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Application of hyphenated analytical techniques in the investigation of selenium speciation of different plants

Doctoral Thesis of

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"And if we want to achieve our goal,

then let us empower ourselves with the weapon of knowledge

and let us shield ourselves with unity and togetherness."

- *Malala Yousafzai*

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List of abbreviations

AAS	Atomic Absorption Spectroscopy
AES	Atomic Emission Spectroscopy
ACN	Acetonitrile
CE	Collision Energy
CID	Collision Induced Dissociation
cps	Counts per Second
Da	Dalton
DW	Dry Weight
ECD	Electron Capture Dissociation
EIC	Extracted Ion Chromatogram
ESI	Electrospray Ionisation
ETD	Electron Transfer Dissociation
FDR	False Discovery Rate
FNR	False Negative Rate
GC	Gas Chromatography
GPx	Glutathione-peroxidase
HILIC	Hydrophilic Interaction Chromatography
HFBA	Heptafluorobutyric acid
HPLC	High Performance Liquid Chromatography
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IDA	Isotope Dilution Assay
IP	Ion Pairing (Chromatography)
IUPAC	International Union for Pure and Applied Chemistry
LA	Laser Ablation
LC	Liquid Chromtography
LOD	Limit of Detection
MALDI	Matrix Assisted Laser Desorption Ionization
MeOH	Methanol
MS	Mass Spectrometry
MSA	Methanesulfonic Acid
MS/MS, MS^n	Tandem Mass Spectrometry
m/z	Mass to Charge Ratio

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OES	Optical Emission Spectroscopy
ppm	Parts per Million
PTFE	Polytetrafluoroethylene
PTM	Post-Translational Modification
Q-TOF-MS	Quadrupole Time of Flight Mass Spectrometry
RDA	Recommended Daily Allowance
RP	Reversed Phase (Chromatography)
SCX	Strong Cation Exchange (Chromatography)
SEC	Size Exclusion Chromatography
SECIS	Selenocysteine Insertion Sequence
SeCys, SeC	Selenocysteine
$SeCys_2$	Selenocystine
SeCysta	Selenocystathionine
SeHCy	Selenohomocysteine
$SeHCys_2$	Selenohomocystine
SeMet	Selenomethionine
Se-Met-SeMet	Methyl-Selenomethionine
S/N	Signal to Noise Ratio
SPE	Solid Phase Extraction
SPME	Solid Phase Microextraction
TIC	Total Ion Chromatogram
γ-Glu	γ-glutamyl, γ-glutamoyl
XANES	X-ray Absorption Near-Edge Structure
μ-XAS	X-ray Absorption Microspectroscopy

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1. INTRODUCTION

Trace elements play a relevant role in metabolic processes as catalytic or structural components of biomolecules. In order to understand the role of trace elements in complex biological systems, various methods have been developed for the investigation of organometallic/metalloid components (Szpunar 2005). The relatively new and rapidly developing interdisciplinary field of trace element research is usually called bioinorganic chemistry since it integrates the methods of biochemistry, analytical and inorganic chemistry (Kaim and Schwederski 1994).

The biological importance of selenium (Se) is due to its essentiality for the majority of organisms - including humans. Among others, it plays an important role in the antioxidant system of many organisms through the incorporation of selenocysteine - the 21st amino acid - into various antioxidant enzymes (Papp et al. 2010). Selenium is thus essential for maintaining the redox homeostasis and also can be utilized in chemoprevention of chronic diseases, such as chronic inflammation, cancer or cardiovascular disease (Hatfield et al. 2014). Dietary selenium supplementation received considerable attention in the last decades, since many studies showed that it is effective in counteracting some negative effects of our modern lifestyle and the so-called Western diet (Rayman 2012).

Since the daily intake of selenium in Hungary is lower than that is necessary for the optimal operation of our body (Szabó et al. 1991), the missing amount of selenium should be ingested in the form of selenium rich or Se-enriched foodstuff or food supplements. However, in case food supplements are utilized for this purpose the amount of selenium supplementation should be carefully optimized (Rayman 2008) since Se can also be toxic and the difference between the essential and toxic level is quite narrow. Moreover, not only the quantity but the quality of supplements is also crucial because the chemical form of selenium also determines its bioavailability and toxicity. Recognizing this fact made speciation analysis substantial in selenium research.

Early speciation studies focused on the development of suitable analytical procedures to measure some targeted selenium species in complex biological matrices. As a result of these works, several routine processes are utilized nowadays in food and feed quality assurance and clinical diagnostics. While the ultimate goal of every life science research is the ability to gain information about life processes, it is still important to note that it would be impossible without an adequate analytical background (Lobinski 1998). The improvement and spreading of molecular mass spectrometry also accelerated the development of speciation analyses. It allowed

the identification of unknown selenocompounds and therefore facilitated the extension of the borders of trace elemental analysis to the investigation of biochemical and physiological processes.

Selenium speciation analysis of plants received significant attention in food and nutrition research because the majority of dietary selenium is provided by plants. They assimilate selenium as the analogue of sulfur depending on the bioavailable sulfur/selenium ratio in the soil (Terry et al. 2000). Consequently, the selenium content of soils determines its quantity in crops and produces. In other words, the level of dietary selenium intake highly depends on the geographical area. In general, plants that contain various sulfur metabolites like *Allium* (*e.g.*, garlic) and *Brassica* species (*e.g.*, radish) are also good sources of dietary selenium (Rayman et al. 2008).

Contrary to animals and humans, selenium is not essential for plants and most plants cannot tolerate seleniferous environments. The assimilation of high amount of selenium creates oxidative stress and its non-selective incorporation into proteins exhibits toxic effects (Van Hoewyk 2013). Some plants, however, have adapted to live on seleniferous soils and developed special selenium metabolic pathways in order to avoid its negative effects. Selenium accumulator plants do not only tolerate, but even have adapted to accumulate high amount of selenium in their tissues and use it in elemental defense mechanisms (Freeman et al. 2006).

The current work addresses the investigation of selenium speciation of some selenium accumulator plants by the application of high resolution mass spectrometry. The comprehensive information gained through the metabolomic and proteomic experiments will hopefully extend our knowledge on the Se metabolism and detoxification mechanisms of selenium accumulator plants. Brazil nut (*Bertholletia excelsa*) and monkeypot nut (*Lecythis minor*) were selected for the analysis since both of them are important sources of dietary selenium as natural food and feed supplements, respectively.

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2. REVIEW OF LITERATURE

„No one lives long enough to learn everything they need to learn beginning from the starting line. To be successful, we absolutely, positively have to find people who have already paid the price to learn the things that we need to learn to achieve our goals."

- *Brian Tracy*

In the second half of the twentieth century, selenium analysis has received considerable attention in bioanalytical research due to the essentiality of this trace element for many living organisms (Schwarz et al. 1957, McConnell 1963, Shamberger and Frost 1969, Schroeder et al. 1970). In the meantime, selenium research became closely related to the concept of speciation. According to the definition of IUPAC (International Union for Pure and Applied Chemistry) a *chemical species* is a specific form of an element defined by its isotopic composition, electronic or oxidation state, and/or complex or molecular structure (Templeton et al. 2000). *Speciation analysis* in analytical chemistry means all the analytical activities of identifying and/or measuring the quantities of one or more individual chemical species in a sample.

Speciation of an element is the distribution of the element amongst defined chemical species in a system (Nordberg et al. 2004). Considering that Se is a trace element which relative abundance in the earth's crust is below the μg g^{-1} level (parts per million, ppm), it is not surprising that the Se content of most biological and environmental samples is also in the trace level. This characteristic makes Se speciation studies and the identification of unknown organic Se species particularly challenging.

Initially the aim of Se speciation studies was the determination of the toxicity of different inorganic and organic species. Subsequently, biochemical, metabolic and nutritional aspects became more prominent and lead to the mapping of various metabolic pathways and the verification of the biological role of several Se species (Stadtman 1977, Mounicou et al. 2009b, Ogra and Anan 2012).

2.1. Analytical approaches in selenium speciation

Principally, there are three main issues that must be carefully considered in order to identify and/or quantify the different species of an element in a biological sample. Primarily, it is crucial to plan an adequate *sample preparation* procedure to ensure that the original species distribution is conserved ("no decomposition" approach). Additionally, complex sample preparation, preconcentration and *separation techniques* are usually required to eliminate matrix

interferences. The choice of an adequate *detection* method is also crucial in order to attain relevant analytical information (Van Loon et al. 1977, Lobinski et al. 2000). Obviously all of these steps influence the result of the whole analytical procedure.

In speciation studies the requested analytical information is related to the chemical form of the species, *i.e.*, the *structure* of the molecules. The characteristics of mass spectrometric techniques such as good selectivity, sensitivity, low detection limits and their capability to deliver structural information can be utilized in the quantitative and qualitative analyses of Se species (Szpunar et al. 2003, Anan et al. 2011). In trace elemental analysis inductively coupled plasma mass spectrometry (IPC-MS) is one of the most widely applied detectors. Its robustness, low detection limits (limit of detection, LOD) and wide linear range made this technique predominant in quantitative analysis of trace elements. However, ICP-MS is an element selective detector that does not provide any direct structural information. Therefore, it has to be coupled to an adequate separation technique (GC, LC or capillary electrophoresis) to fulfill the requirements of Se speciation analysis, *i.e.*, the identification of different chemical forms of Se. The limitation of most methods such as HPLC-ICP-MS is the lack of available standards for most selenium species.

Since each technique has its advantages and shortcomings, in general, the method of choice is determined by the aim of the study and the desired analytical information (Feldmann et al. 2009). For example, from the nutritional or food quality assurance perspective it can be satisfactory to determine and quantify the most abundant Se species (that are available as standards) by HPLC-ICP-MS. On the other hand, from the biochemical point of view it is typical to target the complete characterization of species distribution of selected tissues. This latter process also involves the standardless identification of unknown organic selenium compounds, therefore the application of molecular mass spectrometric methods is inevitable (Casiot et al. 1999, Far et al. 2010).

There are also some cases in physiological studies where the main objective is the direct analysis of intact tissues and moderate speciation information is also acceptable. For instance, X-ray techniques (*e.g.*, X-ray absorption microspectroscopy, μ-XAS and X-ray absorption near-edge structure, XANES) are favorable for their advantages like straightforward sample preparation and they offer the possibility to carry out distributional studies in intact tissues (Freeman et al. 2006). Even if these techniques are limited to provide structural information only about the neighboring atoms of Se and determine its oxidation and coordination number, it can be sufficient to determine if Se is present in the tissue in organic or inorganic forms. This kind of speciation data can become relevant when combined with spatial information (Bulska et al. 2006).

Note, that the spatial resolution capacity of these imaging techniques is a rather important parameter. For instance, recent high resolution µ-XAS data suggest (Freeman et al. 2006) that the inorganic Se content of Se accumulator plants might have been underestimated in earlier bulk XAS studies (Pickering et al. 2003). Another disadvantage of X-ray techniques that their application is usually limited to the analysis of Se hyperaccumulator species because these not particularly sensitive methods require a minimum analyte concentration of several tens of ppm (Freeman et al. 2006, Mounicou et al. 2009b).

Alternatively, laser ablation (LA) is a suitable ion source for the purpose of imaging mass spectrometry. With ICP-MS detection it can provide element or isotope specific distributional analysis (Wu and Becker 2012, Becker et al. 2014), or assist the detection of selenium-containing proteins after two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), while matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) allow the speciation analysis of selenocompounds in intact tissues (Lobinski et al. 2006, Becker et al. 2007). The most important analytical approaches in Se speciation are summarized on Figure 2.1.

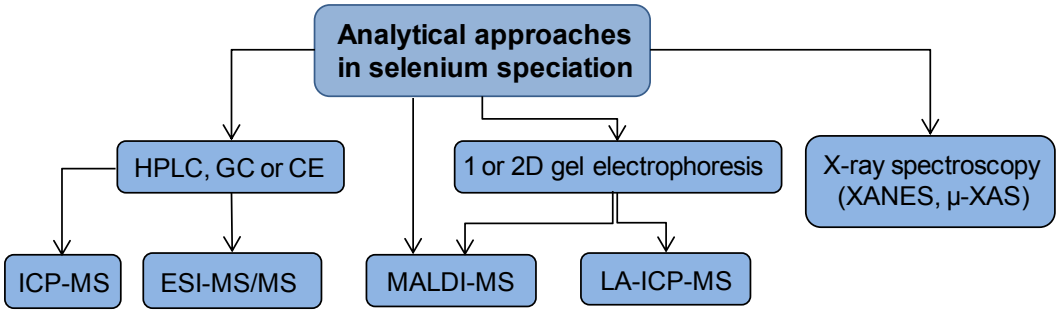

Figure 2.1. Overview of the most common analytical approaches in Se speciation.

2.1.1. Sample preparation and preconcentration

Since most Se species are prone to oxidation and decomposition, the sample preparation procedures applied in Se speciation studies must be carefully considered in order to avoid any alteration in species distribution and the formation of artefact species (that are absent in the original sample) (Dernovics et al. 2006). Principally, mild extraction and separation conditions and the least possible sample preparation steps are recommended. On the other hand, multistep sample purification and preconcentration procedures are inevitable in complex metabolomic studies because the low concentration of Se species in biological samples makes their direct determination impossible by molecular mass spectrometry (Szpunar and Łobiński 2002). Thus,

the sample preparation strategy usually represents a *compromise* that corresponds to the purpose of the analysis.

Besides a few exceptions, most of the samples subjected to Se speciation studies are solid. Since it is a prerequisite in every analytical measurement to assure the homogeneity and representativeness of the sample, the first step of sample preparation of solid samples must be the reduction of their particle size by cutting, shredding, grinding or cryogenic grinding. In most cases the water content of the sample is also eliminated by lyophilization. It is a particularly mild sample preparation step, suitable to be applied in speciation analysis unless the aim of the analysis is the identification of volatile compounds which are also eliminated during lyophilization (Casal et al. 2010). (The extraction techniques for the analysis of volatile compounds will be detailed in section 2.1.1.3.) Lyophilization can also be utilized for the preconcentration of the sample or for solvent/buffer exchange. These techniques are based on the elimination of solvents/volatile buffers and dissolution of the sample in a lower quantity of solvent or in a different solvent, respectively. These techniques became crucial in the non-targeted analysis and identification of very low-abundance Se species (Lobiński and Szpunar 1999).

2.1.1.1. Total selenium analysis

Although, the determination of the total Se content of biological or environmental samples does not provide any information about the speciation of the element, it is still an important part of every speciation studies. The measurement of total Se content provides the basis for the determination of the recovery and also allows the calculation of the ratio of the identified compounds compared to total Se. The aim of sample preparation before total Se determination is to reduce the complexity of the matrix and obtain a homogenous analyte that contains basically only one inorganic Se species. This Se species should be commercially available to be utilized in quantitative analysis. The acquired analyte also has to be compatible with the chosen method of identification.

Microwave assisted acid digestion became the ultimate sample preparation method for total selenium quantification. It is a simple, fast, reliable, standardized and reproducible procedure that is sufficiently robust but also can be optimized for different sample matrices (Campbell and Kanert 1992). As a result of this digestion technique, the entire Se content of the sample is converted into the form of Se (VI) and the concentration of organic matrix substances is substantially reduced by their oxidative decomposition. Both of these characteristics facilitate the

determination of the total Se content of the sample by the most common element selective detection methods, like ICP-OES (optical emission spectrometry) or ICP-MS.

Alternatively, the application of a non-selective detector is also possible if there is a suitable derivatization technique that can make the method selective. For instance, the determination of total selenium content by fluorescence spectroscopy is possible through the detection of a piazselenol complex that is produced in the reaction of Se with 2,3-diaminonaphtalene (Watkinson 1966). This method is still widely used (mainly in clinical analysis), because it is quite simple and does not require any specific instrumentation. The main disadvantage of this method is, however, that its selectivity and sensitivity is lower compared to the above mentioned element selective detection methods.

2.1.1.2. Extraction techniques

The aim of extraction is to reduce the complexity of the matrix by the selective extraction of targeted compounds that are soluble in the applied extraction solvent or adsorbed on the chosen solid phase. In selenium speciation the most frequently used extraction techniques are solid/liquid extractions since the majority of the samples are solid and they are usually extracted by some kind of solvents. The applicability of (i) organic buffers, (ii) dilute acids or alkali, (iii) enzyme solutions and (iv) the mixtures of water and some organic solvents as extraction solvents have been investigated in several studies.

First of all, it was shown that a generally applicable extraction technique does not exist, because the efficiency of different extraction methods is highly dependent on the sample matrix and the expected Se species (Kotrebai et al. 2000, Dernovics et al. 2002). For instance, water extraction fulfills the most important requirements for Se speciation analysis but usually yields in moderate recoveries. Therefore, several studies have been conducted to achieve better extraction efficiencies.

Extraction by dilute acids has been reported to provide better extraction efficiency compared to water extraction for protein-rich samples (*e.g.*, Brazil nut, yeast). It is due to the capability of acids to liberate free and weakly bound amino acids from complex matrices. On the other hand, the application of acids in Se speciation studies is still a contradictory subject because they lead to the interconversion of Se species by the oxidation of inorganic selenocompounds. Despite of this fact, acidic hydrolysis by HCl (>1 mol L^{-1}) or methanesulfonic acid (MSA) is widely used in the analysis of organic Se compounds, predominantly for the analysis of selenoamino acids

because it yields exceptionally high extraction efficiency (Mester et al. 2006, Dernovics et al. 2007a, Goenaga-Infante et al. 2008). In this case, the inorganic Se content of the sample is referred as "total inorganic Se". Nonetheless, the modification of inorganic Se species is not the only issue about these techniques. Our research group has shown that the chiral configuration of SeMet is also altered during MSA hydrolysis that bias the result of quantitative analyses (Egressy-Molnár et al. 2011).

In order to overcome the weaknesses of the above mentioned extraction techniques, enzymatic extraction was also introduced in Se speciation studies. It represents a mild (pH 6-8, 37 °C) and efficient technique that is adequate for a wide range of biological samples. The limitations of this method are usually due to the specificity of enzymes and the relatively long time required for the complete digestion of the sample matrix (16-48 hours). The application of a mixture of enzymes or a crude enzyme isolate with broad enzyme specificity can increase the extraction efficiency. These enzyme combinations can be utilized subsequently like in artificial digestive systems (pepsin, pancreatin and protease XIV), or simultaneously like in the case of pronase E that is a crude protease isolate from *Streptomyces griseus* that contains at least three different types of proteases. The application of the latter in Se speciation was introduced by Gilon and coworkers and it is now extensively applied for a variety of samples (1996) and also referenced in the latest selenium-related EU regulations (*e.g.*, (EC) No. 1831/2003, 609/2013/EU).

