-HPLC-UV chromatogram of the products resulting after the coupling of pentachlorophenol and glyceric acid. The compound eluted at 14.3 min was collected for further characterization and synthesis. (b) ESI-QQQ-MS full scan spectrum of the compound collected from RP-HPLC. The inset presents the theoretical (left) and experimental (right) isotopic pattern of pentachlorophenol-glycerate. (c) MS/MS spectrum of the compound at *m/z* 351.0, together with the proposed fragmentation event

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5.2.3. Coupling of pentachlorophenol -glycerate to selenocystine and the characterization of the (2,3-DHP)- selenocysteine-selenocysteine and di-N-2,3-DHP- selenocysteine species

Active ester coupling to selenocystine yields a mixture of non-derivatized, single and double derivatized species [193], thus requiring a clean-up step. As the free $–NH_2$ groups are bound in the reaction with pentachlorophenol-glycerate the resulting species will show anionic properties even at slightly acidic pH, which enables the SAX-HPLC based purification.

Figure 15a presents the HPLC-ICP-MS chromatogram of the synthesised products, where three selenium containing peaks could be observed: selenocystine arriving close to the dead volume, and the hypothetic (2,3-DHP)-selenocysteine-selenocysteine and di-N-2,3-DHP-selenocysteine species in the order of elution, respectively. The latter two compounds were cleaned-up and characterized with HPLC-ESI-QTOF-MS analyses. Figure 15b shows the relevant TIC and the EICs of the two compounds extracted at their relevant theoretical *m/z* values.

Figure 15c presents the full scan recorded at the related extracted ion chromatogram of the *m/z* 424.93 compound. The accurate mass ($C_9H_{17}O_7N_2Se_2^+$ $[M+H]^+$, *m/z* 424.93607, Δ= -0.64 ppm), isotopic distribution and MS/MS fragments (see Figure 15d) match those of reported by Arnaudguilhem *et al*. [17]. Concerning di-N-2,3-DHP-selenocysteine, the data presented on Figure 16a ($C_{12}H_{21}O_{10}N_2Se_2^+$ $[M+H]^+$, *m/z* 512.95203, Δ= -0.16 ppm) are in agreement with those published by Casal *et al*. [181], while the MS/MS fragments have been presented here for the first time (Figure 16b).

The suggested fragmentation pathways of the two compounds are included in the supplementary information. It should be highlighted that the fragmentation of both species results in the abundant appearance of the couple of *m/z* 255.97 – *m/z* 167.95 fragments that is also characteristic of the conjugate of 2,3-DHP-selenocysteine–glutathione (*m/z* 563.05).

Considering the low efficiency of the 2,3-DHP coupling process, both /single and double/ derivatized compounds were purified and pooled in order to increase the yield of the following conjugation step with glutathione. The proposed fragmentation of the compounds can be seen in Figures 17 and 18.

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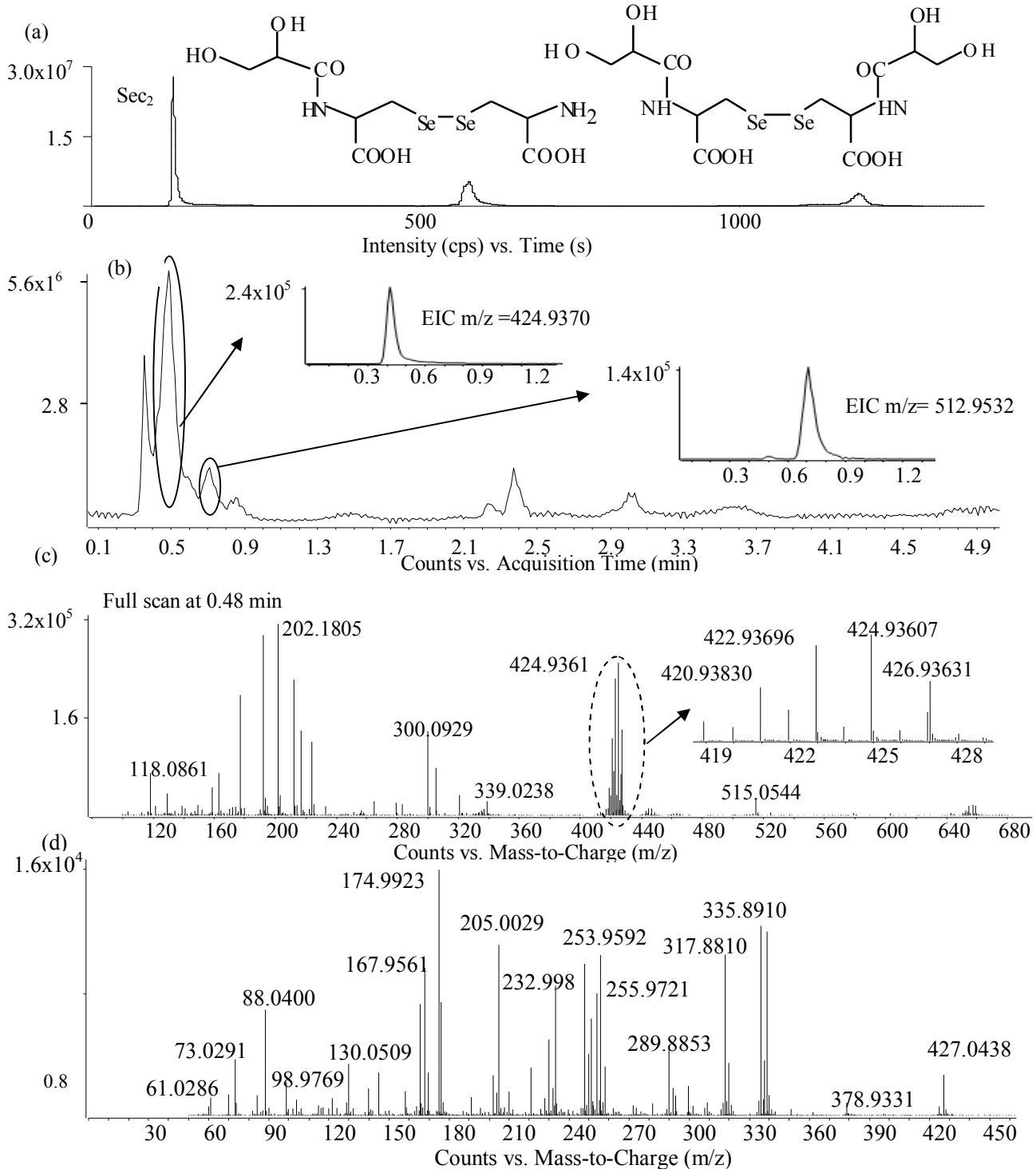

Figure 15 (a) Analytical scale SAX-HPLC-ICP-MS chromatogram of the products arising from the coupling of pentachlorophenol-glycerate and selenocystine. The two peaks marked with the hypothetical compound structures eluting at 605 s and 1230 s were pooled for further characterization and synthesis with preparative scale SAX-HPLC. (b) HPLC-ESI-QTOF-MS based TIC of the compounds collected from SAX-HPLC. The inset presents the EICs for *m/z* 424.9370 and *m/z* 512.9532. (c) Full scan spectrum recorded near the apex of the EIC for *m/z* 424.9370. The inset shows the selenium pattern of the target compound. (d) MS/MS spectrum of the compound at *m/z* 424.9370

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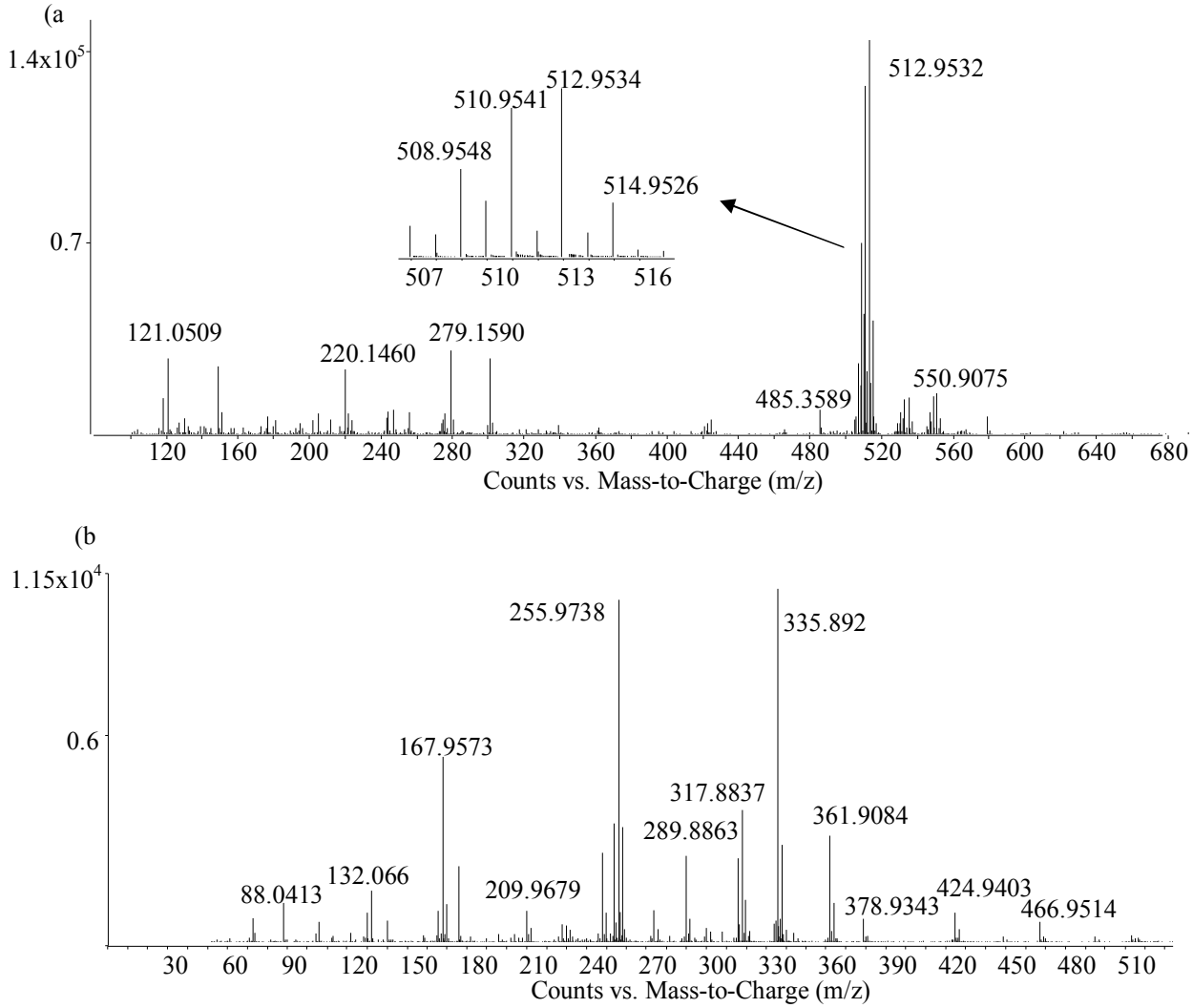

Figure 16: (a) Full scan spectrum recorded near the apex of the EIC for *m/z* 512.9532. The inset shows the selenium pattern of the target compound. (b) MS/MS spectrum of the compound at *m/z* 512.9532

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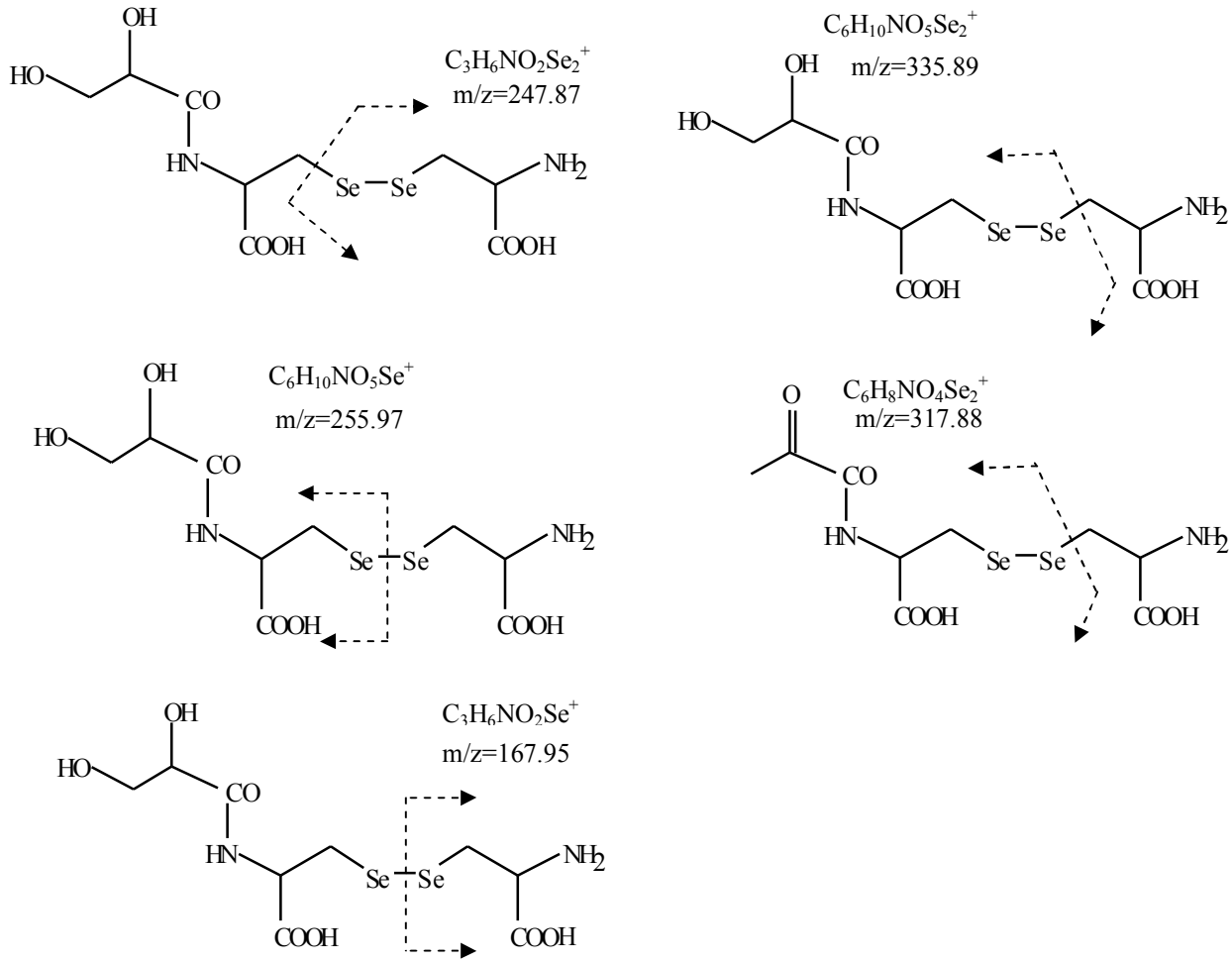

Figure 17 Proposed MS/MS fragmentation mechanisms of the conjugate of 2,3-DHP-selenocysteine and selenocysteine (*m/z* 424.93)

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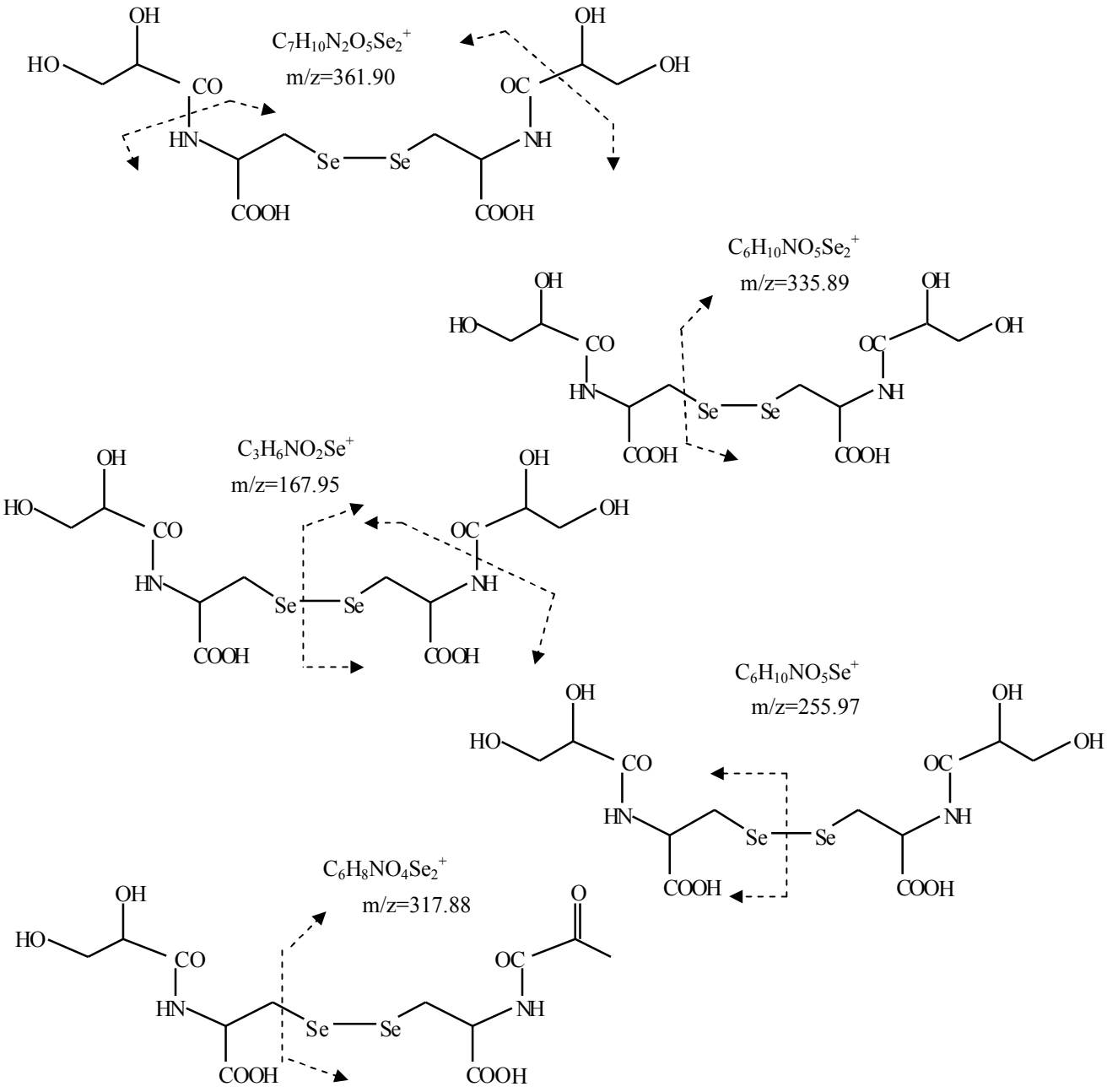

Figure 18: Proposed MS/MS fragmentation mechanisms of di-N-2,3-DHP-selenocysteine (*m/z* 512.95)

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5.2.4 Conjugation and characterization of 2,3-DHP-selenocysteine-glutathione

Combining the optimized processes of selenocysteine-glutathione conjugation and the clean-up of 2,3-DHP-containing selenocystine species were the prerequisites to arrive at a detectable amount of 2,3-DHP-selenocysteine-glutathione. However, this compound is slightly retained on special RP-HPLC columns intended for use with eluents with low organic solvent content [179]; therefore, a more robust clean-up technique with SAX-HPLC was chosen [97]. The fragments of 2,3-DHP-selenocysteine-glutathione match those of reported in literature, and the proposed fragments can be seen in Figure 19.