Despite of the advantages of this method, it is still quite time consuming compared to other extraction processes. Several new approaches have been introduced in order to solve this problem. The combination of enzymatic extraction with ultrasonic or microwave energy led to rather promising results (Montes-Bayón et al. 2006, Reyes et al. 2009). The extraction time could be reduced to a few minutes, while the extraction yield remained the same (Capelo et al. 2004, Cabañero et al. 2005, Yang et al. 2013). The remarkable decrease in time can be attributed to the enhanced contact between cellular components and enzymes.

Ultrasonic energy has been utilized for various analytical purposes (homogenization, dispersion of agglomerates, cell disruption and emulsification) on the basis of cavitation effects. The same effects are utilized in ultrasound assisted enzymatic digestion. Additionally, microwave irradiation was also shown to be effective in enzymatic and chemical reaction acceleration as well as improvement of digestion efficiency since it also provides increased sample exposure (Pramanik et al. 2002, Sun et al. 2006). Both methods have been shown to introduce some species transformation through oxidation, but the level of this unfavorable process is not significantly higher than in the case of other extraction methods (Pedrero et al. 2007a).

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Although, the extraction efficiency can be enhanced by the application of acid hydrolysis or enzymatic digestion, the choice of extraction remains to be a compromise that is determined by the aim of the analysis. Sometimes it is more important in speciation analysis to prevent species transformation than to achieve high Se recovery. In selenometabolomics, when the aim of the analysis is the characterization of low molecular weight compounds, water is the most commonly applied extraction solvent. It became widespread because the majority of Se species are water soluble and it provides mild extraction conditions that preserve the original species distribution (Mounicou et al. 2009b). Water extraction is also generally applied in proteomics studies.

The utilization of polar organic solvents or their mixtures with water offers an interesting alternative for water extraction in the analysis of low molecular weight compounds. Some authors suggest that water extracts can contain some endogenous enzymes that retain their activity owing to mild conditions and might contribute to species transformation (Ouerdane et al. 2013). These enzymatic processes can be avoided by the application of organic solvents since enzymes are usually denatured in such solutions. The most commonly applied extraction solvents in metabolomic studies are methanol, acetonitrile, chloroform, dichloromethane and their mixtures (Canelas et al. 2009).

As regards to non-polar organic solvents, they are normally applied for the extraction of lypophilic compounds. It is worth noting that there have never been any lypophilic selenocompounds reported in the literature. Various studies showed that Se is not present in considerable amounts in the lipid fraction of biological samples. Therefore, the lipid fraction of the samples is usually eliminated by organic solvent extraction without the loss of any Se species. For this purpose, the application of Soxhlet extraction with cyclohexane is preferable because of its good reproducibility. This extraction step is especially important in order to reduce matrix interferences before the analysis by HPLC based hyphenated analytical methods, since lypophilic compounds are normally not compatible with HPLC separation techniques (Bodó et al. 2003).

When the aim of the analysis is the determination of the complete Se speciation of the sample, and "100 % recovery" is required, a combination of various extraction techniques might be necessary. Sequential extraction can be utilized for instance for the determination of the distribution of Se between the different tissues or fractions of the sample (Mounicou et al. 2009a). Another special application of sequential extraction is the investigation of Se bioavailability by artificial digestive systems (Yasumoto et al. 1988).

Liquid samples such as fresh water, seawater, body fluids and different solvent extracts also require some kind of purification or preconcentration before Se speciation analysis (Klein et al. 2011). For this purpose, the application of solid phase extraction (SPE) offers a valuable tool (Bueno and Potin-Gautier 2002). Since the principle of SPE is similar to liquid chromatography it can be used as a fast and convenient sample purification step to reduce matrix interferences before HPLC analysis (Yathavakilla et al. 2005). The process consists of three steps: first (i) the targeted compounds are selectively bound to the stationary phase (the SPE resin), then (ii) the non-targeted matrix compounds are eluted and discarded, finally (iii) the targeted compounds are also eluted and collected for further analysis. The most frequent applications of this technique include the selective preconcentration of fresh water or ground water before mass spectrometric analysis
(Yu et al. 2004) and the elimination of salts before electrospray ionization measurements.

2.1.1.3. Extraction techniques preceding gas chromatography

Gas chromatography is suited for the analysis of volatile compounds, but some non-volatile compounds can also be analyzed after increasing their volatility by derivatization. The extraction techniques for the analysis of volatile compounds are quite different from the above detailed methods. In the case of the most common technique, solid phase microextraction (SPME), volatile selenocompounds are directly extracted from the headspace of the sample prior to GC-MS measurement (Mester et al. 2001, Dietz et al. 2004). The selenium species most frequently analyzed by SPME-GC-ICP-MS or SPME-GC-MS are volatile alkylselenides, such as methylselenol (CH_3SeH), dimethyl-selenide ($(CH_3)_2Se$) and dimethyl-diselenide ($CH_3Se_2CH_3$) (Meija et al. 2002, Bueno and Pannier 2009). This technique is exceptionally beneficial for the analysis of living organisms (plants or microorganisms) owing to its non-destructive nature (Swearingen Jr. et al. 2006, Kubachka et al. 2007).

Besides solid phase extraction, the application of non-polar organic solvent extraction is also common in gas chromatographic sample preparation. However, this extraction procedure is not very typical in selenium speciation studies because of the relative lack of non-polar selenium compounds. Nonetheless, this approach was successfully applied by the group of Szpunar for the analysis of low molecular weight volatile compounds of mustard seeds (Ouerdane et al. 2013). They have applied the mixture of pentane and diethylether as an extraction solvent.

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2.1.1.4. Sample purification by multidimensional HPLC

The separation of selenium containing compounds from each other is a prerequisite in selenium speciation studies. It is especially important in element specific detection methods where the identification of species relies on their retention time matching with standards (Pedrero et al. 2007b). The application of multidimensional HPLC methods became crucial because the separation of selenocompounds is usually impossible in a single chromatographic run (Szpunar and Łobiński 2002). Multidimensional HPLC separations consist of the subsequent application of different separation mechanisms (preferably orthogonally) in order to achieve better separation of target compounds. It also facilitates the elimination of matrix interferences that becomes essential in molecular mass spectrometry applying soft ion sources, where the ionization efficiency critically depends on the number of co-eluting compounds, *i.e.,* the relative purity of the sample.

Our ability to understand life processes that are responsible for the essentiality and toxicity of selenium depends on the identification, characterization and quantification of many different selenium species (Szpunar and Łobiński 2002). Multidimensional approaches are utilized not only for the purification but also for the preconcentration of samples (Gammelgaard et al. 2003). It can be considered as a preparative method because between the subsequent HPLC separations the selenium-containing fractions are heart-cut (collected and preconcentrated by lyophilization). Selenometabolomic studies would not be possible without the application of multidimensional HPLC techniques because of the very high number of selenocompounds and their low concentration in natural samples (Mounicou et al. 2009b).

There are several examples in the literature for the application of multidimensional approaches in selenium speciation. The first applications combined size exclusion chromatography (SEC) with reversed phase (RP) chromatography for the purification of selenium-enriched garlic (McSheehy et al. 2000) and yeast (McSheehy et al. 2001, 2005) before the (partial) identification of Se species by electrospray ionization tandem mass spectrometry (ESI-MS/MS). The improvement of the technique provided the basis to conduct more complete metabolomic studies and realize the large scale identification of selenometabolites (McSheehy et al. 2002a, Aureli et al. 2012, Preud'Homme et al. 2012, Németh et al. 2013, Ouerdane et al. 2013). The utility of multidimensional HPLC for the unambiguous identification of Se species was also demonstrated through the identification of the major selenium metabolite in human urine (*Se*-methyl-*N*-acetylselenohexosamine) (Gammelgaard et al. 2003, Huerta et al. 2003).

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2.1.2. Elemental mass spectrometry

Inductively coupled plasma mass spectrometry (ICP-MS) is a type of inorganic mass spectrometry (Becker 2008) that is frequently termed as *elemental mass spectrometry* since it is an excellent tool for the analysis of nearly all elements of the periodic table (shown in color in Figure 2.2.). The few exceptions include carbon, hydrogen, oxygen, nitrogen, helium, neon, argon and fluorine. The natural isotopic abundances and detection limits of the detectable elements by contemporary instrumentation are also displayed on Figure 2.2.

Traditionally, atomic absorption spectroscopy (AAS) and inductively coupled plasma atomic emission spectroscopy (ICP-AES) have served as suitable devices for trace metal analysis. However, ICP-MS became the instrument of choice for this purpose because of its superior detection capabilities (down to the ng kg^{-1} range for most elements), wide linear range and high analytical productivity (Date and Gray 1985, Zoorob et al. 1998). Other advantages of this technique include the easy coupling to chromatographic and laser ablation techniques and the possibility to carry out isotopic analyses (Gray 1985a). These characteristics allowed ICP-MS to become a unique tool in Se speciation studies.

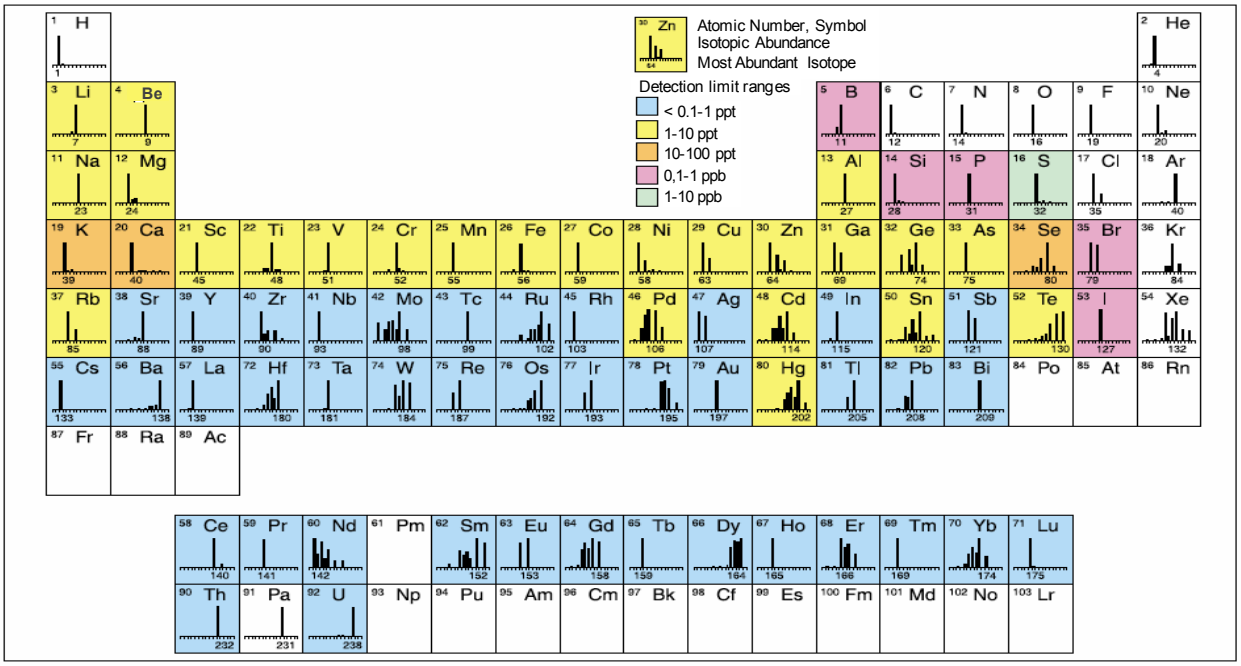

Figure 2.2. Elements determined by ICP-MS, their natural isotopic abundance and their approximate detection capability (PerkinElmer) (Internet 1).

The structure of the instrument and its operation are discussed in chapter 4.2. Here I would like to focus on the principles of ICP ionization and quadrupole mass analysis. In addition, the

most common issues affecting the application of ICP-MS detection will also be assigned in this chapter.

In general, the main constituents of a mass spectrometer are the ion source (where the ions to be analyzed are generated) and the mass analyzer that separates the ions according to their mass-to-charge ratio. In an ICP-MS instrument the inductively coupled plasma serves as the ion source. The plasma is generated by passing argon through a series of concentric quartz tubes (ICP torch) that are surrounded at the end by the radio frequency (RF) coil. As power is supplied to the coil, oscillating electric and magnetic fields are established at the end of the torch (Gray 1985b). When a spark is applied to the argon flowing through the ICP torch, electrons are stripped off the argon atoms, forming argon ions. These ions are caught in the oscillating fields and collide with other argon atoms, forming an argon discharge or plasma (Montaser 1998). The sample is introduced to the 6000 K plasma as an aerosol. Liquid droplets evaporate then atoms are formed, that collide with free electrons, argon cations, and neutral argon atoms. ICP is a so-called hard ionization technique because any molecules initially present in the aerosol are quickly and completely broken down into singly charged cations (Hoffmann and Stroobant 2007). The generated ions are then guided to the quadrupole mass analyzer.

It is worth to note that some of these singly charged cations will recombine with other species in the plasma to create both stable and meta-stable molecular species, which will then be transmitted into the mass analyzer along with the singly charged cations. These polyatomic ions represent the most common interferences in ICP-MS detection (Taylor 2001). For instance, an argon dimer (Ar_2^+) with a mass-to-charge ratio of 80 is an interferent for the major isotope of Se. Furthermore, the measurement of the most abundant isotope of S is also interfered by oxygen dimers (O_2^+, *m/z* 32). The analyses of these elements depended on the measurement of less abundant isotopes, such as ^{82}Se and ^{34}S, resulting in reduced sensitivity and higher limits of detection (see Figure 2.2.) (Gray 1985b).

Recent advances of ICP-MS technique involve the improvement of its selectivity to overcome the problem of interferences. The main trends on this field are the introduction of different reaction cells (Tanner et al. 2002, Bednar et al. 2009, D'Ilio et al. 2011) and the establishment of high resolution (HR) ICP-MS through replacing quadrupole mass analyzers by double focusing magnetic sector field analyzers. In general, sensitivity is inversely proportional to resolution and the aim of the analysis will determine the optimal compromise between these two characteristics. Nonetheless, sensitivity can also be improved through the application of both reaction cells and HR-ICP-MS, since by the elimination of interferences the most abundant isotopes of the elements can be measured. With traditional ICP-MS, detection limits for Se and S

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are around 10 µg L^{-1} and 1 mg L^{-1}, respectively, due to the argon plasma background and polyatomic interferences. Notably, the detection limit for Se can be reduced to < 0.1 µg L^{-1} by reaction or collision cells. Another interesting innovation is the application of multicollector detectors that allow the simultaneous detection of several mass-to-charge values that is particularly useful in high accuracy isotope analysis (Wang et al. 2007).

Quadrupole mass analyzers are still the most widely used mass analyzers for routine ICP-MS analyses. They fit for this purpose because they are simple, robust and affordable. They consist of four perfectly parallel, circular (ideally hyperbolic) rods. Ions travelling along the rods are subjected to a total electric field made up of a quadrupolar alternative field superposed on a constant field (Figure 2.3.). Separation of ions is based on their stable trajectories in this oscillating electric field (Hoffmann and Stroobant 2007).

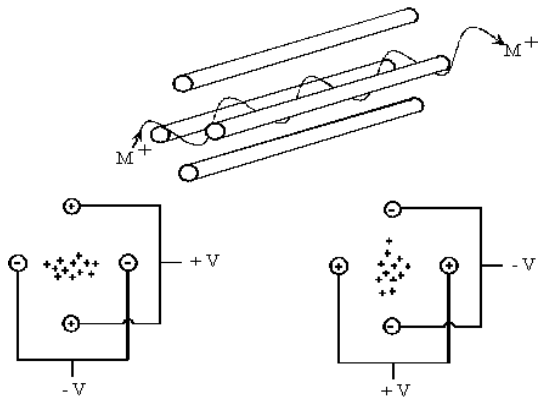

Figure 2.3.: The trajectory of selected ions in the oscillating electric field (Internet 2)**.**

Considering that every mass analyzer has its advantages and limitations, it should be emphasized that none of them are universally applicable. The main characteristics to be considered to evaluate the performance of a mass analyzer are the mass range limit, the analysis speed or scan speed, mass accuracy and resolution. The mass range limit of general purpose quadrupole analyzers is around 4000 Th and they provide a scan speed of 2500 amu s^{-1}. Both of these characteristics fulfill the requirements of traditional ICP-MS measurements. The mass-to-charge ratio is usually expressed as *m/z*, where *m* is given as the relative mass and *z* as the charge number. Mass accuracy is the accuracy of the measured *m/z* (m_{exp}). It is the difference of m_{exp} and the theoretical *m/z* (m_{theo}) and customarily expressed in parts per million (ppm).

Resolution is the ability of the mass analyzer to provide a distinct signal for two ions with a small *m/z* difference. Quadrupole mass spectrometers are low resolution instruments that yield a resolution around 2000 providing a unit mass resolution. Mass accuracy is linked to the resolution of the analyzer. Low resolution mass analyzers are not able to provide high accuracy.

The mass accuracy of quadrupole analyzers is 100 ppm at the full width at half maximum (FWHM) of *m/z* 1000 (Hoffmann and Stroobant 2007).

Despite of the few limitations of ICP-MS it is an unmatched instrument in Se speciation because of its superb detection limits and quantitation capability compared to any other techniques. However, considering the recent development in bioinorganic chemical research, it is increasingly important to gain molecular information of the analyzed species. Nowadays, in complex biochemical research, ICP-MS is mostly used to assist sample preparation (Szpunar and Łobiński 2002) and to detect Se in trace quantities (Cheajesadagul et al. 2014), while the qualitative analysis of the species is usually based on molecular mass spectrometric techniques.

2.1.3. Molecular mass spectrometric techniques

Molecular mass spectrometry covers those MS techniques that apply soft ion sources and allow the analysis of molecular ions. These techniques enable the structural analysis and standardless identification of unknown biomolecules. Therefore, the development of molecular mass spectrometric techniques also opened the way to completely new analytical perspectives in bioinorganic chemistry. The most frequently used ion sources in Se speciation studies are electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI). These two techniques are complimentary since ESI is suitable for the analysis of low molecular weight compounds (up to 1000 Da), while MALDI is better suited for the analysis of high molecular weight biopolymers (even exceeding 100,000 Da).

Versatile mass analyzers can be utilized for the analysis of biomolecules, ranging from quadrupole, time-of-flight (TOF) to Orbitrap and Fourier transform ion-cyclotron resonance (FT-ICR) mass spectrometers. Hybrid instruments are also applied in speciation analysis since they extend the analytical toolkit by combining the strengths of several types of mass analyzers. A potential application of hybrid instruments is tandem mass spectrometry. In the most common tandem mass spectrometry experiment a first analyzer is used to select a precursor ion, which then undergoes a fragmentation to yield product ions and neutral fragments. The second analyzer separates the product ions and a tandem MS or MS-MS spectrum is produced.