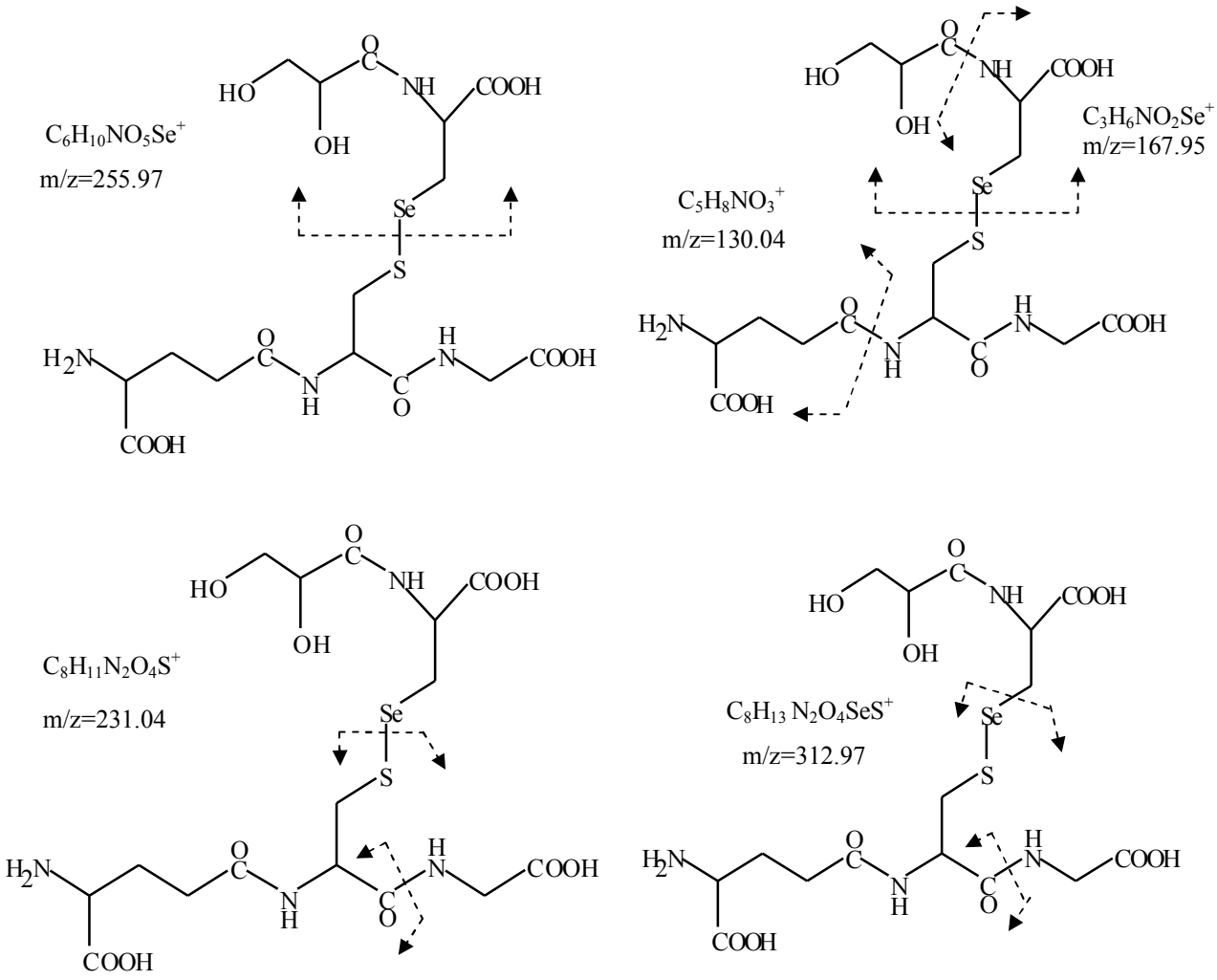

Figure 19: Proposed fragmentation mechanisms of the conjugate of 2,3-DHP-selenocysteine and glutathione (*m/z* 563.05)

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The SAX-HPLC-ICP-MS chromatogram of the reaction products can be seen in Figure 19a. The first Se-containing compound, eluting at 500 s, was identical to the conjugate of selenocysteine-glutathione that was formed in the reaction of non-derivatized selenocysteine residues. The HPLC-ESI-QTOF-MS characterization of the more intense second peak, eluting at 720 s, is presented in Figure 19b. The targeted search for *m/z* 563.05568 resulted in an EIC of a single peak with the full scan shown in Figure 19c and MS/MS fragmentation data shown in Figure 19d.

Both the MS ($C_{16}H_{27}O_{11}N_4SSe^+$ $[M+H]^+$, *m/z* 563.05546 $[M+H]^+$, Δ= -0.39 ppm) and MS/MS data (see Table 11) correspond to the previously reported information on this compound [97], which indicates the synthesised compound matches the genuine, Se-yeast specific 2,3-DHP- selenocysteine -glutathione conjugate.

While some (*e.g.*, the γ-Glu specific) of the MS/MS fragments of selenocysteine -glutathione and 2,3-DHP- selenocysteine -glutathione are shared, the majority of the fragments are different (see Figures 13 and 20). The most significant difference is the high abundance of the SeCys residue that appears both in native (*m/z* 167.95) and 2,3-DHP-derivatised (*m/z* 255.97) forms during the fragmentation of 2,3-DHP- selenocysteine -glutathione but appears only as a minor fragment during the fragmentation of selenocysteine-glutathione. This great difference in fragmentation pattern is unusual, as the two compounds share their basic structure. Indeed, the addition of the 2,3-DHP residue, that can be broken off during fragmentation at the amide bond, could stabilize the SeCys residue and increase its abundance while affecting the bond strength of the S-Se bridge [142]. The high fragmentation event of the S-Se bridge in positive ion mode together with the abundant appearance of the selenocysteine residue is a unique feature of 2,3-DHP-containing glutathione derivatives and it is reported exclusively in such structures [17,194].

Since all the four Se species are Se-yeast specific to the best of our knowledge, their availability might offer an important tool in the quantitative characterization and quality control of Se-yeast production.

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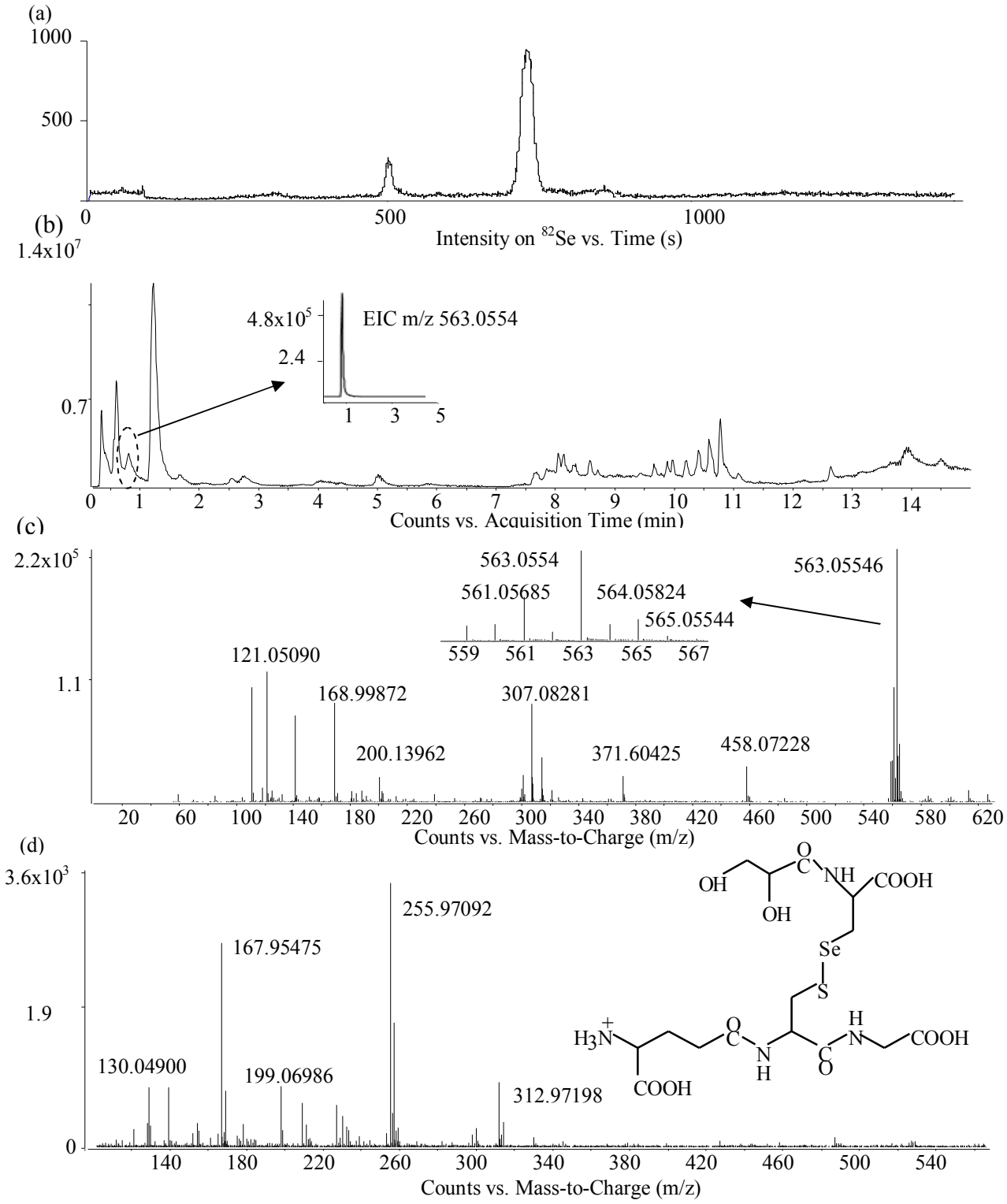

Figure 20: (a) Analytical scale SAX-HPLC-ICP-MS chromatogram of the products arising from the oxidative conjugation of (2,3-DHP)-selenocysteine-selenocysteine and di-N-2,3-DHP-selenocysteine with glutathione. The compound eluting at 720 s was collected for further characterization with preparative scale SAX-HPLC. (b) HPLC-ESI-QTOF-MS based TIC of the compound collected from SAX-HPLC. The inset presents the EIC for *m/z* 563.0554. (c) Full scan spectrum recorded near the apex of the EIC for *m/z* 563.0554. The inset shows the selenium pattern of the target compound. (d) MS/MS spectrum and structure of the compound at *m/z* 563.0554

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5.3. Metabolism of selenium in Hericium erinaceus (lion's mane mushroom)

The aim of our study was to examine the selenium metabolism of *H. erinaceus* cultivated on regular compost fortified with inorganic selenium. Special attention was paid to the less abundant selenium compounds in order to map the influenced metabolic pathways. To acquire reliable information, both HPLC-ICP-MS and HPLC-ESI-QTOF-MS set-ups were used after orthogonal chromatographic clean-up processes.

5.3.1. Quantification of total selenium and selenoamino acid content

The total selenium content was found to be 42.3 μg g^{-1} (d.w.). Compared to usual mushroom enrichment processes this concentration falls into the moderate enrichment range [29,195,196].