It is important to note, that tandem mass spectrometry can be conceived either in space or in time. In the first case, the mass analyzers are physically coupled, like in triple-quadrupole (QqQ) or in quadrupole time-of-flight (Q-TOF) mass spectrometers. Tandem mass spectrometry in time is achieved by the time separation of fragment ions in ion traps, Orbitrap and FT-ICR instruments that contain an ion storage device. Among these instruments, FT-ICR is able to

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provide the most complex MS^n data. Since it is based on the trapping and non-destructive detection of ions it allows the monitoring of a sequential fragmentation process (up to 7-8 fragmentation steps).

The TOF analyzer separates ions (after their initial acceleration with a constant potential) according to their velocities when they drift in a field-free region that is called the flight tube (Hoffmann and Stroobant 2007). Since all ions acquire the same kinetic energy in the acceleration region, their velocity will depend on their masses (*i.e.,* if all ions receive the same energy, smaller ions will fly faster towards the detector). Therefore, their mass-to-charge ratio can be calculated from the measurement of the time that they need to reach the detector (Figure 2.4.).

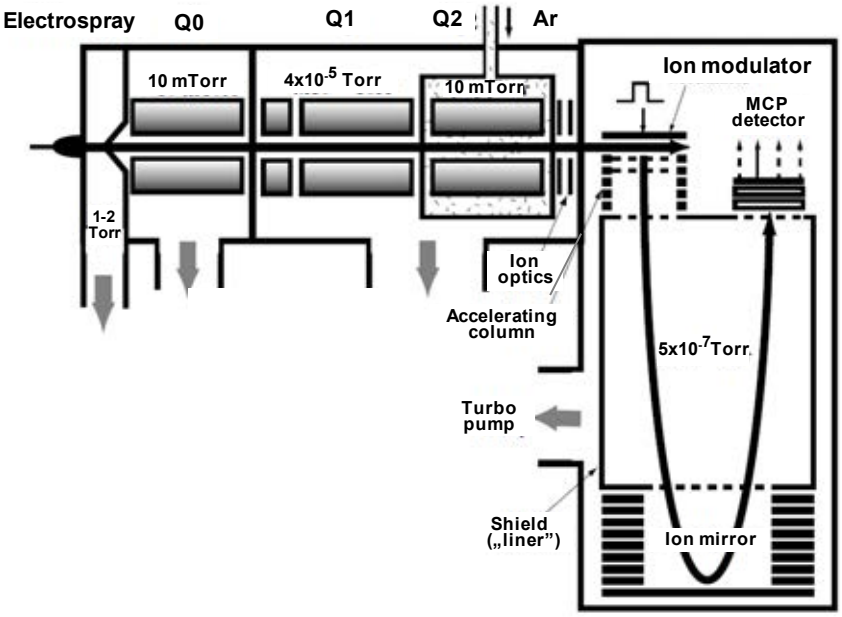

Figure 2.4. Schematic image of a Q-TOF analyzer (Chernushevich et al. 2001).

One of the main advantages of TOF analyzers is that theoretically they do not have an upper mass limit. This characteristic made them rather popular for the analysis of very high molecular weight compounds (up to 300 kDa), but the initial enthusiasm for the technique gradually diminished because of the relatively poor resolution of the first commercially available instruments. Indeed, the mass resolution of a TOF analyzer can be seriously reduced by the factors that create a distribution in flight times of ions with the same *m/z* ratio.

The mass resolution of recent TOF analyzers is improved by the application of delayed pulsed extraction and reflectrons. Both techniques are designed to reduce the initial kinetic energy dispersion among ions with the same m/z ratio. As a result, the state-of-the-art instruments can achieve a mass resolution of over 10,000 (in FWHM of m/z 1000) that allows an exact mass measurement within 5 ppm (in MS mode). In a Q-TOF tandem mass spectrometer the

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targeted compounds can be selected by the quadrupole analyzer (Q1), fragmented in a collision cell (q2) then analyzed by the high resolution TOF analyzer. This combination of accurate mass measurement with MS/MS fragmentation offers a powerful tool for the *de novo* identification of unknown compounds.

In some cases, however, the successful identification of unknown compounds might depend on some additional information such as characteristic isotope distribution. In Se speciation studies, the unique isotope distribution pattern of Se is an excellent tool in the detection and identification of new compounds. For instance, the automatic search for this characteristic isotope pattern would hugely facilitate the detection of these compounds. There were a few partially successful attempts to develop a search algorithm for this objective (Shah et al. 2007), but the issue is still not properly elucidated by the most common searching tools.

Quite recently, the group of Lobinski accomplished the first automated assignment of selenium metabolites in Se-rich yeast (Preud'Homme et al. 2012). They designed an on-line two dimensional chromatographic system with combined ICP-MS and electrospray linear trap-Orbitrap (ESI-MS^n) detection and utilized the enhanced data acquisition and processing capabilities of Frontier™ and MetWorks™ software. MetWorks™ includes such advanced automatic isotope pattern recognition and multiple mass defect filters (Internet 3) that allowed the automated identification of 41 known and 8 previously unknown selenometabolites. These results are very promising but unfortunately, these kind of searching tools are still scarce and manufacturer-dependent. Thus, the detection and identification of selenocompounds are usually achieved by the complementary use of elemental and molecular mass spectrometric techniques.

2.1.4. The complementary use of elemental and molecular mass spectrometry

The previous chapter demonstrated the limitations of both techniques that can be compensated by their complementary use. For the detection of selenocompounds ICP-MS is an unmatched technique due to its robustness, selectivity and very low detection limits. On the other hand, it cannot provide any direct structural information of the analytes that would be crucial in speciation analysis. The coupling of HPLC separation to ICP-MS can provide some indirect structural information by retention time matching, but only for a few selenocompounds that are available as standards. Therefore, a lot of HPLC-ICP-MS based studies were usually concluded with only the partial identification of the detected selenocompounds (Bird et al. 1997b, Zheng et al. 1998).

The application of high resolution mass spectrometric techniques allowed the extension of Se speciation studies by enabling the standardless identification of previously unidentified selenocompounds (Casiot et al. 1999, Mounicou et al. 2009b). Nowadays, the most widespread techniques for *de novo* identification are ESI-Q-TOF-MS and Orbitrap-MS (Gammelgaard et al. 2008). However, the detection of unknown Se species is more complicated by these molecular mass spectrometric techniques because their selectivity and sensitivity is lower compared to ICP-MS. There are a few solutions for this problem, but the most simple and therefore the most frequently applied is the parallel application of elemental and molecular mass spectrometric techniques (Becker and Jakubowski 2009).

In practice, it cannot be accomplished without the harmonization of the two methods. First of all, we have to find a suitable separation that is compatible with both MS techniques (Casal et al. 2010). For instance, the addition of an ion pairing agent to the mobile phase in RP-HPLC-ICP-MS can remarkably improve the separation of polar compounds (Bird et al. 1997a), but it is not recommended to be used with ESI-MS detection because it can drastically reduce its sensitivity (Kotrebai et al. 1999, Szpunar and Lobinski 2003). On the other hand, reversed phase chromatography is commonly coupled to ESI-MS in metabolomic studies, but the application of such high concentration of organic solvents (that are typical in RP-HPLC) can severely reduce the sensitivity of ICP-MS detection (Szpunar and Lobinski 2003). Nonetheless, this technique is usually applied, because the negative effects of organic solvents can be compensated by some technical solutions, such as oxygen introduction, nano-flow HPLC and total consumption nebulizers (Giusti et al. 2006, Preud'Homme et al. 2012). More recently, RP-HPLC is usually replaced by hydrophilic interaction liquid chromatography (HILIC), because it enables the more effective separation of polar selenometabolites (Far et al. 2010, Arnaudguilhem et al. 2012, Ouerdane et al. 2013). The resolution of polar compounds by HILIC can achieve or even exceed the resolution of traditionally applied ion exchange techniques.

Besides the parallel application of elemental and molecular MS, the two techniques can also complement each other in different phases of speciation analysis. For example, the above mentioned multidimensional HPLC separations are assisted by ICP-MS detection, while the identification of selenocompounds from the adequately purified and concentrated fractions is delivered by ESI-MS techniques (Dernovics et al. 2007a). Another interesting application of these techniques is the detection of selenium containing proteins on two-dimensional gels by laser ablation (LA) ICP-MS, then the identification of these proteins by high resolution ESI-MS (Bianga et al. 2013, Bierla et al. 2013, Cheajesadagul et al. 2014).

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2.1.5. Development of hyphenated analytical techniques and their application in speciation analysis

In speciation analysis mass spectrometric detection is always preceded by some kind of separation that enables the qualitative and quantitative analysis of the different species by separating them from each other and also from matrix interferences. This kind of techniques are called coupled or hyphenated analytical techniques (Hirschfeld 1980). They consist of two main parts: a separation technique (GC, HPLC or electrophoresis) and a detector (UV, AAS, ICP-MS or ESI-MS) that are connected by an interface. In selenium speciation studies, the most frequently applied coupled analytical techniques are HPLC-ICP-MS, HPLC-ESI-Q-TOF-MS and HPLC-Orbitrap-MS (Dressler et al. 2011). In the followings I would like to focus on coupled mass spectrometric techniques, since these were utilized throughout my research work.

Following their introduction, the application of coupled analytical techniques became exceptionally popular due to their unmatched advantages. For instance, the separation of target compounds from matrix interferences can remarkably improve the signal-to-noise ratio and therefore enables lower detection limits. In this way, absolute detection limits as low as sub-picogram level in GC and femtogram level in LC separations can be achieved. (Szpunar and Lobinski 2003). In addition to improved quantification, the information about the retention time of target compounds allows their qualitative analysis.

The coupling of GC to mass spectrometry was one of the first coupled analytical techniques (Holmes and Morrell 1957). It offered a unique opportunity for the analysis of volatile or volatilizable compounds. It was first applied in Se speciation for the detection of SeMet in soybean proteins after its volatilization by *N,O*-bis(trimethylsilyl)acetamide (Yasumoto et al. 1988) (see Table 2.1). GC-MS was also applied for the analysis of volatile selenocompounds of sediments by Gómez-Ariza and coworkers (1998). On the other hand, the coupling of HPLC separation to mass spectrometry for the analysis of non-volatile compounds remained to be unresolved for decades due to technical issues. Developing an adequate interface for the efficient ionization of non-volatile and thermally instable compounds and overcoming the problems of solvent and flow rate incompatibility of HPLC and MS turned out to be quite a challenge (Arpino 1982).

Actually, the coupling of elemental mass spectrometry to HPLC was less complicated than the development of HPLC coupled molecular mass spectrometric techniques. Soon after the development of ICP-MS (Houk 1980), the HPLC-ICP-MS coupling was also introduced by Dean and coworkers (1987). It was possible because the flow rate of HPLC separations was

compatible with ICP nebulizers that made the direct introduction of the HPLC eluent feasible. As regards to solvent compatibility, high organic solvent ratios are not very favorable for ICP ionization, but they can also be handled by the addition of oxygen to the carrier gas and the application of robust radio frequency generators.

The first application of HPLC-ICP-MS in Se speciation (Table 2.1.) involved the investigation of selenomethionine incorporation into cyanobacterial metallothioneins (Takatera and Osaki 1994). This coupled technique is still extensively applied in Se speciation (mainly for quantitative analyses) due to its unmatched sensitivity and selectivity. However, in HPLC-ICP-MS the qualitative analysis of different Se species relies on their retention time matching with standards. This approach proved to be limited by the lack of available standards and also turned out to be quite unreliable in some cases. For instance, selenocystine used to be inaccurately quantified in biological matrices by the application of anion-exchange HPLC-ICP-MS (Pedrero and Madrid 2009). In this system $SeCys_2$ is not sufficiently retained and other co-eluting Se species are usually also quantified as $SeCys_2$ based on the retention time matching with the standard.

Table 2.1. The application of different coupled mass spectrometric techniques in Se speciation

Year	Technique	Matrix/target compounds	Reference
1988	GC-MS	SeMet from soybean protein	(Yasumoto et al. 1988)
1994	HPLC-ICP-MS	Cyanobacterial metallothioneins	(Takatera and Osaki 1994)
1995	CE-ICP-MS	Inorganic Se species	(Liu et al. 1995)
1998	GC-MS	Volatile and inorganic Se species of sediments	(Gómez-Ariza et al. 1998)
2000	HPLC-ESI-MS	Se-enriched and natural plant samples and yeast	(Kotrebai et al. 2000)
2002	GC-ICP-MS	Volatile Se species of plants	(Meija et al. 2002)
2002	Offline ESI-Q-TOF-MS	Selenized yeast	(McSheehy et al. 2002b)
2003	Low ion strength SEC-ICP-MS and offline MALDI-TOF-MS and ESI-Q-TOF-MS	Selenized yeast	(Encinar et al. 2003)
2006	nanoHPLC-ICP-MS and nanoHPLC-ESI-MS	Selenized yeast	(Giusti et al. 2006)
2008	HPLC-ESI-Orbitrap-MS^n	Yeast-based food supplements	(Dernovics and Lobinski 2008)
2013	2D-PAGE LA-ICP-MS and parallel capHPLC-ICP-MS and ESI-trap/Orbitrap-MS	Wheat proteins	(Bianga et al. 2013)

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Nowadays, Se speciation studies predominantly focus on qualitative analysis, thus the application of HPLC-ICP-MS and LA-ICP-MS became a complementary tool for the detection of Se compounds before their qualitative identification by molecular mass spectrometric techniques (Bianga et al. 2013, Cheajesadagul et al. 2014). The introduction of different coupled molecular mass spectrometric techniques to Se speciation is summarized in Table 2.1. The first application of HPLC-ESI-MS for selenium speciation was reported by Kotrebai and co-workers (2000). Nonetheless, for a few years molecular mass spectrometry remained to be an offline tool for the identification of purified Se species (McSheehy et al. 2002b, Encinar et al. 2003) then finally became the ultimate tool of qualitative analysis in Se speciation (Giusti et al. 2006, Dernovics and Lobinski 2008).

2.2. Selenium speciation in plants

Selenium is not classified as an essential element for plants. Plants normally do not possess any selenium specific metabolic pathways, but they take up and metabolize selenium as the analogue of sulfur, based on the chemical similarities of the two elements. In general, the ratio of bioavailable Se and sulfur in the soil determines the amount of Se metabolized by plants. Therefore, the Se content of edible plants also depends on this ratio. For instance, North American soils are relatively rich in Se (Gustavsson et al. 2001) and cereals absorb and store a reasonable amount of it. For this reason, in the United States wheat is recognized as a good source of dietary Se (Alexander et al. 1983) and the adequate intake (AI) of Se can be easily gained through normal diet. This is not the case in Europe (Lintschinger et al. 2000, Rayman et al. 2008), where the Se content of soils is considerably lower and Se supplementation is advisable in order to reach the AI (Adams et al. 2002, Broadley et al. 2006). To demonstrate the consequence of this difference, it is worth mentioning, that according to the British Nutrition Foundation, the decline of total Se intake in the UK in the last decades can be associated with the trend of importing wheat from Europe instead of North America (Internet 4, Rayman 2000).

Although Se is not considered to be essential for plants, moderate soil selenium content is proved to be beneficial for them (Kaur et al. 2014). It was shown in Finland, that Se supplementation of soil (by the fortification of fertilizers with Se) was not only a successful way of increasing dietary Se intake, but also increased cereal yields (Hartikainen 2005, Alfthan et al. 2014). Selenium was found to promote the growth of lettuce seedlings (Xue et al. 2001) and to increase the tolerance of plants against abiotic stresses such as drought, high temperature, high

UV-B radiation, salinity and heavy metals (Xue and Hartikainen 2000, He et al. 2004, Djanaguiraman et al. 2010, Hasanuzzaman et al. 2013). The exact molecular mechanisms responsible for this protective characteristic remain to be elucidated (Hasanuzzaman et al. 2010, Feng et al. 2013).

Indeed, these kinds of abiotic stresses generate oxidative damage and lipid peroxidation that is presumably compensated by the antioxidant effect of Se (Xue and Hartikainen 2000, Djanaguiraman et al. 2005). For instance, Hartikainen and coworkers showed that moderate level of Se (up to 1 mg kg^{-1} soil) increased glutathione-peroxidase activity in ryegrass, but also concluded that the dose-effect relationship of Se in plants resembles the pattern observed in animals: low amount Se acts as an antioxidant but becomes pro-oxidant in elevated quantities (2000). Certainly, increased soil Se levels create such an oxidative stress that most plants cannot tolerate (Iskandar and Kirkham 2001).

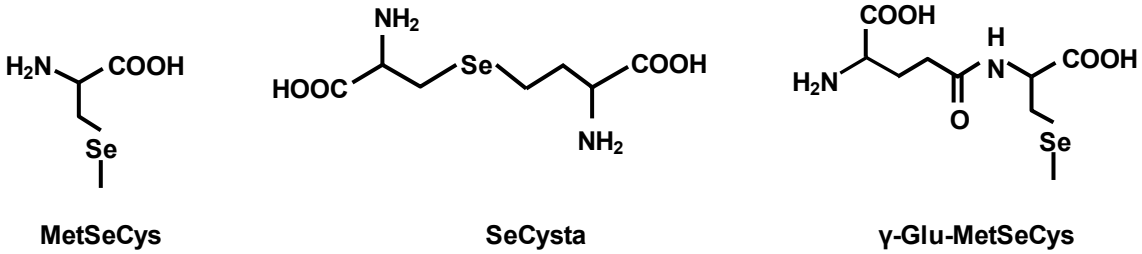
Some plant species, however, adapted to high Se levels and they are not only able to survive on selenium-rich soils, but can selectively absorb and accumulate Se in their shoots. The storage of Se in strategic plant tissues provides defense against pathogens and herbivores, furthermore facilitates reproduction of the plants (Freeman et al. 2006). Regarding the level of Se accumulation, plants can be classified as non-accumulator (10-100 μg Se g^{-1} DW), secondary/facultative accumulator (100-1000 μg Se g^{-1} DW) and hyperaccumulator (or primary accumulator) species (1000-15,000 μg Se g^{-1} DW) (Terry et al. 2000).

2.2.1. The fate of selenium in non-accumulator plants

Non-accumulator plants represent the majority of plant species. They assimilate and metabolize selenium through sulfur-specific metabolic pathways that results in the non-specific incorporation of Se. From the speciation point of view non-accumulator plants are characterized by their relatively high inorganic Se content and the prevalence of Se-analogues of sulfur metabolites (Terry et al. 2000, Whanger 2002). The more developed the sulfur metabolism of the plant is, the more Se it will contain (Leustek 2002). Plants from the *Allium* and *Brassica* genera like garlic, green onion, radish, broccoli or different kinds of cabbages contain a variety of selenium species depending on the Se content of the soil. These characteristics made them the ideal targets for biofortification in order to provide dietary Se from natural sources (Ogra et al. 2005, 2007, Larsen et al. 2006, Kápolna and Fodor 2007, Cheajesadagul et al. 2014).