The IP-RP-ICP-MS chromatogram acquired after enzymatic digestion can be seen in Figure 21. The two most abundant peaks could be identified and quantified with standard addition as selenomethionine (20.7 μg g^{-1} d.w.) and *Se*-methylselenocysteine (0.3 μg g^{-1} d.w.).

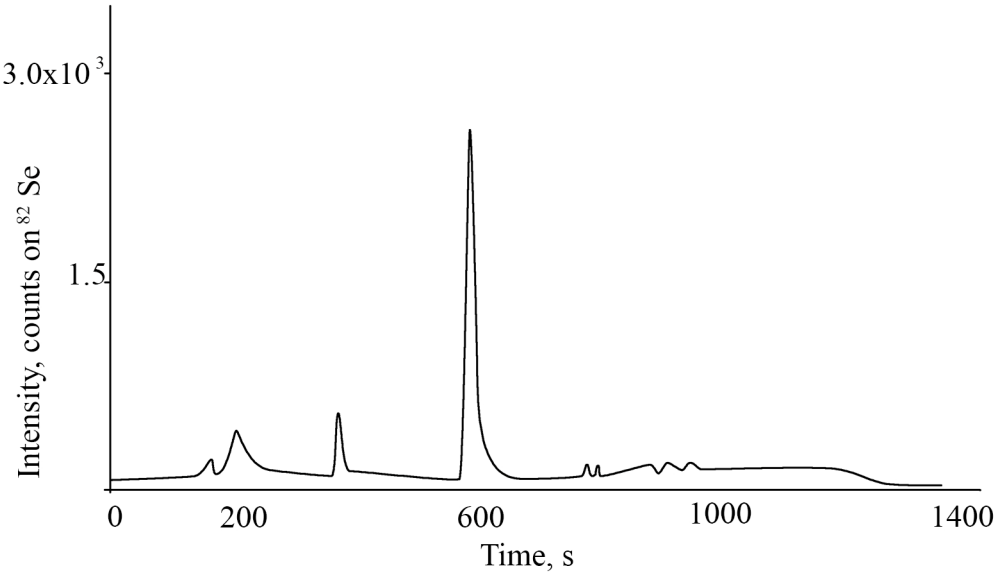

Figure 21: IP-RP-ICP-MS chromatogram of the enzymatically digested mushroom sample

However, the presence of these two selenium species has already been described in mushroom samples [22,197], their cumulated amount almost reaches 50% of total selenium content, which is unprecedented in any classic button type mushrooms grown on inorganic selenium source. This suggests that the metabolism of *H. erinaceus* is atypical of what is known of the pathways of *Agaricomycetes,* and more similar to that of yeast, *S. cerevisiae*. To support

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this theory, further investigations were carried out in search for typical yeast selenium metabolites, especially for seleno-adenosyl compounds that are the indicators of the partly sulphur analogous selenium metabolism in yeast.

5.3.2. SEC separation and IP-RP-HPLC based clean-up of selenium species

According to the protocol of low ionic strength SEC separation of Encinar *et al.* [136] developed for selenised yeast samples, the most abundant and the late eluting fractions of the water extractable part of selenium were collected and further purified with IP-RP-HPLC separation with ICP-MS monitoring on ^{77}Se (Figure 22a-c).

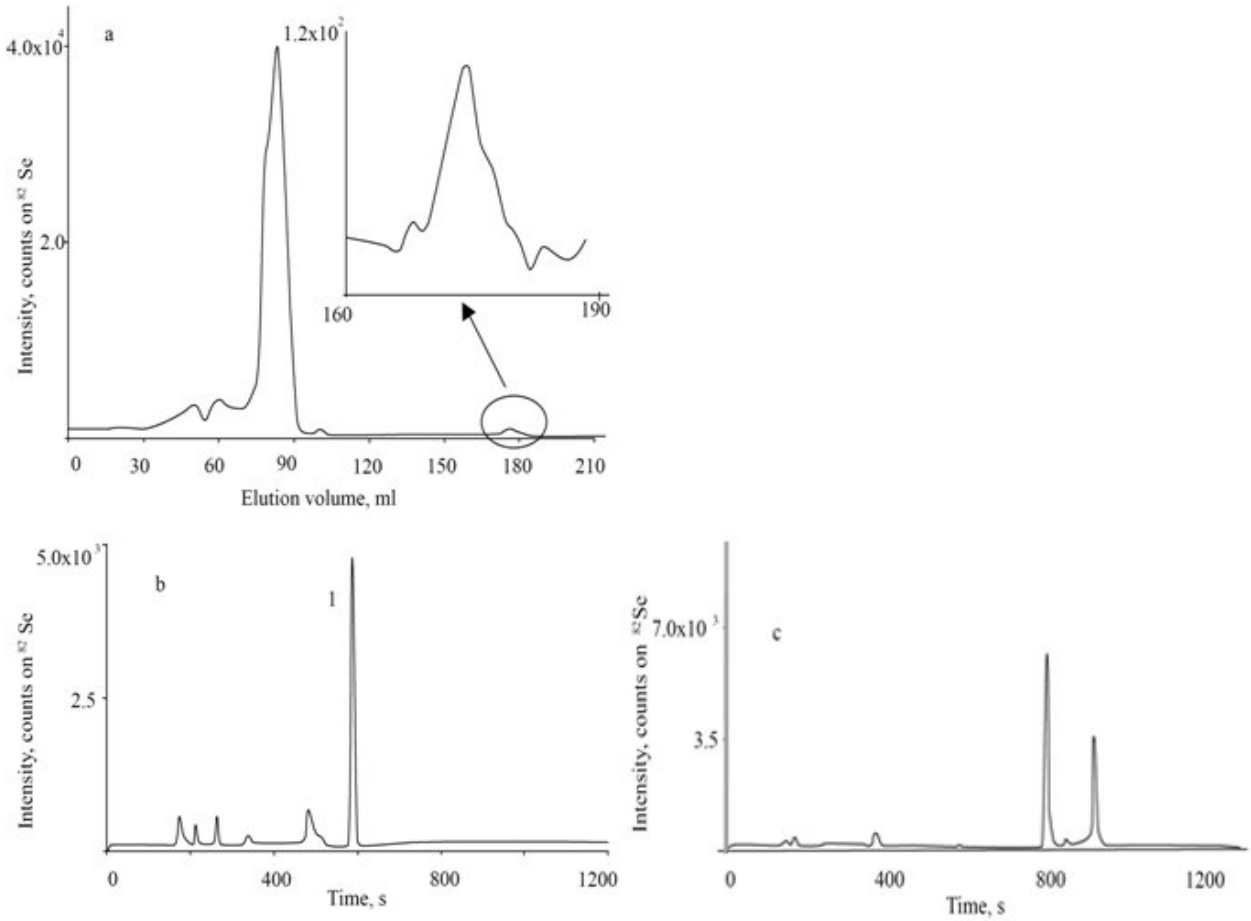

Figure 22: (a) SEC-ICP-MS chromatogram of the ultrasonic aqueous extract of the mushroom sample. The inset shows the late eluting low molecular weight fraction. (b) IP-RP-ICP-MS chromatogram of the most abundant peak of the SEC separation; '1' denotes to selenomethionine. (c) IP-RP-ICP-MS chromatogram of the late eluting low molecular weight fraction.

According to IP-RP based separation the most abundant fraction contained only one significant compound which could be identified as selenomethionine with standard addition (Figure 22b). In the late eluting low molecular weight fraction (Figure 22c) two peaks could be

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detected with retention times not matching that of any standards available, therefore they were collected, pooled and subjected to HPLC-ESI-QTOF-MS analyses.

5.3.3. HPLC-ESI-QTOF- MS analysis of the late eluting SEC fraction

The sample injected to the HPLC-ESI-QTOF-MS system yielded in positive ion mode the TIC that can be seen in Figure 23. Three selenium-containing components were found by manual scanning for the characteristic selenium isotope (and also isotopologue) patterns with the mass-to-charge ratios (*m/z*) of 362.03623, 360.05707 and 346.04163, respectively.

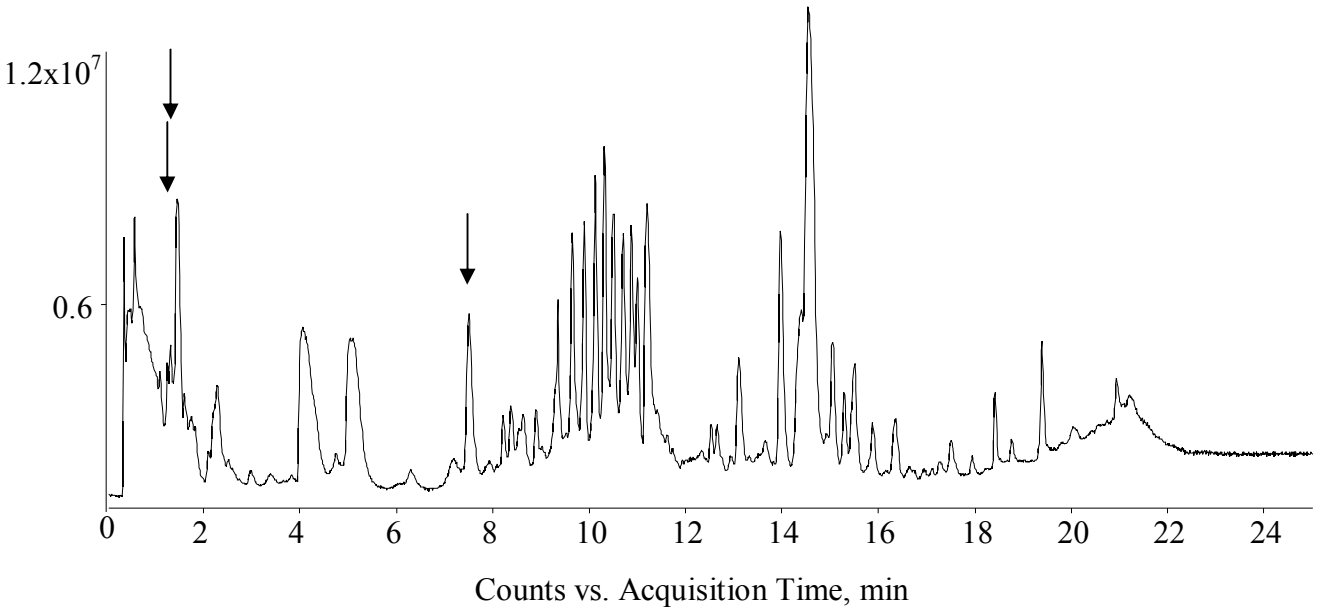

Figure 23: HPLC - ESI - QTOF MS TIC of the fraction collected from IP - RP - ICP-MS (see Figure. 22c). The arrows indicate the elution of the three assigned compounds, *m/z* 362.03623, 360.05707 and 346.04163, respectively

The most abundant one of these, *m/z* 346.04163 eluted at 7.5 minutes. Figure 24a shows the recorded full scan with the inset of the selenium-containing isotopic pattern. The targeted MS/MS fragmentation of this compound (Figure 24b) indicated it to be *Se*-methyl-5-selenoadenosine, a compound detected first in yeast selenium metabolism by McSheehy *et al*. [92]. Not only the accurate mass data ($C_{11}H_{16}N_5O_3Se^+$, theoretical *m/z* 346.0413; δ + 0.95 ppm, $[M+H]^+$) and the fragmentation pattern support this observation, but the detection of its oxidized product at t_R=1.2 min with *m/z* 362.03623 /$C_{11}H_{16}N_5O_5Se^+$, theoretical *m/z* 362.0362, δ + 0.08 ppm, $[M+H]^+$/ (Figure 25) also proves this theory.