The non-specific incorporation of Se into selenoamino acids also occurs in non-accumulator plants. Considering the toxicity of selenium, its storage in the form of protein-bound

selenomethionine is considered to be harmless. For instance, selenomethionine (SeMet) is one of the major Se species found in selenium-enriched cereals (Stadlober et al. 2001, Cubadda et al. 2010, Cheajesadagul et al. 2014). On the other hand, selenocysteine (SeCys or SeC) has a much more toxic effect through its non-selective incorporation into proteins. The most possible explanation for this characteristic is that the crucial roles of cysteine in protein folding and the activity of enzymes are hindered by the replacement of cysteine by SeC (Goldenberg 1992, Dimkovikj et al. 2015). Therefore, the transformation of SeC into non-protein amino acids (Figure 2.5.) such as *Se*-methyl-selenocysteine (MeSeCys), selenocystathionine (SeCysta) and γ-glutamyl-MeSeCys (γ-Glu-MeSeCys) is a key mechanism in Se tolerance (Brown and Shrift 1981).



2.5. The structure of some non-protein seleno-amino acids.

Although protein misfolding seems to be an obvious consequence of SeC incorporation, the exact biochemical mechanism of toxicity of this process was confirmed only nowadays. Van Hoewyk and coworkers successfully proved that the incorporation of SeC into proteins results in malformed proteins that are eliminated by the ubiquitine-proteasome system (Sabbagh and Van Hoewyk 2012). They have also suggested that if SeC concentration reaches a toxic level (Dimkovikj et al. 2015), the impairment of ubiquitine-proteasome system can potentially lead to cellular disregulation and cell death because of the aggregates formed by ubiquitinated misfolded SeC-containing proteins (Bence et al. 2001).

2.2.2. Adaptation to high selenium levels

Some plant species were found to contain surprisingly high concentration of Se (Beath et al. 1934, Virupaksha and Shrift 1965). These plants are called Se-hyperaccumulators (for the analogy of other metal hyperaccumulator species) because they are able to accumulate two orders of magnitude higher concentration of Se in their shoots compared to other plants, growing on the same site (Baker and Brooks 1989). While most plants contain less than 25 μg Se g^{-1} dry

weight (DW), Se hyperaccumulators can incorporate up to 1000-15,000 μg Se g^{-1} DW into their shoots (Terry et al. 2000).

These plants are also called indicator plants, since they preferably grow on Se-rich soils (Beath et al. 1934). They normally assimilate such a high concentration of Se that results in toxicosis when ingested by animals. One of the most famous stories describing Se intoxication of animals is found in the book "Description of the World" written by Marco Polo. He reported that his horses lost hair and hooves because they fed on poisonous grass at the highlands of Tibet. These "grass" were later identified to be the members of the *Astragalus* genus (Böck 2007). This type of toxicosis was later named as "alkali disease" (Draize and Beath 1935).

Indeed, hyperaccumulator plants obtained the following important selective advantages:

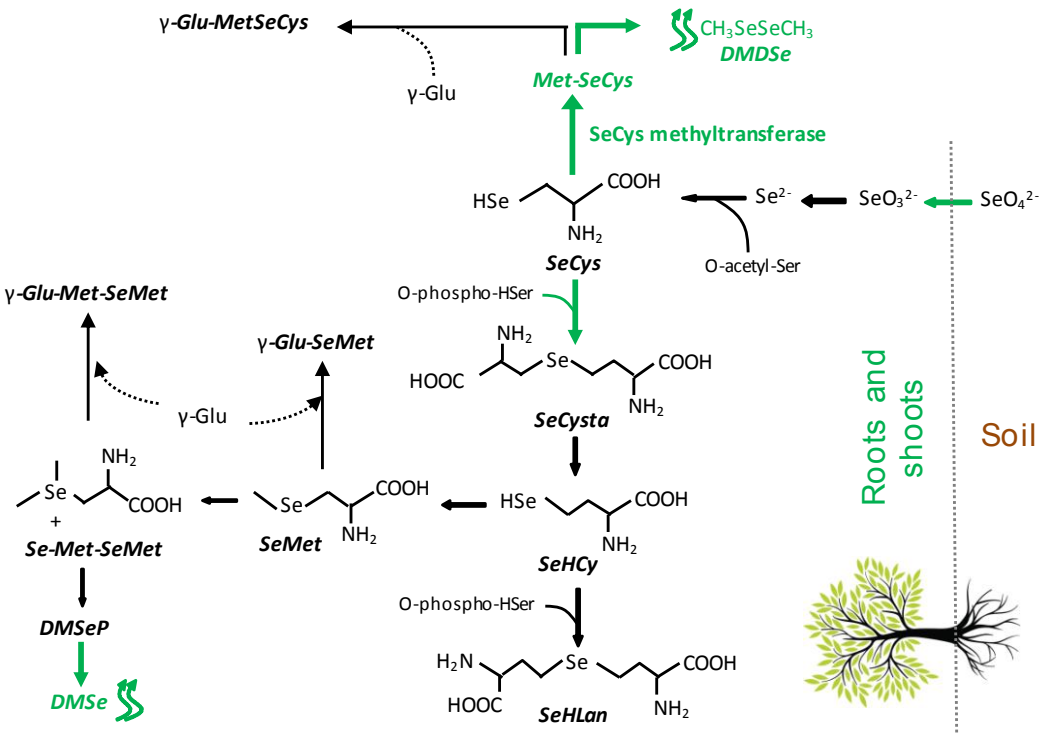
- by tolerating high soil selenium levels they became able to survive on seleniferous soils and
- by utilizing Se in so-called elemental defense mechanisms they are able to defend themselves against herbivory and pathogens (Hanson et al. 2004, Freeman et al. 2007).

Selenium was thought to be essential for Se-accumulator plants because they show reduced growth in its absence (Trelease and Trelease 1938). Despite of this fact, until now, selenium could not be proved to be essential for any plants (Birringer et al. 2002, Sors et al. 2005b, Feng et al. 2013). Its beneficial effects are more likely to play an important role in the long-term survival of plants due to its role in defense mechanisms (Freeman et al. 2006).

Speciation studies of Se accumulator plants contributed to the understanding of mechanisms of Se uptake, metabolism and adaptation. The application of both classical biochemical and molecular biological tools (such as genetic transformation) played an important role in the exploration of Se metabolic pathways in plants (Van Hoewyk et al. 2008, Zhu et al. 2009). For example, the sulfate transporter genes that are responsible for the uptake of sulfur (and Se) were identified by selecting selenium-resistant *Arabidopsis thaliana* mutants (Shibagaki et al. 2002). However, the relative difficulty of the identification of minor selenium species still limits the complete elucidation of these pathways. Figure 2.6. shows a currently recognized outline of Se metabolism in plants (Ogra and Anan 2009). The most relevant, rate limiting steps of Se tolerance are highlighted. Some molecular mechanisms underlying Se tolerance in accumulator plants concerning the whole plant metabolic system were found to include:

- an increased rate of detoxification of Se by volatilization (mostly in the form of dimethyl-selenide, dimethyl-diselenide and dimethyl-selenoniopropionate) (De Souza et al. 1998, Meija et al. 2002, Kubachka et al. 2007) and
- increased antioxidant capacity that protects the plant against the pro-oxidant characteristics of Se (Freeman et al. 2010).

The first step of Se accumulation is indeed, the uptake of Se from the soil. Selenium is absorbed through sulfate transporters that differ in their selectivity for selenate and sulfate (Sors et al. 2005a). Accumulator plants contain so-called "high-affinity sulfate transporters" that are responsible for the selective accumulation of Se over S (Parker et al. 1992, White et al. 2007). In addition, Se assimilation is up-regulated in Se accumulator plants by elevated concentrations of defense-related phytohormones (Tamaoki et al. 2008). The uptake of Se is further facilitated because the roots of hyperaccumulator plants preferentially grow toward Se enriched soils as a result of an elemental chemotaxis (Goodson et al. 2003).



List of abbreviations	
DMDSe	dimethyl-diselenide
DMSe	dimethyl-selenide
DMSeP	dimethyl-seleno-phosphate
MetSeCys	methyl-selenocysteine
O-acetyl-Ser	O-acetyl-serine
O-phospho-Hser	O-phospho-homoserine
SeCys	selenocysteine
SeCysta	selenocystathionine
SeHCy	selenohomocysteine
SeHLan	selenohomolanthionine
SeMet	selenomethionine
Se-Met-SeMet	seleno-methyl-selenomethionine
γ-Glu	γ-glutamyl group
γ-Glu-MetSeCys	γ-glutamyl-methyl-selenocysteine
γ-Glu-Met-SeMet	γ-glutamyl-methyl-selenomethionine
γ-Glu-SeMet	γ-glutamyl-selenomethionine

Figure 2.6. Selenium metabolic pathways in plants (reproduced from Ogra and Anan 2009).

After absorption, first rate limiting step of Se metabolism is the conversion of selenate (SeO_4^{2-}) into selenite (SeO_3^{2-}) (Li et al. 2008). Selenite is then converted into SeCys and other organic selenium species through hydrogen-selenide (H_2Se or Se^{2-}). The toxic effects of SeCys in accumulator plants are avoided by its effective conversion into other non-toxic Se species. The pivotal function of SeCys methyltransferase enzyme was demonstrated by several studies (Neuhierl and Böck 1996, Ellis et al. 2004, LeDuc et al. 2006). These findings were also confirmed by the investigations of Sors and coworkers, who found that the level of the expression of SeCys methyltransferase enzyme correlates well with the level of Se accumulation in *Astragalus* species (2005a).

The formation of non-toxic metabolites of a toxic element is a very effective but not the only possible way of detoxification. In the case of other metal hyperaccumulators (Ni and Cd) it was found that detoxification by metal ligands, such as metallothioneins and phytochelatins play an important role in their metal tolerance (Callahan et al. 2006). However, these kinds of metal ligands have not been observed in Se-hyperaccumulators. The possible explanation for this fact is the oxidation state (it forms inorganic anions) and redox behavior of Se that favors the catalysis of the formation of disulfide bonds from the thiol groups of metallothioneins (by oxidation) instead of forming metal complexes with them through reduction (Chen and Maret 2001).

2.2.3. Selenium speciation in selenium hyperaccumulator plants

The well-known representatives of Se accumulator plants are mostly the members of the *Brassicaceae* family like *Astragalus bisulcatus* (two-grooved poison vetch), *Stanleya pinnata* (desert princeplume) or *Brassica nigra* (black mustard). The potential of these plants to be utilized in phytoremediation or as functional foods stimulated research interest in the elucidation of their Se metabolism (Montes-Bayón et al. 2002, Zhu et al. 2009, Barillas et al. 2011). In our study we chose to investigate the Se speciation of the secondary accumulator Brazil nut (*Bertholletia excelsa*) and its close taxonomical relative, the Se-hyperaccumulator monkeypot nut (*Lecythis minor*). The latter is a rather unique Se-hyperaccumulator that has received more attention since its seed proved to be suitable to be utilized as feed supplement.

The previous chapter revealed that basically the whole metabolic system of selenium hyperaccumulator plants is altered in order to tolerate such high amounts of Se (up to 1% of plant dry weight). It is important to note that there are two interrelated mechanisms: accumulation and detoxification. On the one hand, high amount of Se is accumulated by the

plant in order to be utilized in elemental defense mechanisms, but on the other hand without detoxification it could easily become toxic for the plant itself. These two mechanisms determine the characteristic speciation pattern and the spatial distribution of Se in hyperaccumulator plants.

With respect to their speciation, it was shown that they incorporate Se mainly in the organic form. Protein-bound SeMet may account for the majority of the total selenium content of hyperaccumulator plants. On the other hand, the non-specific incorporation of SeCys is avoided by its effective methylation and transformation into different non-protein amino acid derivatives. Therefore, the main non-protein bound organic Se compounds found in higher plants are methylated Se species like MetSeCys and non-protein amino acid derivatives like SeCysta and γ-Glu-MetSeCys (Yathavakilla et al. 2005).

The relative amount of these organic forms highly depends on the plant species, the accumulating tissues and the availability of Se in soil. To demonstrate the differences, let us see an example from the *Brassicaceae* family. The trichomes (leaf hair) of *Astragalus bisulcatus* (that are the main depositories of sequestered Se in this plant) contain only two organic Se species: MeSeCys (53%) and γ-Glu-MeSeCys (47%). The rest of the leaves contain around 30% of inorganic selenium (20% of selenate and 10% of selenite) and 70% of MeSeCys (Freeman et al. 2006). Unfortunately, detailed Se speciation data of Se-accumulator species are still missing because most plant physiological studies focus only on the determination of the most abundant Se species. Since the insight into the complete Se speciation of different plant species would also offer the opportunity to uncover the molecular mechanisms responsible for the differences, it is increasingly important to develop adequate analytical techniques for this purpose (Ouerdane et al. 2013, Rigby et al. 2014).

Considering the spatial distribution of Se in plants, non-accumulator and accumulator species are remarkably different from each other. It was shown by XANES that in non-accumulators selenium metabolites are normally co-localized with their sulfur analogues, proving that these plants cannot distinguish between the two elements. On the other hand, in Se hyperaccumulator plants Se was found to be concentrated in some specific parts of the plant. It is predominantly stored in strategic plant tissues such as flowers, seeds and the edges and tip of young leaves (Freeman et al. 2006, Prins et al. 2011, Quinn et al. 2011). This phenomenon is typical to other metal hyperaccumulator plants too (Boyd 2007), because it provides the most effective defense against pathogens and herbivores and facilitates the reproduction of the plant (Quinn et al. 2010). For example, it was shown that young leaves contain more Se than mature ones, probably because they are more likely to be attacked by herbivores. Additionally, Se is predominantly stored in the seeds during the reproductive stage of growth (Ferri et al. 2004) that

provides a direct protection for them. For example monkeypot nuts cannot be directly consumed because they contain such a high amount of Se that they can cause acute toxicity.

2.3. Selenoproteomics

Selenoproteomics covers the investigation of two types of proteins: selenoproteins and Se-containing proteins. The incorporation of SeC into selenoproteins is genetically encoded, while Se-containing proteins are formed by the non-specific substitution of methionine by SeMet. (Note, that the methionines in selenoproteins can also be replaced by SeMet, resulting in the intersection of the two groups.) Selenoproteins can be found in all the three domains of life (eukarya, archaea, and eubacteria) that indicates their ancient origin. However, the number of selenoproteins varies considerably between different species. For instance, the highest number of selenoproteins were found in aquatic animals, while they are completely absent in some insects, fungi and higher plants. The lack of selenoproteins and the SeC insertion sequence (SECIS) in these organisms is probably the consequence of a loss of a physiological function during their adaptation to terrestrial habitats (Lobanov et al. 2007).

The important role of selenoproteins in human and animal health is the main reason for the essentiality of Se. Table 2.2. shows the classification of human selenoproteins and their main functions (Papp et al. 2007). Most of them are involved in redox-reactions owing to the unique chemical properties of SeC (in comparison, the sulfur analogues of these proteins usually exhibit significantly lower enzyme activity (Kim et al. 2006)). Although selenoproteins are normally regarded as antioxidant enzymes, their specific functions can also involve hormone activation and inactivation, redox signalling, Se transport or repair of oxidatively damaged proteins (Labunskyy et al. 2014).

The first selenoprotein identified was the mammalian glutathione peroxidise 1 (GPx1) (Flohe et al. 1973, Rotruck et al. 1973). Provided that glutathione-peroxidase activity showed a strong correlation with erythrocyte Se concentration, it became the first biomarker for the evaluation of Se status (Hafeman et al. 1974). It is the most abundant cytosolic selenoenzyme that plays an important role in the maintenance of redox homeostasis by catalysing the glutathione-dependent reduction of hydrogen-peroxide (H_2O_2).

However, recent studies also showed that the overexpression of GPx1 can also have some negative effects. For example, it can become pro-oxidative due to its high catalytic activity. Moreover, by the reduction of H_2O_2, GPx1 can also disrupt its signaling activity (D'Autréaux and Toledano 2007). It can have serious consequences, since H_2O_2 signaling has a relevant role

in the regulation of insulin secretion, apoptosis and even cell proliferation (Mukherjee et al. 1978, McClung et al. 2004). These processes are still not fully understood, but they might be responsible for the inconsistent results of Se chemoprevention trials (Lippman et al. 2009, Platz and Lippman 2009) and the correlation between increased Se level and the incidence of type 2 diabetes (Hatfield et al. 2011).

Table 2.2. Classification of the human selenoproteome (reproduced after Papp et al. 2010). In the abbreviations "Sel" denotes to "selenoprotein".

Antioxidant enzymes	GPx1	GPx1 is the most abundant cytosolic selenoenzyme that plays an important role in the maintenance of redox homeostasis by catalysing the glutathione-dependent reduction of hydrogen-peroxide into water. GPx4 can directly reduce phospholipid- and cholesterol-hydroperoxides (Imai and Nakagawa 2003). It contributes to male fertility. Interestingly, GPx6 is a selenoenzyme in humans but not in rodents (Dear et al. 1991).
	GPx2	
	GPx3	
	GPx4	
	GPx6	
	SelK	
	SelR	
	SelW	
Redox signalling	TrxR1	Thioredoxine reductases (TrxRs) catalyse the NADPH-dependent reduction of thioredoxin 1 (Trx1). This reaction is the basis of the disulfide reduction system of the cell, since Trx1 is the major protein disulfide reductase with various physiological functions (Arnér and Holmgren 2000).
	TrxR2	
	TrxR3	
Thyroid hormone metabolism	DI1	The iodothyronin deiodinase (DI) family is involved in the regulation of thyroid hormone activity. They have a complex function in maintaining adequate levels of thyroid hormones: DI1 and DI2 catalyse the deiodination of thyroxin into its active from (3,3',5-triiodothyronine), but they also assist in its degradation.
	DI2	
	DI3	
Se synthesis	SPS2	Selenophosphate synthetase 2 has a basic role in selenoprotein synthesis. It is responsible for the synthesis of selenophosphate that is an active form of Se used in SeC biosynthesis (Xu et al. 2007).
Transport and storage of Se	SelP	Selenoprotein P accounts for more than 50 % of total Se in the plasma. It is an especially Se-rich protein (containing more than 10 SeC residues) that transports Se to peripheral organs, such as brain, testes and bones (Read et al. 1990, Pietschmann et al. 2014).
Protein folding (potential)	Sep15	Sep15 is present in the brain, lung, testis, liver, thyroid, and kidney (Kumaraswamy et al. 2000). Its exact function is still unknown but it was shown to have a potential role in protein folding (Korotkov et al. 2001).
	SelN	
	SelM	
	SelS	
Unknown function	SelH	
	SelI	
	SelO	
	SelT	
	SelV	

2.3.1. The fundamentals of proteomics

Proteomics is one of the so called "OMICS" sciences that cover a wide range of bioanalytical investigations. Genomics is the well-established discipline of DNA sequence analysis. It is based on the investigation of pure genetic information, while transcriptomics analyze RNAs that are responsible for the specific expression of this information. Undoubtedly, these types of analyses are fundamental, but the investigation of proteins and metabolites that are actually present in the organism offer a better insight into biological systems (Cox and Mann 2011). Indeed, proteomics and metabolomics provide more direct biological information that can be effectively utilized in biochemical and biotechnological research.

Despite of the advantages of proteomic analyses from the biological point of view, their ability to provide reliable and valuable data (in the analytical sense) is seriously influenced by the applied analytical and bioinformatic techniques. The recent development in the field of mass spectrometry provides the basis for the rapid development of proteomics (Han et al. 2008). Both the quality and the quantity of proteomics data increased dramatically by the spread of high throughput high resolution mass spectrometric techniques (Mann et al. 2013). On the other hand the continuous improvement and revision of proteomic databases and the refinement of data evaluation tools are crucial in order to achieve more reliable results (Mallick and Kuster 2010).

In general, proteomics covers the investigation of the protein fraction of any biological samples. There are different approaches that target the characterization of even (i) a complex mixture of proteins, (ii) a limited number of proteins with similar characteristics or (iii) a single protein. Principally, the number of proteins analyzed is inversely proportional to the extent and the accuracy of the analytical information that can be acquired.

Depending on the aim of the investigation there are three main types of proteomic studies (Figure 2.7.). *Top-down* proteomics enable the most complete characterization of a selected protein (Kelleher et al. 1999, McLafferty et al. 2007). It consists of the separation and purification of one targeted protein followed by a sophisticated mass spectrometric analysis. The selected protein is directly introduced to the mass spectrometer, where the intact protein ion is subjected to gas phase fragmentation. The combination of different fragmentation methods such as collision induced dissociation (CID), electron capture dissociation (ECD) and electron transfer dissociation (ETD) provides comprehensive data on the sequence of the protein as well as the post-translational modifications (Zubarev et al. 1998, Syka et al. 2004).

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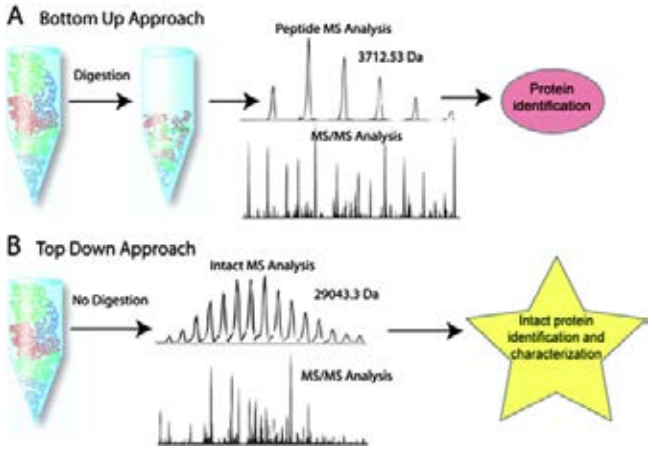

Figure 2.7. The main approaches in proteomics: bottom-up and top-down (Kellie et al. 2010).

In *bottom-up* proteomics the selected protein is subjected to some kind of enzymatic digestion (most frequently tryptic digestion) and the obtained peptide mixture is analyzed by mass spectrometry. The combination of top-down and bottom-up approaches by applying different mass spectrometric techniques for the characterization of the whole protein and the peptides gained through several enzymatic digestions can provide extensive analytical data (McLafferty et al. 2007). This kind of proteomic study is widely applied in the pharmaceutical industry because the complete characterization of pharmaceutical proteins and biomarkers is doubtlessly inevitable (Reinders 2013).

When the aim of a study is not the complete characterization of one or a few selected proteins but the fingerprinting of a complex mixture of proteins, the application of the *shotgun* proteomics approach is suitable (Pappin et al. 1993). It is actually a type of bottom-up proteomics, where a complex protein fraction is obtained through a simple sample preparation procedure and its tryptic digest is analyzed by high resolution mass spectrometry preceeded by a one or two dimensional HPLC separation (Figure 2.8).

The identification of proteins is based on the identification of the peptides derived from them. This shotgun method enables the identification of a higher number of proteins, usually with a relatively lower coverage compared to top-down approaches (Michalski et al. 2011). The peptide-based shotgun method is the far most widely used and the most generic proteomic approach (Cravatt et al. 2007, Cox and Mann 2011). Why is it so popular in spite of the relatively low proteome coverage that it can provide?

It would be expected that reducing the complexity of the analyzed protein fraction by additional purification and fractionation would substantially improve the quality of LC-MS data and facilitate the identification of proteins.

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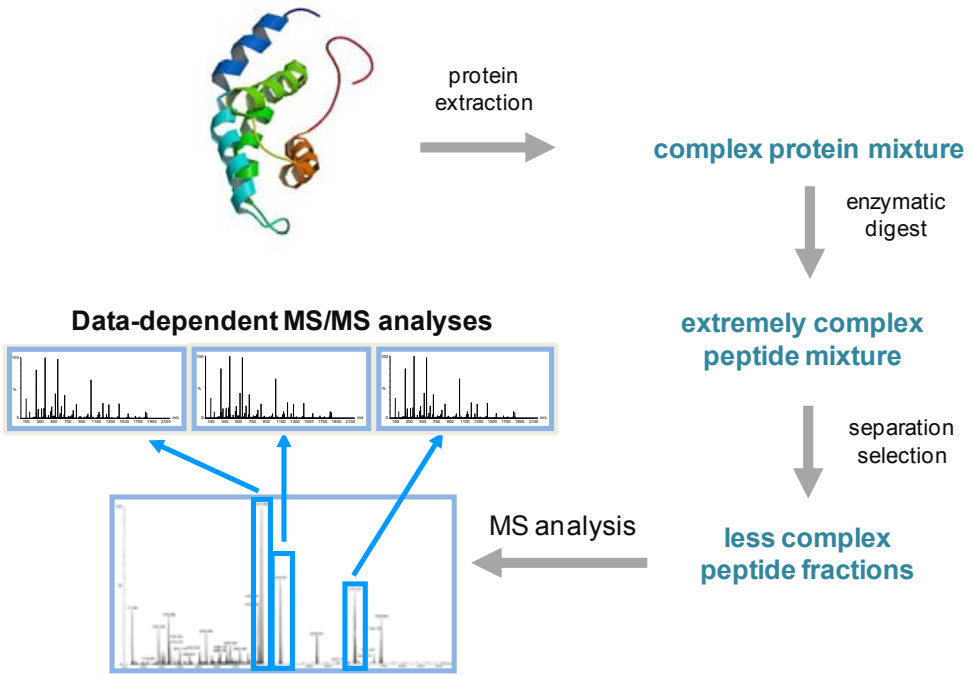

2.8. Experimental design of a shotgun proteomic analysis (adopted from the lecture of Lennart Martens at the Proteomics Bioinformatics Workshop at the Wellcome Trust Sanger Institute, Hinxton, 2013).

But this strategy proved to be impractical, because the extensive fractionation on the proteome level greatly increases the overall analysis time without providing any significant improvement in protein identification (Cox and Mann 2011, Michalski et al. 2011). The improvement of HPLC separation and MS detection seems to be a much more promising strategy (Makarov et al. 2006, Mann et al. 2013).

2.3.2. The application of bioinformatics in protein identification

Indeed, the identification of proteins is impossible without the elucidation of their amino acid sequence. The fragmentation of polypeptides in tandem mass spectrometers is a rather reproducible process that offers the opportunity of MS-based protein sequencing utilizing highly automated bioinformatic tools. There are three main fragmentation types: ECD, CID and ETD that act at different parts of the peptide bond, resulting in a/x, b/y or c/z fragment ions, respectively (Figure 2.9.).

The sequence of the protein can be determined from the MS/MS spectrum of the peptide, where the mass difference between two neighboring fragment ions (like b_2 and b_3) defines an

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amino acid (Senn et al. 1966, Dawkins et al. 1978). This procedure is called *de novo* sequencing and it is usually applied, if we do not possess any initial knowledge about the target protein. However, nowadays there is a lot of publicly accessible information in international databases that can also be utilized in protein identification.

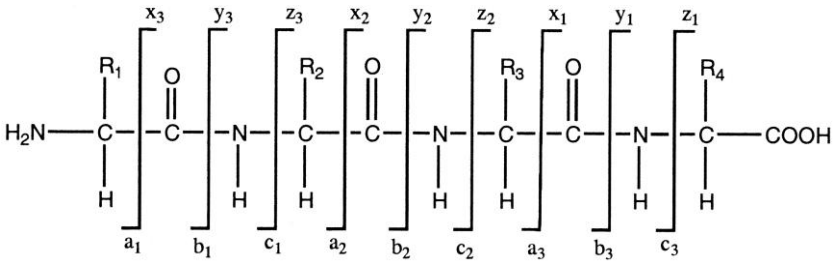

Figure 2.9.: Peptide fragmentation.

The accumulation of huge amount of biological data through large-scale genomic studies raised the need to develop appropriate computational tools to analyze them. Initially, most bioinformatic tools were designed to browse the publicly available data in international genomic repositories. In general, bioinformatics is the application of computer technology for the analysis of molecular biological information. Principally, bioinformatics is based on the utilization of public databases and enables to set our own results in a systems biology context. The recent development of various other large-scale biology methods (proteomics, metabolomics, nutrigenomics, *etc.*) resulted in the exponential expansion of biological data. The application of bioinformatic tools for data-mining became crucial in these research areas too.

The automated, database-dependent identification of proteins is based on the comparison of experimental data with the data derived from a protein database containing a known set of proteins. In practice, every protein identification process begins with the establishment of a specific protein database of possible proteins that can be expected in the particular organism or tissue. This database should be extracted from an international protein sequence database such as UniProt that is supervised and curated by the European Bioinformatics Institute (EBI). It is very important to keep our "working database" as specific and as simple as possible in order to facilitate data analysis and avoid generating abundant false positive results.

It was mentioned before that MS-based protein identification is usually based on the identification of peptides. Peptides are normally obtained by the tryptic digestion of proteins. Trypsin is a universally applied endopeptidase because it can very reproducibly and specifically cleave oligopeptides at the carboxyl side of arginine and lysine residues. Taking into account these characteristics of trypsin, the peptides obtained by the tryptic digestion of known proteins can be easily predicted by *in silico* digestion.

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Every peptide produces a characteristic MS/MS spectrum depending on its amino acid sequence. As a result of the most frequently applied ESI ionization and CID fragmentation, the MS/MS spectrum of peptides will consist of b and y fragment ions. This rule can also be applied for *in silico* peptides to predict their MS/MS spectrum. Finally, the identification of peptides is accomplished by comparing the experimental and the *in silico* generated spectra and assigning the most probable peptide from the database to each experimental spectrum (Figure 2.10.). Since these peptides are derived from known proteins, the outcome of the whole process will be a list of assigned proteins with varying coverage and confidence of identification.

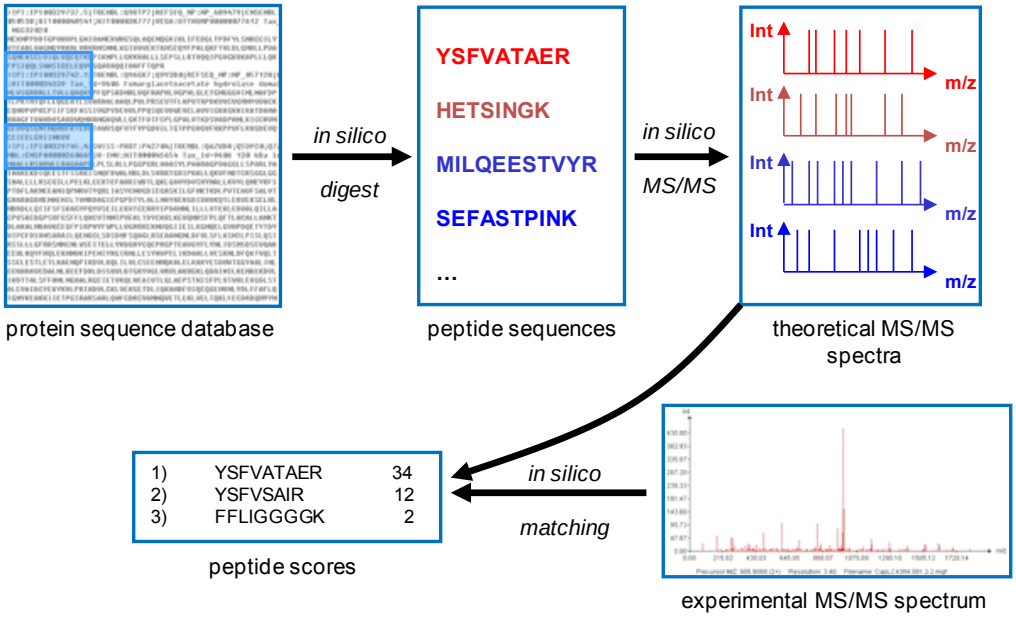

Figure 2.10. The workflow of database-dependent protein identification (adopted from the lecture of Lennart Martens at the Proteomics Bioinformatics Workshop at the Wellcome Trust Sanger Institute, Hinxton, 2013).

To tell the truth, the bioinformatic workflow is still not finished here. The identified proteins have to be validated. It is a crucial part of data analysis because *in silico* digestion and fragmentation cannot account for the actual variability of "real peptides" (Michalski et al. 2011). Therefore, the variability of experimental results and the unavoidably discrepancies in the process of assigning peptides to experimental spectra can significantly bias the results (Vaudel et al. 2014). The best way to propagate this problem is the manual check of each assigned peptides and exclude false positive results (Nilsson et al. 2010). Of course, working with huge datasets where thousands of proteins are identified in an experiment, does not allow the manual validation of every single peptide. We need to apply some statistical tools to increase the reliability of our results.

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The reliability of identification will depend on the number of peptides assigned for the particular protein, the number of identified amino acids compared to the total number of amino acids of the protein (coverage) and the specificity of the detected peptides. Theoretically, one specific peptide is enough to identify a protein with 100% confidence. In practice, it can mean that only a small fraction of the whole protein is actually found in the sample (*e.g.*, less than 5% coverage). Nevertheless, these protein identification results are usually validated, because statistical confidence is considered to be the most important validation parameter. I would like to point out here, that from the analytical point of view, coverage seems to be a more critical parameter and the possible ways of increasing it should be addressed in future studies (Mann et al. 2013).

2.3.3. Special challenges in selenoproteomics

The basic difference between classical proteomic studies and the investigation of the selenoproteome is the possibility to apply elemental screening procedures for the detection of Se-containing proteins and peptides. The ICP-MS based elemental screening can assist each step of protein analysis. The application of LA-ICP-MS is particularly useful for the mapping of 2D-PAGE gels. In the first step of a bottom-up analysis (which is designed to separate the targeted proteins based on some of their special properties), LA-ICP-MS can be used to detect the selenoprotein containing spots on the gel. Moreover, the complementary use of elemental and molecular mass spectrometry can also facilitate the detection of Se-containing proteins, peptides and their fragments in each type of proteomic analysis (Figure 2.11.).

Until quite recently, selenoproteomic investigations were mostly limited to the characterization of selenoproteins of different animal species. With regard to plants, most studies identify Se-containing proteins as “protein-bound selenomethionine”. There are only a few examples for the complete selenoproteomic characterization of plants.

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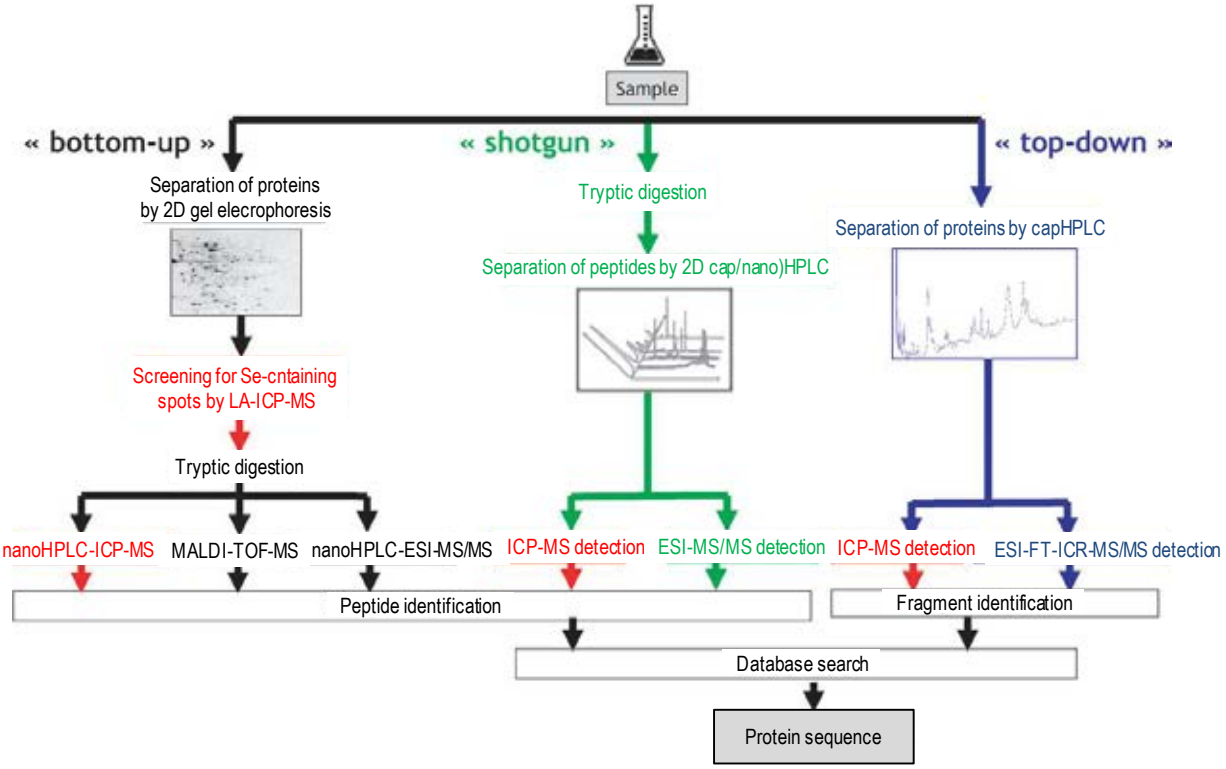

Figure 2.11. The application of ICP-MS detection for elemental screening in selenoproteomics (Mounicou et al. 2009b).