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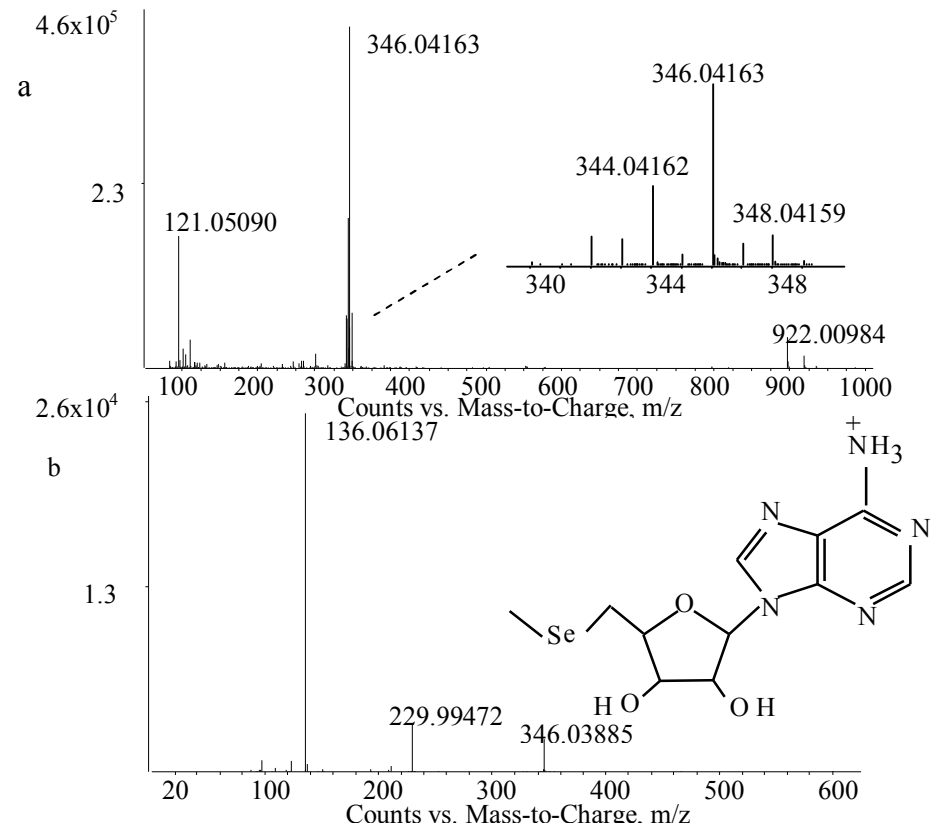

Figure 24: (a) ESI-QTOF-MS full scan spectrum of molecule *m/z*=346.04163 with the isotopic pattern of the molecule in the inset. (b) ESI-QTOF-MS/MS fragmentation spectrum and the structure of the identified molecule, *Se*-methyl-5-selenoadenosine

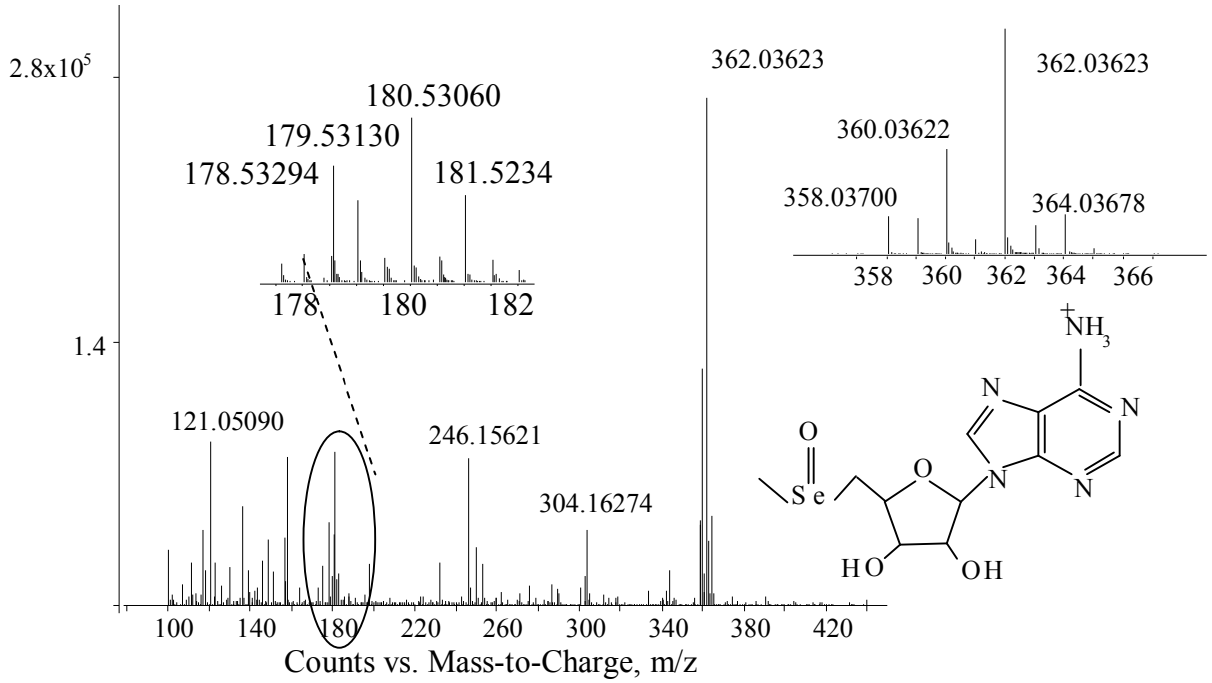

Figure 25: ESI-QTOF-MS full scan spectrum of molecule *m/z*=362.03623, oxidised *Se*-methyl-5-selenoadenosine, with the isotopic pattern and the molecule structure in the insets

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Table 12: Mass accuracy of discovered compounds and fragments

	Measured, *m/z*	Calculated, *m/z*	ppm difference
positive mode			
$[C_{11}H_{15}N_5O_3Se+ H]^+$	346.04163	346.0413	0.95
$[C_{12}H_{18}N_5O_3Se]^+$	360.05707	360.0569	0.47
$[C_{11}H_{15}N_5O_4Se+H]^+$	362.03623	362.0362	0.08
$[C_{11}H_{15}N_5O_3S+ H]^+$	298.09473	298.0986	-6.91
$[C_{12}H_{17}N_5O_3S+H]^+$	312.11182	312.1124	-1.85
$[C_{12}H_{17}N_5O_3S+2H]^{2+}$	156.55989	156.5598	0.57
negative mode			
$[C_{11}H_{15}O_3N_5Se-H]^-$	344.02880	344.0267	6.10
$[C_{11}H_{15}O_3N_5Se+ HCOOH-H]^-$	390.03257	390.0322	0.95
MS/MS fragments, positive mode			
$[C_5H_6N_5]^+$	136.06137	136.0618	-3.16
$[C_{10}H_{12}N_5O_3]^+$	250.09454	250.094015	2.09
$[C_5H_5O_2]^+$	97.02761	97.0284	-2.52
$[C_8H_{10}N_2OSe]^+$	229.99472	229.9953	-8.14

The identification of the compound eluting at t_R=1.3 min with *m/z* 360.05707 (Figure 26 a-b) is not as straightforward as that of *Se*-methyl-5-selenoadenosine. According to the elemental composition, there is only one possible composition: an additional CH_2 group compared to *Se*-methyl-5-selenoadenosine, that is, $C_{12}H_{18}N_5O_3Se^+$ /theoretical *m/z* 360.0569; δ + 0.47 ppm/. The MS/MS data indicated the losses of adenosyl, adenine and ribose groups. This information may relate basically to two possible structures, *Se*-ethyl-5-selenoadenosine [17] and *Se*-dimethyl-5-selenonium-adenosine, a cationic compound that has not been described before. The mass accuracy of the compounds and fragments can be seen in Table 12.