Actually, it is a rather challenging task, because the selenoproteomes of plants are very diverse due to the non-selective incorporation of SeMet. As a consequence of the heterogeneity and low abundance of Se-containing proteins of plant, both their separation and their detection by HPLC-ESI-MS are complicated. Fortunately, the application of elemental screening techniques can greatly contribute to the success of their investigation and offers a new platform for large-scale plant selenoproteomics (Bianga et al. 2013, Cheajesadagul et al. 2014).

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3. OBJECTIVES

Selenium speciation is a relatively long-established but still developing discipline that is utilized in various research areas, such as human and animal nutrition, chemoprevention and plant physiology. The assessment of the role of selenium in diverse biological systems would not be possible without the development of adequate analytical methods. Recent studies are based on the application of high resolution mass spectrometric methods that enable the large-scale identification of selenocompounds. Apart from the practical considerations of the identification of new metabolites, the need to set these results in a complex biological context led to the exponentially growing field of metallomics.

In my doctoral study I intended to carry out the comprehensive selenium speciation analysis of the seeds of Brazil nut (*Bertholletia excelsa*) and monkeypot nut (*Lecythis minor*). The main objectives of my work were the followings:

For the analysis of low molecular weight, water extractable selenium compounds of the seeds of *B. excelsa* and *L. minor* I intended*:*

- to design an adequate ICP-MS assisted multidimensional chromatographic separation procedure for the purification of selenium containing fractions
- to build a database of known selenometabolites to be utilized in the database-dependent identification of Se containing compounds of *B. excelsa* and *L. minor*
- to extend the database by previously unreported Se species (based on Se-S analogy)
- to identify new selenometabolites based on their accurate mass, chromatographic behavior and MS/MS fragmentation pattern
- to look for the sulfur analogues of detected selenocompounds
- to confirm the identification of the main new metabolites through synthesis
- to compare the characteristic selenium speciation pattern of the two plants.

For the proteomic analysis of *L. minor* I intended:

- to design a HPLC-ESI-Q-TOF-MS-based shotgun proteomic experiment
- to assign selenium containing peptides and their sulfur analogues

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- to obtain high-quality MS/MS spectra of the selected peptides
- to create a protein database for the database-dependent identification of proteins, carry out the identification and validate the results
- to carry out the *de novo* sequencing of unidentified selenopeptides.

My results will hopefully contribute to the:

- better understanding of Se metabolic pathways in selenium accumulator plants
- offer an interesting insight into the mechanisms contributing to Se tolerance of hyperaccumulator plants
- extend the current knowledge available on the proteomics of *Lecythidaceae*.

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4. EXPERIMENTAL

4.1. Reagents and samples

All reagents were of at least analytical grade (otherwise stated). For chromatographic purposes methanol (HPLC gradient grade), formic acid (98-100%) and acetonitrile (≥99.0%) were purchased from VWR International (Radnor, PA, USA). Iodoacetamide, dithiothreitol, proteomic grade trypsin, heptafluorobutyric acid (HFBA; ≥99.5%), tris(hydroxymethyl)-aminomethane (Tris, ≥99.8%), sodium selenide (CDS00983) and D,L-homocysteine were obtained from the Sigma-Aldrich group (Schnelldorf, Germany). Sodium iodide, glacial acetic acid, hydrogen chloride and hydrogen peroxide were obtained from Merck (Darmstadt, Germany), while acetone, cyclohexane (≥95%), pyridine (≥99%), ammonium acetate (≥98.0%), ammonium hydroxide and sodium acetate were obtained from Reanal (Budapest, Hungary). Milli-Q water (18.2 MΩ cm, Merck-Millipore, Molsheim, France) was used throughout. For the determination of total selenium and sulphur, HNO_3 (a.r., ≥65%), and the 1.000 g L^{-1} standards of Se, S and Rh were obtained from Merck.

A commercially available monkeypot nut (*Lecythis minor*) sample was analyzed. It was partially defatted and ground by the producer (Dr Winfried Behr, Bonn, Germany). Brazil nut (*Betholletia excelsa*) sample was purchased in bulk in Munich, Germany. The samples were cleaned, defatted and homogenized as described by Bodó and coworkers (2003).

4.2. Determination of total selenium and sulfur content

In this study, an Agilent 7500ce ICP-MS was used for the determination of total Se content of the samples and as a detector in the multidimensional chromatographic clean-up procedure. Figure 4.1. demonstrates the general structure of an ICP-MS instrument.

The *sample introduction system* is composed of a peristaltic pump and a nebulizer followed by a spray chamber. The role of the nebulizer is to create a fine aerosol from the sample that can be introduced into the ion source. The *plasma* is generated by passing argon through a series of concentric quartz tubes (ICP torch) that are wrapped at the end by the radio frequency (RF) coil. The temperature of the plasma is around 6000 K and it serves as an ion source. The ionization process and the operation of the *quadrupole mass analyzer* are detailed in chapter 2.1.2.

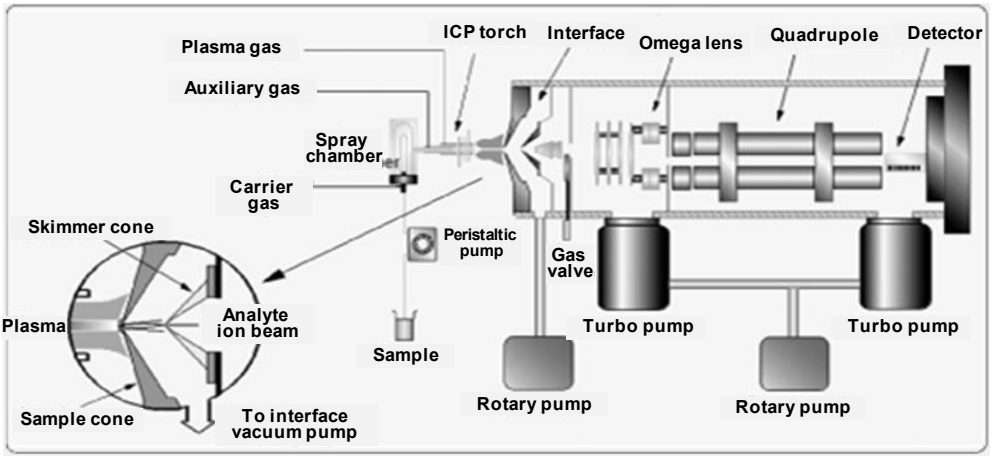

Figure 4.1. Schematic image of an ICP-MS instrument (Day et al. 2002).

The *detector* is basically an electron multiplier that counts individual ions exiting the quadrupole and generates a measurable electric signal. Accordingly, the intensity of selected ions is expressed in counts per second (cps). ICP-MS measurement conditions (nebulizer gas flow, RF power and lens voltage) were optimized daily for the highest intensity of the ^{89}Y signal.

For the determination of total selenium and sulfur content, the digestion of samples was carried out in a CEM Mars-5 microwave digestion system (CEM; Matthews, NC, USA). The sample (5 g) was mixed with 5.0 ml of concentrated HNO_3 in PTFE digestion tubes. The pressure was raised to 250 psi over 20 min and held for 20 min.

Total Se concentration was determined with an Agilent 7500ce ICP-MS on the ^{77}Se and ^{82}Se isotopes by the method of standard addition using rhodium (^{103}Rh) as an internal standard. The total S concentration was determined with a Perkin Elmer Optima 8000 ICP-OES instrument (Waltham, MA, USA) with external calibration.

The concentration of methionine and cysteine was determined according to EN ISO 6498:2012 and Commission Regulation (EC) No 152/2009, while SeCys and SeMet were quantified with HPLC-ICP-MS after proteolytic digestion according to the method by Bierla and coworkers (2008). Each quantitation experiments were carried out in triplicates.

4.3. Analysis of low molecular weight selenometabolites

4.3.1. Ultrasound assisted water extraction

The samples (0.5 g) were extracted with 10.0 mL deionised water with an ultrasonic probe (UP100H, Hielscher Ultrasound Technology, Teltow, Germany) at ambient temperature. The ultrasonic probe was applied three subsequent times for 0.5 minutes in each step. The extract was

centrifuged at 4000 *g* for 15 min. The supernatant was lyophilized and stored at -18 °C until analysis. The extraction recoveries were determined by comparing the total Se content of the original samples and the water extracts.

4.3.2. Size-exclusion chromatography

Lyophilized water extracts were dissolved in 1.2 mL 0.01 M ammonium acetate (pH 7.5; adjusted with NH_4OH). The solutions were centrifuged at 10,000 *g* for 10 min. The supernatants were filtered through 0.45 µm PTFE disposable syringe filters then fractionated by size-exclusion chromatography (SEC). Sample aliquots were injected onto a preparative scale SEC column (HiLoad 16/60 Superdex 30 pg; Amersham Biosciences, Uppsala, Sweden).

The elution was carried out with 0.01 M ammonium acetate (pH 7.5) at 0.8 mL min^{-1}. The eluate was collected every 1 min for 3 hours. Selenium (^{77}Se, ^{82}Se) and sulfur were determined selectively in each fraction by ICP-MS. The SEC–ICP-MS ^{82}Se and ^{34}S profiles were then established. Fractions selected for further analysis were pooled, frozen and lyophilized.

4.3.3. HPLC-ICP-MS experiments

The normal bore HPLC–ICP-MS coupling was achieved by using an Agilent 1200 HPLC system (Agilent Technologies) connected to an Agilent 7500ce ICP-MS for element-specific detection of ^{34}S, ^{77}Se, ^{82}Se and ^{88}Sr.

The chromatographic parameters are summarized in Table 4.1.

Table 4.1. Chromatographic parameters applied in the multidimensional sample clean-up procedure.

	Column	Buffer A	Buffer B	Flow rate (mL min^{-1})	Gradient program	
					Time (min)	Composition
SCX	Zorbax 300 SCX 4.6 x 150 mm x 5 µm (Agilent)	1 mM pyridine formate in H_2O	40 mM pyridine formate in H_2O (pH 2.75)	1.0	0-4 35 40 60	0% B 40% B 100% B 100% B
IP-RP	XTerra MS C18 4.6 x 150 mm x 5 µm (Waters)	5 v/v % methanol and 0.1 v/v % HFBA in H_2O	0.1 v/v % HFBA in methanol	1.0	0-5 15 20 21 27	0% B 50% B 50% B 100% B 100% B
RP	XDB C18 2.1 x 50 mm x 3.5 µm (Agilent)	0.1% formic acid in H_2O	acetonitrile with 0.1% formic acid	0.35	0-5 15 20 25 32 37	5% B 50% B 100% B 100% B 5% B 5% B

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For cation-exchange chromatography (SCX), the sample was dissolved in 1 mL of 1 mM pyridine formate (buffer A). The injection volumes were individually optimized for the fraction collection depending on the Se concentration of the sample. The selenium containing fractions were collected, frozen and lyophilized.

For the ion-pairing reversed-phase chromatography (IP-RP) the sample was dissolved in 500 µL of water that contained 5 v/v % methanol and 0.1 v/v % heptafluorobutyric acid (eluent A). The instrument was operated in oxygen mode with the addition of 5 % oxygen to the nebulizer gas flow (and utilizing platinum sample cones) to compensate for the relatively high ratio of organic solvent in the eluent. Injection volumes and fraction collection were identical as for the cation-exchange purification. The fractions of interest were pooled, frozen and lyophilized.

4.3.4. Reversed-phase chromatography coupled to ESI-Q-TOF-MS

For the HPLC–ESI-MS experiments, a 6530 Accurate Mass ESI-Q-TOF LC-MS system (Agilent Technologies) was applied. The ESI-Q-TOF-MS instrument was operated with an Agilent 6220 instrument derived dual ESI ion source in positive and negative ionization modes. ESI-Q-TOF-MS-MS experiments were carried out in the range m/z 50–800. MS-MS experiments were accomplished with collision energies individually optimized for each Se containing compounds.

Table 4.2. Operating parameters of the ESI-Q-TOF instrument.

ESI source	Dual ESI (Agilent)
Precursor ion selection in MS/MS mode	medium (4 m/z)
Mass accuracy	< 2 ppm
Mass resolution	> 10000
Detection frequency	4 GHz
Fragmentor voltage	150 V/-150 V
Curtain voltage	65 V/ -65 V
Drying gas	13 L/min
Capillary voltage	800 V
Nebulizer pressure	40 psig
Gas temperature	325 ºC
Data analysis software	Mass Hunter Acquisition B.02.01 with SP3
	Mass Hunter Qualitative Analysis B.03.01 with SP3

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The mass accuracy of the instrument was calibrated with Agilent Technologies ESI-L low concentration tuning mix before the experiments. The MS settings are described in Table 4.2. The samples were dissolved in 200 µL of water before injecting to the column. The injection volume was 20 µL for the MS-MS experiments. HPLC parameters are listed in Table 4.1.

4.4. Synthesis of organic polychalcogenides

For the synthesis of polychalcogenides 10 mg of D,L-homocysteine was dissolved in 10 mL 0.2 M sodium acetate buffer (pH 5.5). Before the addition of 5 mg sodium selenide (Na_2Se) to the solution, 2 mg of sodium iodide (NaI) was also added in order to act as a catalyst in the mild oxidation of thiols into polychalcogenides. Then 20 µl of hydrogen peroxide (H_2O_2) was added in order to ensure oxidative conditions. The resulting mixed Se-S polychalcogenides containing -S-Se-S-, -S-Se-Se-S- and -S-Se-Se-Se-S- chains were analyzed by HPLC-ESI-Q-TOF-MS-MS experiments.

4.5. Proteomic analysis

4.5.1. Separation of the protein fraction

The partially defatted and ground *Lecythis minor* sample was defatted by Soxhlet extraction with cyclohexane then the defatted sample (0.2 g) was extracted with 2 mL deionized water with an ultrasonic probe (UP100H, Hielscher Ultrasound Technology) three times for 0.5 min. The extract was centrifuged at 4000 *g* for 15 min.

Ice cold acetone (7 mL) was added to the supernatant to precipitate the protein fraction. The solution was vortexed then kept at -18 °C for 1 hour. Then the solution was centrifuged at 10,000 *g* for 10 min then the supernatant was discarded. This process was repeated for three times. Before the tryptic digestion of the protein fraction, the sample was kept at 37 °C for 2 hours in order to evaporate acetone residues.

4.5.2. Tryptic digestion

A subsample of the isolated crude protein extract (3.5 mg) was dissolved in 100 µL of Tris buffer (100 mM, pH 7.8). The intramolecular disulfide bonds of the proteins were released by the addition of 5 µL of 200 mM dithiothreitol (DTT). This reaction was carried out for 1 hour at

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room temperature. The cysteine residues of the proteins were derivatized by the addition of 20 µL of 200 mM iodoacetamide (IAA). The reaction was carried out for 1 hour at room temperature in dark. Then 20 µL of DTT was added to react with the excess of IAA (1 hour, room temperature). For the enzymatic digestion 100 µL of trypsin solution (0.6 mg mL^{-1} in Tris buffer) was added to the derivatized proteins. After incubation at 37 °C for 16 hours, the reaction was stopped by the addition of 8 µL glacial acetic acid (to decrease the pH under 4).

4.5.3. Sample purification and mass spectrometric analysis

Before the analysis by the HPLC-MS system, the peptides were separated from salts and other matrix components by the application of a C_{18} SPE resin (Zip tips, Merck-Millipore). The resin was first wetted with 10 µL ACN then conditioned with water then the sample was aspired ten times in order to bind the targeted peptides to the resin. The salts and other highly polar compounds were washed away with water. Finally, the targeted compounds were eluted with 10 µL 0.1 % formic acid in 50 % ACN. Before injecting to the HPLC column, 30 µL of water with 0.1 % formic acid was added to the sample. In order to characterize a wider range of peptides, a second SPE separation was also carried out where 70 % ACN was applied for the elution of the sample from the resin. This procedure allowed the analysis of higher molecular weight (more apolar) peptides.

The ESI-Q-TOF-MS experiments were carried out in the range m/z 50–1100 that was suitable for the detection of multiply charged peptides (z = 2-3). While MS-MS experiments were accomplished in the range m/z 100-3500, that also allowed the detection of singly charged parent and fragment ions. The collision energies were automatically optimized for each peptide. The chromatographic parameters are described in Table 4.3. The injection volume was 10 µL for the MS-MS experiments.

Table 4.3. Chromatographic parameters applied in the proteomic analysis.

Colunm	XDB C_{18}, 2.1 x 50 mm x 3.5 µm (Agilent)	
Injection volume (µL)	10	
Flow rate (mL min^{-1})	0.35	
Buffer A	0.1% formic acid in H_2O	
Buffer B	acetonitrile with 0.1% formic acid	
Gradient program	Time (min)	Composition
	0-2	10% B
	4	20% B
	10	45% B
	15	100% B
	20	100% B
	22	10% B

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4.5.4. Protein database search

The identification of proteins was carried out by the utilization of publicly available software SearchGUI-1.15.0. and PeptideShaker1-0.23.0 (Barsnes et al. 2011, Vaudel et al. 2011). These software provide a graphical user interface for the evaluation of the results generated by OMSSA (Open Mass Spectrometry Search Algorithm) and X!Tandem search engines. The details about the database search and validation settings are presented in Table 4.4.

Table 4.4. Settings utilized for database-dependent protein identification.

Name and version of the program	PeptideShaker1-0.23.0
Enzyme	Trypsin
Fixed modification	Carbamidomethyl cysteine
Variable modifications	Phosphorylation of serin or tyrosine
	Oxidation of methionine
	Selenomethionine
	Selenocysteine
Precursor mass tolerance	10 ppm
Fragment ion types	b and y
Maximum missed cleavages	2
Fragment mass tolerance	0.05 mDa
Precursor charge	2-3
Validation settings	
Validated hits	8
Confidence	95%
Lowest confidence of validated results	100%
FDR	1
FNR	1

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In accordance with the sample preparation procedure carbamidomethyl cysteine was assumed as a fixed modification since (theoretically) all cysteine residues were derivatized by IAA. Regarding variable modifications, in addition to the substitution of sulfur by selenium in methionine and cysteine the most common post-translational modifications (PTMs) were also taken into account: the phosphorylation of serine and tyrosine and the oxidation of methionine. The number of PTMs considered in a database search should be minimized because these variable modifications usually result in increased rate of false positive hits.

Concerning the database of choice, ideally, a unique protein database should be created from reviewed UniProtKB entries of the studied organism. It is an easy task in case of model organisms (*i.e.*, human, rat or *Arabidopsis thaliana*) but becomes more complicated with non-model, less investigated organisms especially with forest trees (Neale and Kremer 2011). As

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regards to *Lecythidaceae*, there are 441 known proteins, only 5 of them are reviewed. Finally, the tandem MS spectra were searched against a database utilizing the UniProtKB entries for *Ericales* (15114 proteins, 104 of which are reviewed, 1.8.2014).

The *in-silico* tryptic digestion of the proteins was carried out in SearchGUI-1.15.0 with allowing maximum 2 missed cleavages. A so-called concatenated database was derived from the resulting peptide database. It contains two sets of the peptides: the original peptides and a set of false peptides that have reversed sequences compared to the original ones. This concatenated database is applied for validation purposes and facilitates the estimation of the rate of false negative and false positive hits. In order to further improve the validation of our results and avoid false positive hits, both false discovery rate (FDR) and false negative rate (FNR) were set to 1%.

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5. RESULTS AND DISCUSSION

5.1. Investigation of selenometabolites

In this experiment we intended to analyze the unknown, non-protein bound, low molecular weight, water extractable selenium compounds of the seeds of *Bertholletia excelsa* and *Lecythis minor*. These plants belong to the same family, *Lecythidaceae*, the morphology of the fruits (nuts) are analogous and the concentration of selenium in the soil of the production areas (in Brazil and Venezuela) is equally extreme (Ferri et al. 2004, Gabos et al. 2014).

On the other hand, the available speciation data are remarkably different concerning their non-protein bound compounds. *L. minor* contains about 5% of selenium in the form of SeCysta (Dernovics et al. 2007a), while up to now only protein bound SeMet has been identified in *B. excelsa* nut samples, accounting for up to 96% of total selenium (Dumont et al. 2006b). Identifying the uncharacterized, presumably minor selenium compounds in both species can lead to a better understanding of Se metabolic pathways in selenium accumulator plants.

The total Se concentration of the defatted *B. excelsa* and *L. minor* sample was found to be 127 ± 1 μg Se g^{-1} DW and 4480 ± 22 μg Se g^{-1} DW, respectively. If the fat content of the nuts is assumed to be around 70 % the total Se concentration of the whole nuts correspond to 38 μg g^{-1} (*B. excelsa*) and 1464 μg g^{-1} (*L. minor*). These results agree with relevant literature data (Secor and Lisk 1989, Chang et al. 1995, Vonderheide et al. 2002, Ferri et al. 2004). The total S concentration of the defatted *L. minor* was found to be 2560 ± 12 μg S g^{-1} DW.

5.1.1. Sample purification procedure

5.1.1.1. Water extraction and fractionation by size exclusion chromatography

Water extraction was chosen because the targeted low molecular weight compounds are water soluble and it provides mild extraction conditions that ensure minimal species transformation. The efficiency of the extraction was enhanced by the application of an ultrasonic probe that facilitated the disruption of the matrix of the nut samples. The aqueous extract of *B. excelsa* and *L. minor* seeds contained 37.7 ± 0.1 % and 72.2 ± 2.6 % of Se originally presented in the samples, respectively. I would like to note here that the recovery of extraction was determined by comparing the total Se content of the original samples and the water extracts. This procedure is preferred in Se speciation analysis because unlike the most common spiking

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experiments that are used to characterize the efficiency of the chosen extraction technique, it also provides some information about speciation. For instance, the recovery of water extraction can characterize the ratio of "free" and (protein-)bound Se species. The extraction recovery of *B. excelsa* might seem a bit low but it is acceptable considering that our target compounds are the minor low molecular weight, non-protein bound Se species while the vast majority of Se is stored in the proteins of Brazil nut (Kannamkumarath et al. 2002, Vonderheide et al. 2002, Chunhieng et al. 2004).

The Se concentration of the SEC fractions was monitored by ICP-MS. Fractions corresponding to the Se containing peaks shown in Figure 5.1 were collected for further analysis. The SEC-ICP-MS chromatograms of the two samples are quite similar, but the low molecular weight fraction of Brazil nut is considerably less intense compared to the monkeypot nut sample.

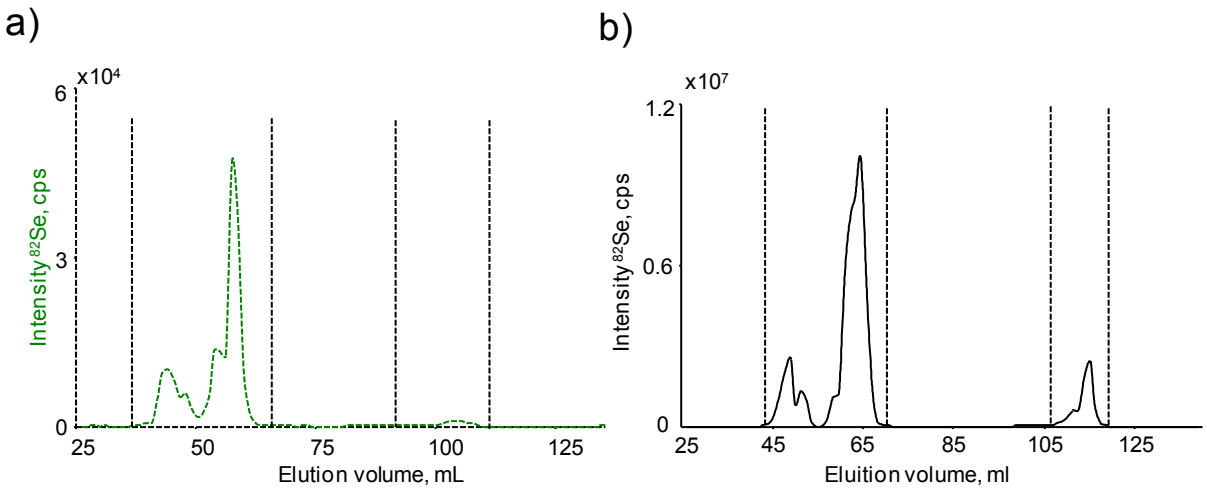

Figure 5.1. SEC-ICP-MS chromatogram of the water extract of defatted *B. excelsa* (a) and *L. minor* (b). The vertical lines indicate the collected fractions.
***Note*: 25 ml corresponds to the dead volume of the applied SEC column.**

5.1.1.2. Multidimensional clean-up procedure

In the followings, I would like to summarize the sample preparation procedure through the example of one relevant newly identified compound from each nut samples. Additional chromatograms and spectra are provided in the appendix. The orthogonal design of the process was a key factor that ensured the elimination of as many matrix interferences as possible. We chose size exclusion chromatography as a first dimension because with ICP-MS coupling it is an excellent tool for the crude separation of Se containing fractions. The following three dimensions

were ion exchange, ion-paring and reversed phase chromatography. From these three techniques RP is the best compatible with ESI ionization therefore it was chosen to be the last step.

Considering ion-exchange chromatography, strong cation exchange chromatography is the method of choice for Se speciation analysis because the application of anion exchange chromatography might require the utilization of buffers at pH 9-11 that was shown to introduce species transformation (Woller et al. 1998). The majority of Se containing compounds of Brazil nut eluted close to the void that implies that these compounds are mostly anionic or neutral. However, a small but well-retained fraction could also be collected that had similar retention to fraction IV. of *L. minor*. Since HPLC-ICP-MS is a destructive technique that does not allow the collection of target compounds after analysis, it was applied only to select retention time intervals corresponding to the Se-containing peaks. All the Se-containing peaks (assigned with roman numerals on Figure 5.2.) were collected by a heart-cut type offline HPLC fraction collection.

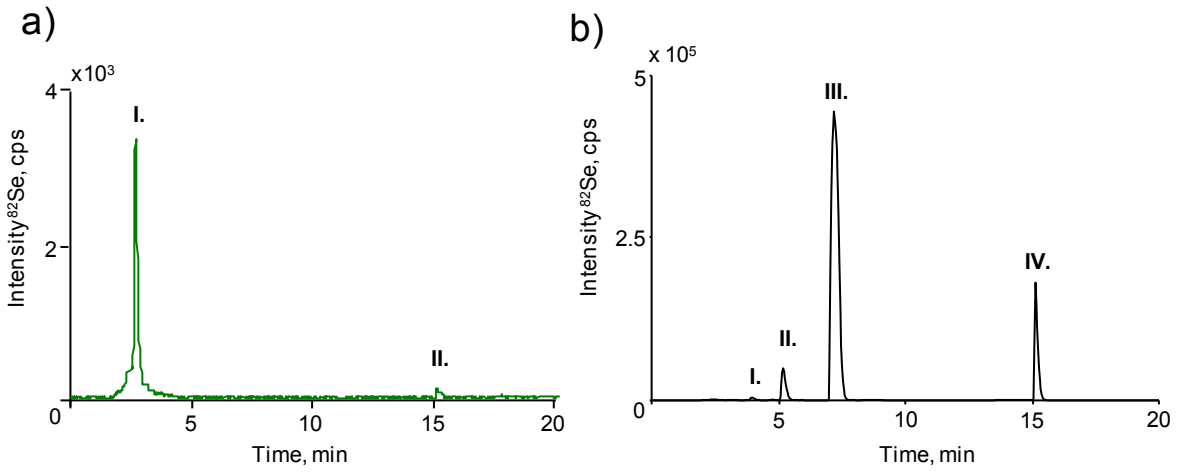

Figure 5.2. SCX-ICP-MS chromatograms of the fractions of *B. excelsa* (a) and *L. minor* (b) collected in the previous step. All Se-containing peaks were collected for further analysis.

Figure 5.3. demonstrates the IP-RP-ICP-MS chromatograms of peak I. of *B. excelsa* (a) and peak III. of *L. minor* (b). The Se containing peaks (fraction I./1, 2 and fraction III./1, 2 and 3) were collected and further analyzed by RP-HPLC-ESI-Q-TOF-MS. It is worth noting that the injection volume for each fraction collection was individually optimized depending on the Se concentration of the analyzed fraction. For instance, the injection volume was set to 60 µl in the IP-RP separation of the Brazil nut fraction I., while 20 µl was the maximum amount that could be injected from the *L. minor* fraction III. without overloading the HPLC column.

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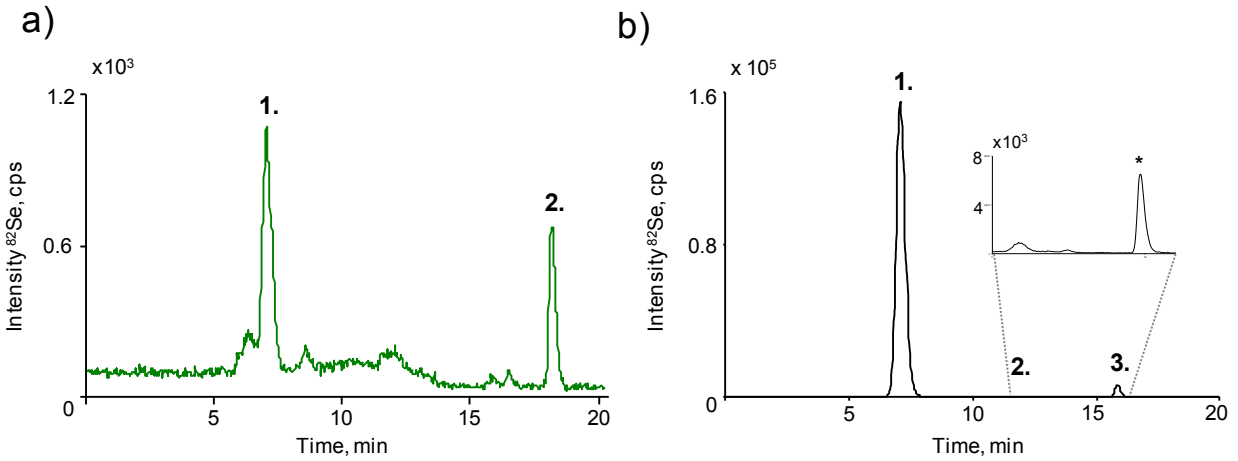

Figure 5.3. IP-RP-ICP-MS chromatograms peak I. of *B. excelsa* (a) and peak III. of *L. minor* (b) collected from the SCX separation.

5.1.2. Identification of selenocompounds

This complex sample clean-up procedure assured the elimination of matrix interferences and the pre-concentration of the analyte necessary for the ESI-Q-TOF-MS analysis. Fractions collected by IP-RP-HPLC were introduced to the ESI-Q-TOF-MS instrument by means of an RP-HPLC system that provided an additional separation to achieve better signal-to-noise (S/N) ratios. An integrated data processing strategy was required for processing the data acquired throughout the LC-MS experiments.

Our strategy for the scanning of the chromatograms consisted of three main approaches. Namely, after applying (i) database search for Se containing compounds previously described in the literature, the chromatograms were also (ii) screened for diagnostic in-source fragments. The rest of the compounds were (iii) screened by manual pattern exploration.

The identification of selenocompounds was principally based on accurate mass measurement and the analysis of MS-MS spectra. Accurate mass measurement offers the opportunity to calculate the elemental composition of the molecule. Although it is a very powerful tool, the number of possible elemental compositions increases exponentially with increasing mass-to-charge ratio. In such cases false hits can be excluded by considering the nitrogen rule, comparing the theoretical and experimental isotopic distribution and excluding the improbable elemental compositions.

Fortunately, the Se content of a molecule can be easily determined from its isotopic distribution and the elemental composition calculations can be simplified by taking the number

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of selenium atoms into account. However, the complex isotopic pattern of the molecules makes the identification of their monoisotopic mass (used for the calculations) a bit more complicated compared to other metabolites that consist of monoisotopic elements. Figure 5.4. shows the characteristic isotopic distribution of compounds that contain one to four Se atoms. The isotopologues that correspond to the monoisotopic mass are highlighted (in green).

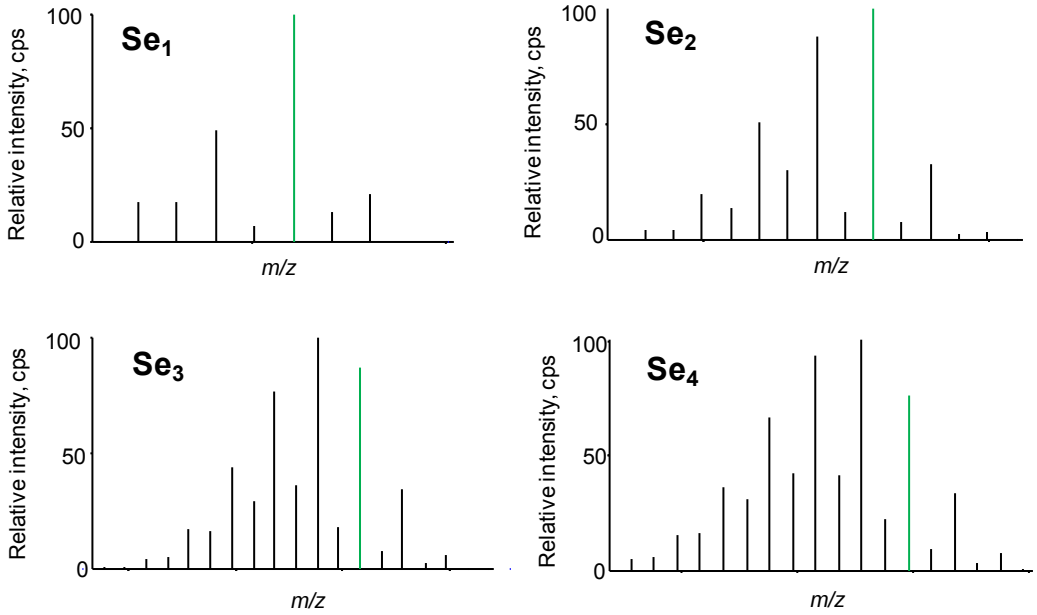

Figure 5.4. The characteristic isotopic distribution of Se containing compounds. The highlighted isotopologues correspond to the monoisotopic mass.

5.1.2.1. Database search for previously reported Se species

The challenge in building up a database for the scanning of Se compounds in samples of plant origin is that the number of Se species observed in plants is still limited (Birringer et al. 2002, Whanger 2002, Sors et al. 2005b, Ogra and Anan 2009). Moreover, there are only a few examples for compounds identified in microorganisms and animal tissues that are also present in plants. This is basically the consequence of the non-essentiality of Se in plants and its non-specific incorporation.

An accurate mass database was established (Table A1 in the appendix) utilizing the previously identified Se species from plants and some other common derivatives from yeast and animal origin (Terry et al. 2000, Freeman et al. 2006, Ogra and Anan 2009, Far et al. 2010, Aureli et al. 2012). As the result of the database search, seven selenocompounds could be

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identified from *L. minor*, including non-protein selenoamino acids, conjugates and natural derivatives (Table 5.1.). Most of these seven compounds have been detected in *Brassica juncea* (Grant et al. 2004, Yathavakilla et al. 2005, Ogra and Anan 2012) and *Allium* species before (Dumont et al. 2006a). Regarding the *B. excelsa* sample, only γ-Glu-SeMet could be detected by the database search, together with the previously described SeMet. γ-Glu-SeMet was first observed in garlic samples and was characterized by Larsen and coworkers (2006).

Table 5.1. List of the selenocompounds identified by database search from *L. minor* and *B. excelsa*. The compounds are listed in the increasing order of their relative abundance in *L. minor*.

Name	Elemental composition $[M+H]^+$	Abbreviation	Theoretical *m/z*	Experimental *m/z*	Δppm	*Lecythis minor*	*Bertholletia excelsa*
Methyl-selenomethionine	$C_6H_{14}NO_2Se^+$	Se-Met-SeMet	212.01843	212.01851	0.38	+	-
Selenocystine	$C_6H_{13}N_2O_4Se_2^+$	$SeCys_2$	336.96195	336.96193	-0.06	+	-
γ-glutamyl-*Se*-methyl-selenocysteine	$C_9H_{17}N_2O_5Se^+$	γ-Glu-Met-SeCys	313.03289	313.03287	-0.06	+	-
γ-glutamyl-selenomethionine	$C_{10}H_{19}N_2O_5Se^+$	γ-Glu-SeMet	327.04537	327.04537	0	+	+
Selenocystathionine	$C_7H_{15}N_2O_4Se^+$	SeCysta	271.01916	271.01915	-0.04	+	-
Selenomethionine	$C_5H_{12}NO_2Se^+$	SeMet	198.0028	198.00275	-0.25	+	+
Selenohomocystine	$C_8H_{17}N_2O_4Se_2^+$	$SeHCy_2$	364.95133	364.95139	0.16	+	-

The relatively low amount of Se-Met-SeMet implies that Se metabolism in *L. minor* is preferably directed to the storage of SeMet instead of the excretion of Se through volatile Se species. The very low abundance of $SeCys_2$ and the dominance of γ-Glu derivatives and SeCysta confirm the effective metabolism of SeCys into non-amino acid derivatives that is a common detoxification mechanism in Se accumulator plants. Selenohomocystine was present in an exceptionally high abundance (approximately twice more than SeCysta or SeMet) that seems to be a unique characteristic of *L. minor*.

5.1.2.2. In-source fragment based data mining

Despite of the soft ionization process in the ESI ion source some in-source fragments can be formed from the precursor ions. This feature can be utilized for the screening of possible derivatives of known fragments. In addition, the mass accuracy of these fragments measured in the TOF mode is usually better than that of the Q-TOF fragments that also facilitates the screening. The reason for this characteristic is that in the TOF mode the reference masses (*m/z* 121.0509 and *m/z* 922.0098) are constantly measured and the acquired experimental data are steadily corrected.