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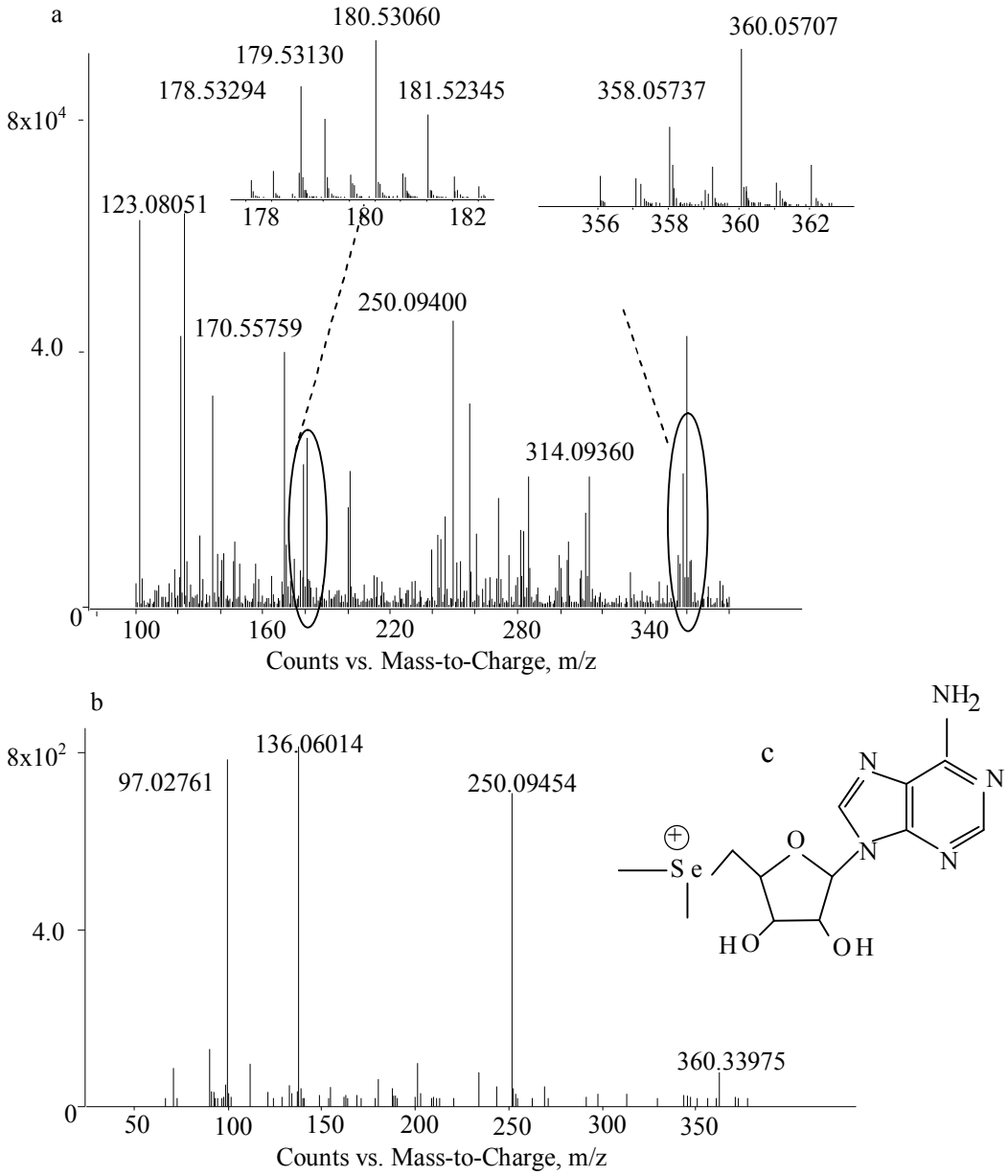

Figure 26: (a) ESI-QTOF-MS full scan spectrum of the molecule *m/z*=360.05705, with the isotopic patterns of the single and doubly charged pseudomolecular ions in the insets. The double charged ion is interfered with the ion *m/z* 179.07043. (b) ESI-QTOF-MS/MS fragmentation spectrum (c) the assigned structure of the molecule, *Se*-dimethyl-selenonium-adenosine

As stable cations are known among selenium species (such as trimethylselenonium ion, *Se*-adenosyl-selenomethionine and *Se*-methylselenomethionine), none of the possible structures can be excluded based on accurate mass and MS/MS results. Therefore, additional information must be taken into account: (1) the retention time of the *m/z* 360.05707 compound was close to the void, while an ethyl group on the Se moiety would not result in a retention so much shorter

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than that of *Se*-methyl-5-selenoadenosine (t_R=7.5 min *vs*. t_R=1.3 min); (2) while *Se*-methyl-5-selenoadenosine was found in oxidized form in the sample, no compound was found at the theoretical mass of *m/z* 376.0519, which could have been expected from *Se*-ethyl-5-selenoadenosine, indicating a different proneness to oxidation of the *m/z* 360.05707 compound; (3) while *Se*-methyl-5-selenoadenosine was not found in doubly charged form neither in the actual ESI full scan spectra at any level of abundance, nor in the first related study [92], the compound at *m/z* 360.05707 was continuously present in single and doubly charged states, which suggests an ease of second ionisation probably due to the stable cationic structure; (4) in negative mode ESI (Figure 27) only *Se*-methyl-5-selenoadenosine could be detected as the $[M-H]^-$ pseudomolecular ion and as a formic acid adduct, while no such analogous ions could be detected for the new compound. All these observations indirectly prove the compound with *m/z* 360.05707 is *Se*-dimethyl-5-selenonium-adenosine. However, the ultimate identification would require the synthesis of the molecule in question.

The full scan spectrum acquired with HPLC-ESI-QTOF-MS in negative mode of the pooled fraction collected after IP-RP-HPLC separation can be seen in Figure 27. The insets show *Se*-methyl-5-selenoadenosine (*m/z* 344.02880, $[M-H]^-$) and its HCOOH adduct (*m/z* 390.03257) Interestingly, the background (co-eluting matrix constituents) are dominating the full scan spectrum, so the location of the target compounds would not have been possible without the previously evaluated data obtained in positive ion mode. This observation is an example of the rare situation featuring more crowded full scan spectrum in negative ion mode than in positive ion mode.

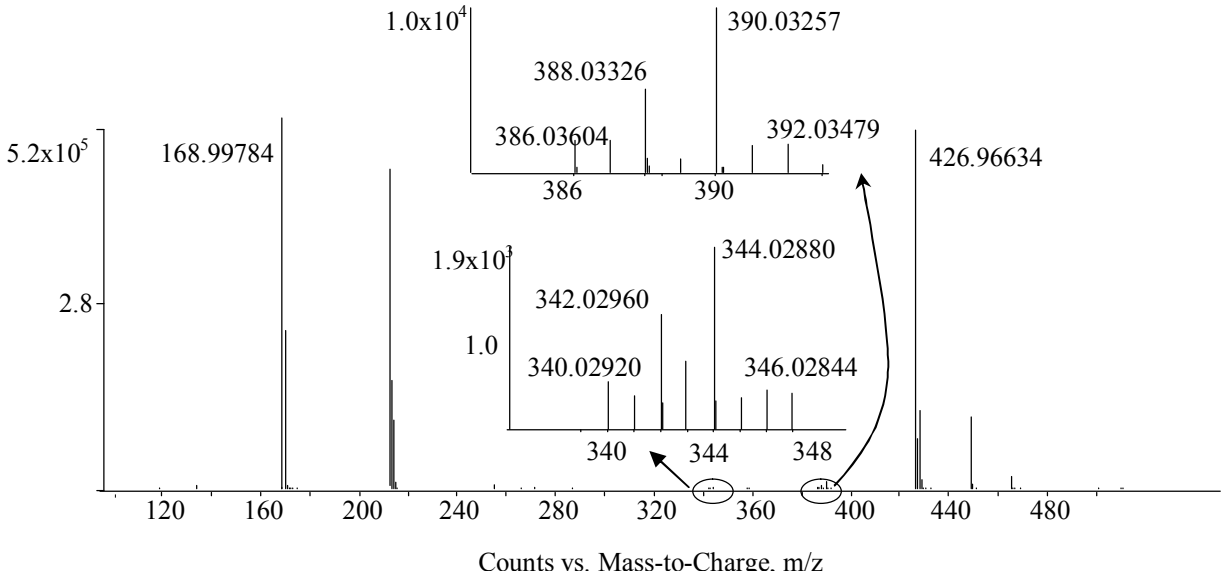

Figure 27: Full scan spectrum acquired with HPLC-ESI-QTOF-MS in negative mode of the pooled fraction collected after IP-RP-HPLC separation. The insets show *Se*-methyl-5-selenoadenosine (*m/z* 344.02880, $[M-H]^-$) and its HCOOH adduct (*m/z* 390.03257)

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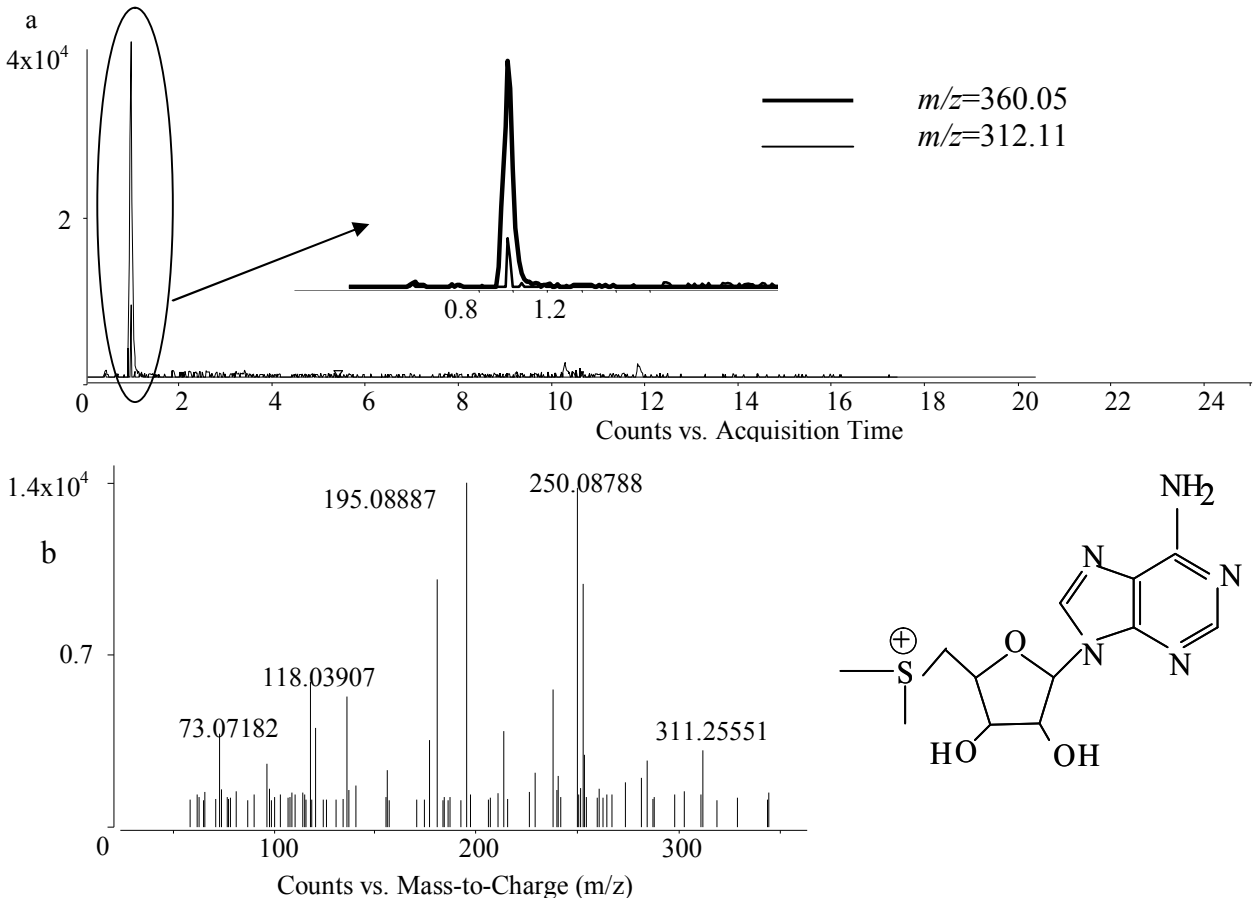

Figure 28: a, The coelution of *Se*-dimethyl-selenonium-adenosine and *S*-dimethyl-adenosine; b, the fragmentation pattern of *S*-dimethyl-adenosine

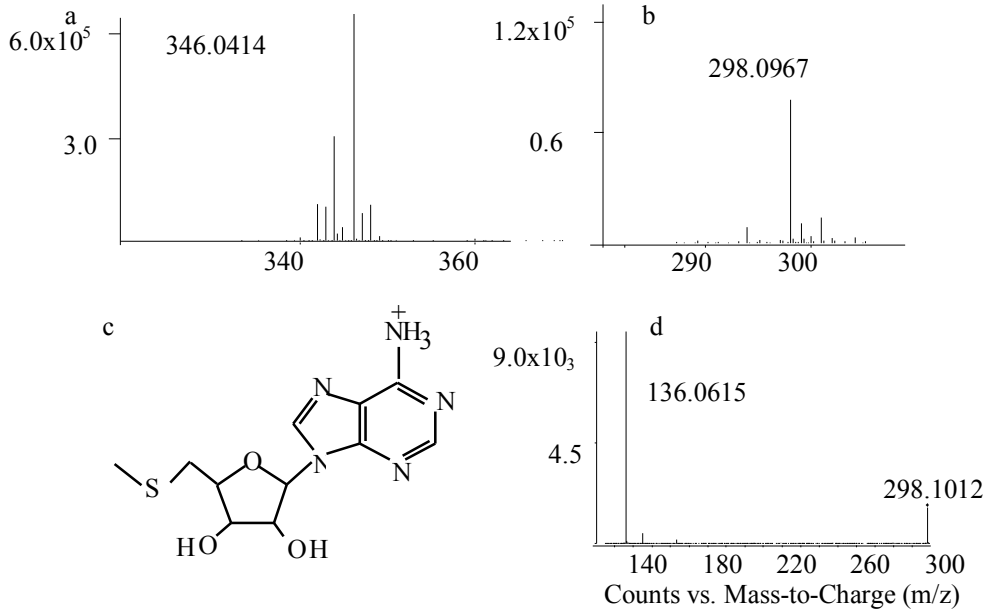

Figure 29: a, the isotopologue pattern of *Se*-methyl-selenonium-adenosine; b, the isotopologue pattern of *S*-methyl-adenosine, c, molecule structure of *S*-methyl-adenosine, d, fragmentation pattern of *S*-methyl-adenosine

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The S analogue of *Se*-dimethyl-selenonium-adenosine was also detected, eluting at the same time. The elution peaks and the fragmentation pattern of *S*-dimethyl-adenosine can be seen in Figure 28. The S analogues of *Se*-methyl-selenonium-adenosine and *Se*-methyl-5-selenoadenosine-*Se*-oxide were also detected and fragmented. The results can be seen in Figures 29 and 30.

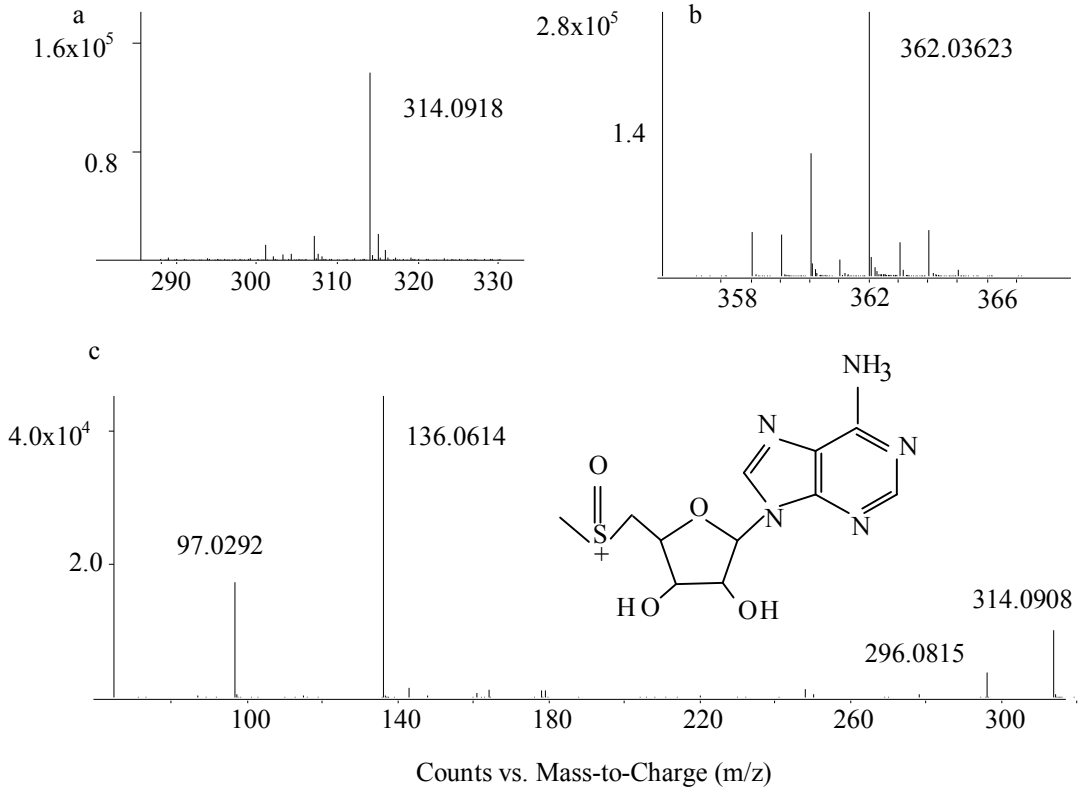

Figure 30: a, the isotopologue pattern of *S*-methyl-5-adenosine-*S*-oxide b, the isotope pattern of *Se*-methyl-5-selenoadenosine-*Se*-oxide, c, molecule structure and fragmentation pattern of *S*-methyl-5-adenosine-*S*-oxide

Interestingly, the most abundant seleno-adenosyl species of *S. cerevisiae*, *Se*-adenosyl-selenohomocysteine, [92,132,167] has not been detected in the sample. In Figure 31 the S metabolic pathway of yeast from KEGGS can be seen, with the position of the S-analogues of *Se*-methyl-5-selenoadenosine and *Se*-adenosyl-selenohomocysteine marked.

Whether the lack of *Se*-adenosyl-selenohomocysteine is caused by significantly different metabolic pathways or merely the lower level of selenium enrichment (*i.e.*, 2000 mg kg^{-1} *vs.* 43 mg kg^{-1}) still needs to be confirmed. On the other hand, the high level of contribution of selenomethionine and methyl-selenocysteine to the total selenium content and the presence of seleno-adenosyl compounds definitely make this mushroom unique among the *Agaricomycetes*

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group. This unusual metabolism might offer the possibility for the development of a functional food product.

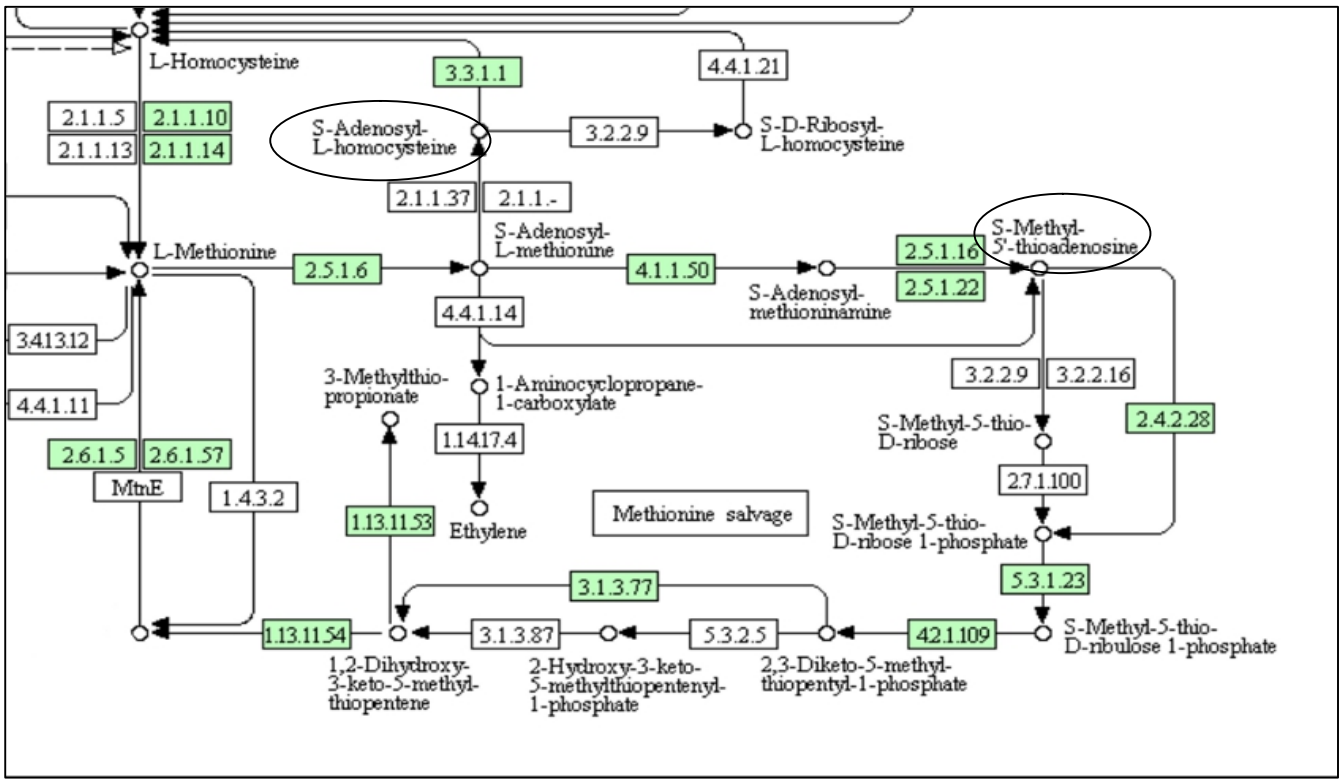

Figure 31: S metabolic pathway of yeast from KEGGS (http://www.genome.jp/kegg/pathway/sce/sce00270.png)

It must be underlined that our approach, partly targeting seleno-adenosyl compounds has not been addressed previously in the case of mushroom samples, therefore the presence of the these Se species in other mushrooms cannot be excluded.

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6. Conclusions

In our research to determine the effect of sample preparation methods on the D,L-enantiomer ratio of extracted selenomethionine we have not only compared two different methods of sample preparations: we have also developed a reliable, non-matrix dependant method that can be used for the determination of accurate D/L-selenomethionine enantiomer ratio. This method might be used for supervising the quality of feed supplements and dietary supplements. Currently the Hungarian market is flooded with such products with no control. Using the developed method expert laboratories can reveal if given products actually contain the claimed amount of selenised yeast, or if they are fully or partially replaced by racemic selenomethionine. In case of a forgery it could also reveal the extent of cheating.

In our work for the validation of the 2,3-dihydroxy-propionyl group in selenium speciation by chemical synthesis and LC - MS analyses, we have successfully completed the synthesis of four seleno-glutathiones. The yield of the current method is about 1-2%, but it should be suitable for preparative laboratory scale production after careful optimisation. The most crucial step would be the activation of the glyceric acid, as this component is prone to polymer-formation, resulting in a large number of different by-products that can no longer be used for the synthesis. On the other hand even with these four compounds becoming commercially available, more than 60 more selenium species discovered just from selenised yeast remain unavailable and unverified. There are more groups of selenium-species that also still need to be synthesised, such as the recently discovered a distinct group of lypophilic selenium-containing compounds. [114]

In our research about the metabolism of selenium in *Hericium erinaceus* (lion's mane mushroom) we have found a link between the selenium metabolic pathways of yeasts and *Hericium erinaceus*. However, while the main selenium-containing metabolite in yeast is *Se*-adenosyl-selenohomocysteine, in our sample it was not detected. This metabolic relationship could be further investigated by repeating the process with different levels of selenium enrichment with a focused search for the mentioned metabolite. Definitely, other – more common – mushroom species should also be considered.

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7. Summary

Selenium is an essential micro-element for humans. As its poisonous and necessary levels are very close its supplementation has to be monitored very closely.

The only European Food Safety Authority-approved organic selenium supplementation source is selenised yeast, which contains about 80% of its selenium content in the form of selenomethionine. Therefore the enantiomer separation and ratio determination can be of utmost importance in case of selenomethionine, as it would be easy to add the cheaper racemic selenomethionine in the food and feed mixture rather than the enriched yeast. Different sample preparation methods can influence the measured D,L-enantiomer ration though. Our study revealed significant differences in the D,L-enantiomer ratio of selenomethionine depending on the sample preparation method from two highly selenised samples, the CRM SELM-1 and the protein fraction of monkeypot nut. Namely, the ratio of D-selenomethionine was determined to be 2.2-2.7% and 0.5-0.6% after MSA based and enzymatic hydrolyses, respectively. Besides, at least in case of the applied derivatisation technique, highly differing sensitivities were found between two selenomethionine enantiomers when addressing HPLC - ICP-MS. Regarding the fact that the use of MSA digestion is widespread in selenium speciation and applied in certification campaigns with or without subsequent derivatisation techniques [28-30], special care should be taken of quantitative selenomethionine determination. Namely, calibration processes carried out with a selenomethionine standard the enantiomer ratio of which is not inherently identical to what is obtained after the sample preparation steps may lead to biased results. The difference is not negligible and might have remained hidden in previous studies because of the relatively high uncertainty. According to our results, these differences should be taken into account in the evaluation of results because their extent is comparable to measurement uncertainties.

As selenised yeast is the most wildly used source of selenium supplementations, not only possible forgery with racemic selenomethionine has to be prevented, but the retraceability is also important, so bathes from different yeast strains and producers can be identified. Both the quality control and the quantitative characterization of selenised yeast batches require standards to monitor stability and to identify sample origin. As non-Se-yeast-specific selenium compounds (namely, selenomethionine, selenocysteine and inorganic selenium species) specified by the Commission Regulation (EC) No 1170/2009 cannot provide customized options for these purposes, the newly synthesised 2,3-DHP containing species and the conjugate of selenocysteine – glutathione may offer a viable solution. On the other hand, the more than 50 Se-species

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discovered during the last five years from plant and yeast samples call attention to the evident lag in the number of available standards that may be caught up stepwise with an approach similar to our method, that is, with a grouped batch of synthesis.

While selenised yeast is a good source for selenium supplementation, it is not suitable for direct consumption. Most selenium-enriched functional foods contain the supplemented selenium in inorganic forms, which is either less-bioavailble or can have carcinogen side effects. The ideal functional food for selenium supplementation would be a mushroom with yeast-like metabolism: converting the inorganic selenium to organic forms but still suitable for direct consumption. During the experiments about the metabolism of selenium in *Hericium erinaceus* (lion's mane mushroom) five selenium species could be identified. Four of the compounds (selenomethionine, *Se*-methylselenocysteine, *Se*-methyl-5-selenoadenosine and *Se*-methyl-5-selenoadenosine Se-oxide) are common in the selenised yeast *S. cerevisiae* to such qualitative and quantitative extents that a new analogy can be established between the selenium metabolism of budding yeast and this mushroom species of *Agaricales*. It is to note that the sulphur analogue of *Se*-methyl-5-selenoadenosine is a key compound in the general methionine salvage process – in mushroom species, the salvage of all amino acids is a preferred biochemical process in order to facilitate fast protein turnover during fruit development [167,198]. The fifth compound, *Se*-dimethyl-5-selenonium-adenosine, has not been identified before, and may indicate a previously undetected methyltransferase activity. The presence of these compounds and the large amount of selenomethionine proves the similarity between selenised yeast and *Hericium erinaceus*, thus opening up possibilities for creating a new functional food.

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8. Scientific statements

1. I have determined the rate of selenomethionine racemisation occurring during methanesulphonic acid (MSA) and pronase E based protein hydrolytic processes. I have concluded that the enzymatic hydrolysis does not result in racemisation, and I have compared the results acquired though the two different methods. I have determined that more than 2% of selenomethionine appears as the D-enantiomer after MSA digestion, regardless whether the source of protein was of plant or mushroom origin.

2. I have concluded that the HPLC-ICP-MS system has significantly different sensitivities for OPA- and NIBC- derivatized D- and L-Se-methionine when quantified through isocratic RP-HPLC-ICP-MS setup.

3. I have developed and executed a method for the synthesis of 2,3-dihydroxy-propionyl-selenocysteine-glutathione, 2,3-dihydroxy-propionyl-selenocysteine-selenocysteine, di-N-2,3-dihydroxy-propionyl-selenocysteine and selenocysteine-glutathione.

4. I have demonstrated the existence of a selenometabolomic level relationship between Se-enriched yeast and Se-enriched *Hericium erinaceus*. I have extracted and described a new Se-species, *Se*-dimethyl-5-selenonium-adenosine with HPLC-ESI-QTOF-MS experiments.

9. Appendix I: References

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10. Appendix II: List of relevant publications

Full papers:

1. Orsolya Egressy-Molnár, Anna Magyar, Attila Gyepes and Mihály Dernovics, 2014, Validation of the 2,3-dihydroxy-propionyl group in selenium speciation by chemical synthesis and LC-MS analyses, RSC Advances Issue 52,4, 27532-27540
2. Egressy-Molnár, O., Vass, A., Németh, A., García-Reyes, J.F., Dernovics, M, 2011, Effect of sample preparation methods on the D,L-enantiomer ratio of extracted selenomethionine, Analytical and Bioanalytical Chemistry, 401 (1) , pp. 373-380

International Conference lectures:

1. Orsolya Egressy-Molnár, Attila Gyepes, Anna Magyar Mihály Dernovics, Validation of the 2,3-dihydroxi-propionyl group in selenium speciation by chemical synthesis and LC-MS analyses, 2014, Pau, 8th International Franco-Spanish Workshop pp 28
2. Egressy-Molnár O., Vass A, Dernovics M, TEFC konferencia, 2012, Visegrád, előadás: Unique metabolism of selenium in *Hericium erinaceus* (lions's mane mushroom) pp. 27
3. Dernovics Mihály - Egressy-Molnár Orsolya - Juan Francisco García-Reyes - Németh Anikó - Shuxun Shao (2011): Food-Related Phytoremediation Initiative in the Seleniferous Area of Jianshi County, Enshi T.M.A.P., China: Challenges for Selenium Speciation and LC/MS Based Food Analysis. Chinese-European Cooperation for a Long-Term Sustainability – International Conference at the Corvinus University of Budapest. 2011. november 10-11., Budapest

Hungarian Conference lectures:

1. Dr. Dernovics Mihály - Németh Anikó - Egressy-Molnár Orsolya (2011): Kapcsolt tömegspektrometriai rendszerek szerepe az újonnan felfedezett szelénmódosulatok azonosításában. Mikroelem Miniszimpózium. 2011. október 18., Budapest.
2. Németh Anikó - Egressy-Molnár Orsolya - Winfried Behr - Juan F. García-Reyes - Dernovics Mihály (2011): Növényi kén- és szelénanyagcsere folyamatok analog intermedierjeinek azonosítása ortogonális és kapcsolt tömegspektrometriai módszerekkel. HCS 1st National Conference /MKE 1. Nemzeti Konferencia/. 2011. május 22-25., Sopron.

Conference poster:

1. Orsolya Egressy-Molnár, József Lénárt, Júlia Győrfi, Mihály Dernovics, Hericium erinaceus: a mushroom with yeast-like Se-metabolism, 2013, Krakow, European Winter Conference on Plasma Spectrochemistry, poster award MP-46, 47. oldal

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