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The search for the in-source fragment *m/z* 181.9715 ($C_4H_8NO_2Se^+$), characteristic for the SeHCy residue provided a useful tool for the identification of various selenocompounds. Figure 5.5.a shows the extracted ion chromatogram (EIC) of *m/z* 181.9715 from the RP-HPLC-ESI-TOF chromatogram of the *L. minor* fraction III/3. The analysis of the full scan spectrum at 1.75 min (Figure 5.5.b) revealed the presence of an abundant selenocompound (*m/z* 444.86818).

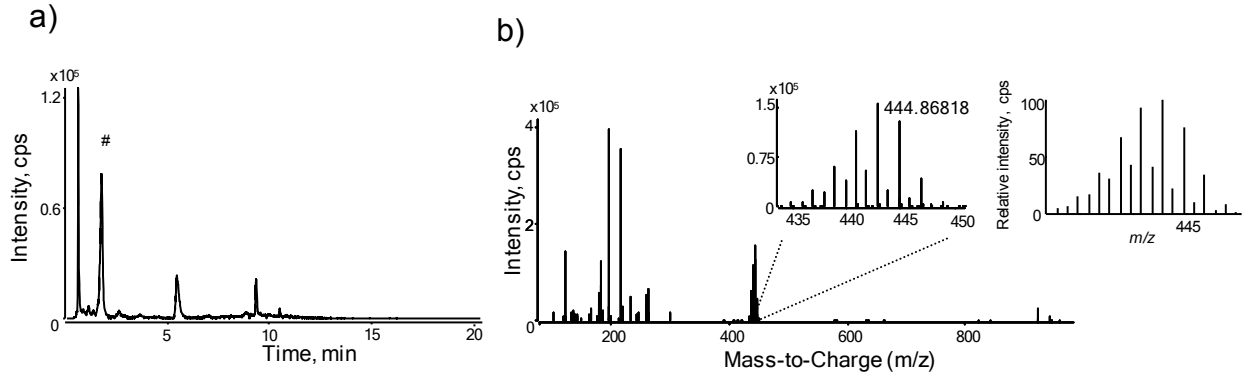

Figure 5.5. : RP-HPLC-ESI-Q-TOF-MS data of the *L. minor* SCX fraction III/ 3. a) Extracted ion chromatogram (EIC) of the ion-source fragment m/z 181.9715. b) Full-scan spectrum obtained at the apex of the peak eluting at 1.75 min (indicated with '#') with the experimental and theoretical isotopic distributions of compound m/z 444.86818 inserted.

The structure of the molecule was elucidated based on its exact mass, isotopic pattern, mass defect and MS-MS fragmentation pattern. The isotopic pattern was characteristic for a molecule containing three Se atoms (see the theoretical isotopic distribution of the compound on Figure 5.5). This concept was further supported by the value of the mass defect. Taking into account that the compound contains three selenium atoms the only possible calculated formula for the compound within 1.5 ppm mass accuracy was $C_8H_{17}N_2O_4Se_3^+$ ($[M+H]^+$). Indeed, the MS-MS spectrum (Figure 5.6) contained the fragment *m/z* 181.97029 ($C_4H_8NO_2Se^+$, Δppm -6.56) characteristic for a SeHCy residue. The product ion *m/z* 135.96643 ($C_3H_6NSe^+$, Δppm -3.2) is formed by the loss of the carboxyl group from SeHCy. The fragment *m/z* 263.90396 ($C_4H_{10}NO_2Se_2^+$, Δppm -1.18) corresponding to the loss of the SeHCy residue from the molecule ion was also present.

These detailed structural information together with the accurate mass measurement data were sufficient to construct the structure of Se-selenohomocysteinyl-diseleno-homocysteine shown on Figure 5.6. Regarding the Se-Se-Se bond in the center of the molecule, the structure of the compound is analogous to the structure of polyselenides and polysulfides known from the literature (Westlake Jr. et al. 1950, Möckel et al. 1985).

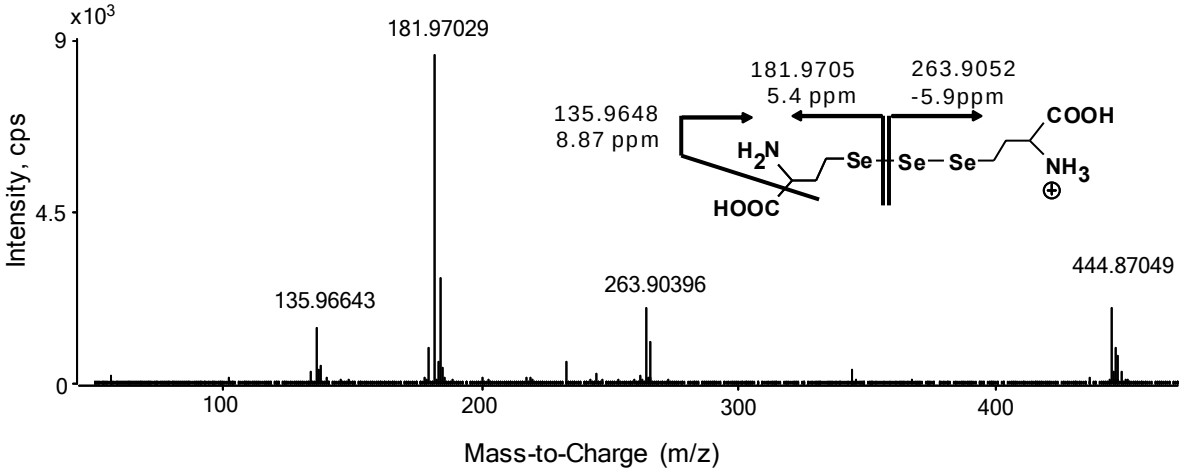

Figure 5.6. The MS-MS fragmentation spectrum of the pseudomolecular ion m/z 444.86818 with the proposed molecular structure and fragments.

5.1.2.3. Identification of polyselenides

Both polysulfides and polyselenides have a non-branched structure where the S and Se atoms take place in the center of the molecules (Woodrow et al. 1951, Möckel et al. 1985). Polysulfides are the main aroma components of *Brassicaceae* and *Allium* (Münchberg et al. 2007) species. There are different mechanisms for the formation of polysulfides but it is suggested that the biological formation involves the reaction of hydrogen sulfide with other precursor sulfides (Westlake Jr. et al. 1950, Pickering et al. 1967).

The mixed Se-S analogues of di- and trisulfides containing up to three Se atoms had been detected from *Brassicaceae* species (Meija et al. 2002, Kubachka et al. 2007) and also from bacterial origin (Burra et al. 2010). A recent study investigating Se detoxification pathways in genetically modified *Escherichia coli* also demonstrates the importance of these compounds (Swearingen Jr. et al. 2006).

The newly identified Se-selenohomocysteinyl-diseleno-homocysteine is analogous to selenohomolanthionine (SeHLan) and selenohomocystine ($SeHCy_2$) but contains three Se atoms. Since two members of this homologous series were actually the most dominant low-molecular weight Se species of monkeypot nut, we decided to search our experimental data for the further compounds of this series. Finally the compounds containing four, five and six Se atoms were also detected (with decreasing intensities) and identified. These polyselenides were found to represent an abundant, distinctive group of the water soluble Se compounds of *L. minor* seeds. Their molecular structures are shown on Figure 5.7. Mass spectrometric results are provided in Table 5.2. and in the appendix (Figure A1).

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Table 5.2. Summary of the mass spectrometric results of polyselenides.

Name	m/z_{exp}	t_R	Elemental composition $[M+H]^+$	m/z_{theor}	Δppm	MS^2	Elemental composition [M]	m/z_{theor}	Δppm
Se-selenohomocysteinyl-diseleno-homocysteine	**444.8682**	1.8	$C_8H_{17}N_2O_4Se_3^+$	444.8678	-0.79	343.8178	$C_4H_9NO_2Se_3$	343.8202	6.92
						263.9052	$C_4H_9NO_2Se_2$	263.9036	-5.90
						181.9705	$C_4H_8NO_2Se$	181.9705	5.40
						135.9648	C_3H_5NSe	135.9660	8.87
Se-selenohomocysteinyl-triseleno-homocysteine	**524.7853**	5.8	$C_8H_{17}N_2O_4Se_4^+$	524.7844	-1.71	343.8204	$C_4H_9NO_2Se_3$	343.8202	-0.67
						261.8862	$C_4H_7NO_2Se_2$	261.8880	6.90
						244.8608	$C_4H_4O_2Se_2$	244.8614	2.67
						181.9705	$C_4H_8NO_2Se$	181.9705	5.40
						135.9648	C_3H_5NSe	135.9660	8.87
Se-selenohomocysteinyl-tetraseleno-homocysteine	**604.7024**	9.3	$C_8H_{17}N_2O_4Se_5^+$	604.7009	2.49	444.8577	$C_8H_{16}N_2O_4Se_3$	444.8678	22.87
						263.9078	$C_4H_9NO_2Se_2$	263.9036	-15.80
						181.9723	$C_4H_8NO_2Se$	181.9705	-4.55
						135.9635	C_3H_5NSe	135.9660	18.51
						102.0557	$C_4H_7NO_2$	102.0549	-7.37
Se-selenohomocysteinyl-pentaseleno-homocysteine	**684.6191**	10.7	$C_8H_{17}N_2O_4Se_6^+$	684.6174	-2.46	181.9716	$C_4H_8NO_2Se$	181.9705	0.57
						262.8967	$C_4H_8NO_2Se_2$	262.8958	-3.31

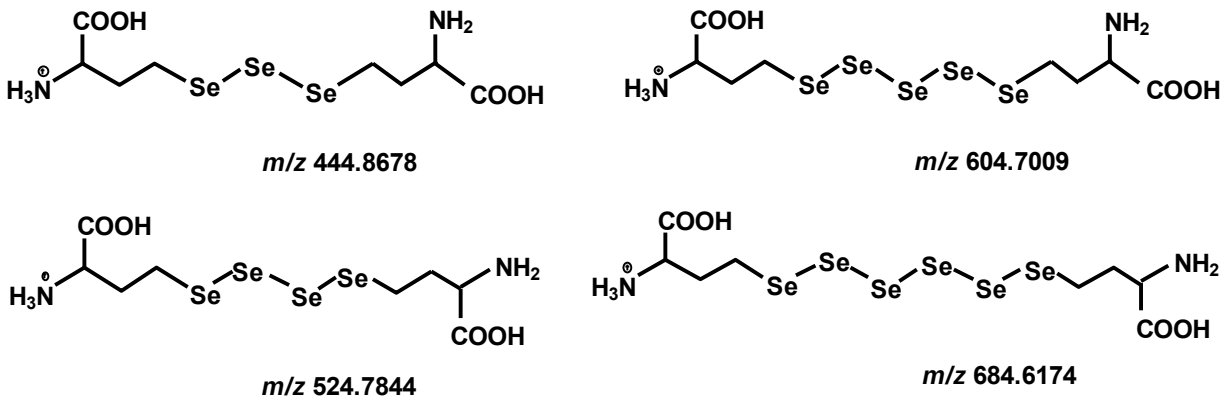

Figure 5.7. Homologous series of selenohomocysteinyl-polyselenides from *L. minor*.

It can be seen in Table 5.2. that the "diseleno-homocysteine" fragment that contains two Se atoms appeared in the MS/MS spectrum of each polyselenides, but its formula was not constant (Figure 5.8.a). This kind of difference in the fragmentation of the SeHCy residue was previously observed in the case of selenocystathionine and selenohomolanthionine that also form different fragment ions (Figure 5.8.b). This phenomenon is not very well-characterized but can be related to the symmetry of the molecules.

Selenocystathionine is an asymmetric molecule and a SeHCys fragment with the formula $C_4H_8NO_2Se^+$ (*m/z* 182) is formed through its fragmentation. In this case, the positive charge will be on the Se atom. On the other hand, selenohomolanthionine is symmetric molecule (where the

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Se atom is in the axis of symmetry) and a SeHCys fragment with the formula $C_4H_{10}NO_2Se^+$ (*m/z* 184) is formed through its fragmentation. In this case, a –SeH moiety will be formed and the positive charge will be on the $–NH_2$ residue.

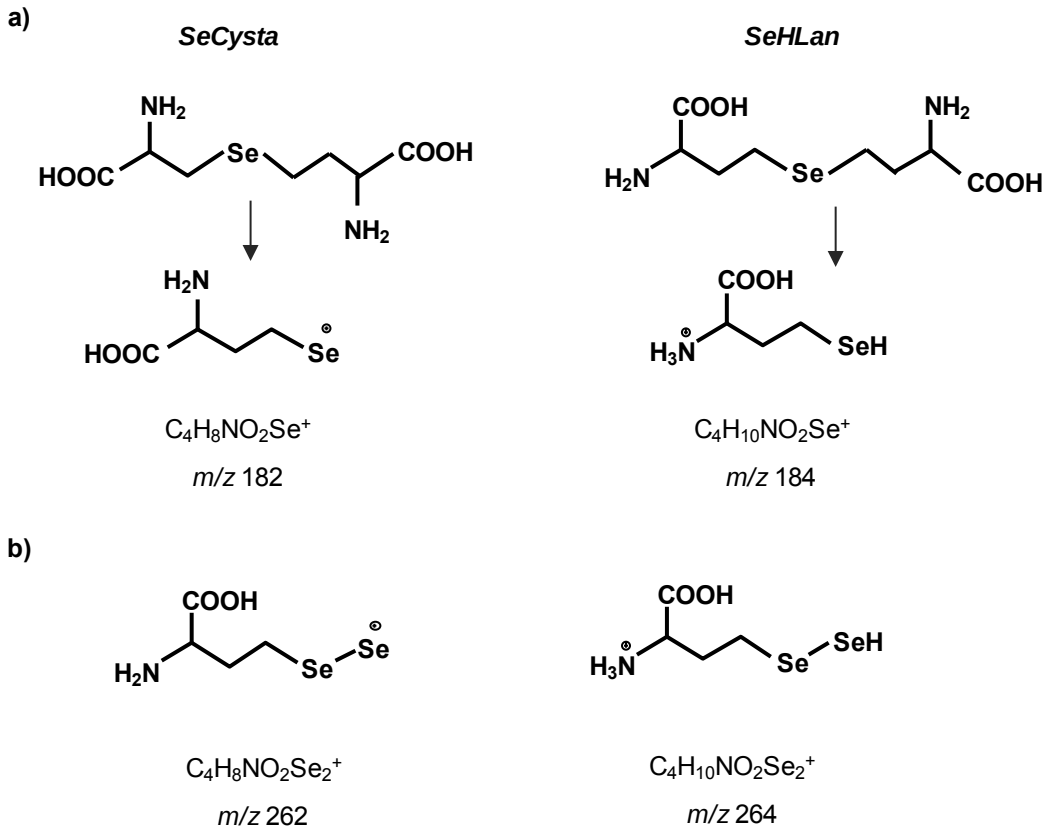

Figure 5.8. Possible ways of fragmentation of the SeHCy residue (a) and its diseleno alalogue (b).

As regards to polyselenides, the axis of symmetry goes through a Se atom in case if there are odd numbers of Se atoms. The fragmentation of these molecules resulted in the fragment $C_4H_{10}NO_2Se_2^+$ (*m/z* 264), while the rest of polyselenides fragmented forming a $C_4H_8NO_2Se_2^+$ fragment ion (*m/z* 262). This theory on the relationship between the fragmentation and symmetry of polyselenides could be considered for further theoretical and experimental studies. Note, that unfortunately the concentration of Se-selenohomocysteinyl-pentaseleno-homocysteine was not enough to carry out a successful MS/MS experiment, therefore only the ion-source fragments could be displayed on Table 5.2. This fact might be the reason why it does not fit into the pattern.

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Table 5.3. Mass spectrometric results of other selenohomocysteine and selenocysteine derivatives.

Name	m/z_{exp}	t_R	Elemental composition $[M+H]^+$	m/z_{theor}	Δppm	MS^2	Elemental composition [M]	m/z_{theor}	Δppm
Se-4-hydroxy-butyl-selenohomocysteine	**256.0444**	1.3	$C_8H_{18}NO_3Se^+$	256.0446	0.95	181.9708	$C_4H_7NO_2Se$	181.9705	3.74
						135.9654	C_3H_5NSe	135.9660	4.43
N-glycyl-diselenomethionine	**320.9263**	0.6	$C_6H_{13}N_2O_3Se_2^+$	320.9251	-3.7	181.9711	$C_4H_7NO_2Se$	181.9705	-2.6
						135.9641	C_3H_5NSe	135.9660	14.06
						84.0501	C_4H_5NO	84.0444	-68.8
						56.0495	C_3H_5N	56.0495	0.96
Se-(3-aminopropionyl)-diseleno-homocysteine	**334.9405**	1.1	$C_7H_{15}N_2O_3Se_2^+$	334.9408	0.79	181.9712	$C_4H_7NO_2Se$	181.9705	-1.5
						135.9661	C_3H_5NSe	135.9660	0.7
Se-(3-hydroxypropionyl)-diseleno-homocysteine	**335.9249**	3.6	$C_7H_{14}NO_4Se_2^+$	335.9248	-0.36	181.9715	$C_4H_7NO_2Se$	181.9705	0.13
						135.9681	C_3H_5NSe	135.9660	-15.6
Se-(3-aminopropionyl)-triseleno-homocysteine	**414.8574**	4.6	$C_7H_{15}N_2O_3Se_3^+$	414.8573	-0.28	244.8638	$C_4H_4O_2Se_2$	244.8614	-9.64
						181.9703	$C_4H_7NO_2Se$	181.9705	-6.4
						135.9657	C_3H_5NSe	135.9660	-2.1
						71.0855	C_5H_{10}	71.0855	0.38
Se-(3-hydroxypropionyl)-triseleno-homocysteine	**415.8416**	8.7	$C_7H_{14}NO_4Se_3^+$	415.8413	-0.72	261.8895	$C_4H_7NO_2Se_2$	261.8880	-5.75
						244.8592	$C_4H_4O_2Se_2$	244.8614	9.23
						181.971	$C_4H_7NO_2Se$	181.9705	2.64
						135.9654	C_3H_5NSe	135.9660	4.43
						56.0525	C_3H_5N	56.0495	-39.6
Se-[(*N*-acetyl)-selenocysteyl]-diseleno-homocysteine	**472.8626**	8.6	$C_9H_{17}N_2O_5Se_3^+$	472.8628	0.35	261.8819	$C_4H_7NO_2Se_2$	261.8880	23.38
						244.8637	$C_4H_4O_2Se_2$	244.8614	-9.23
						181.9706	$C_4H_7NO_2Se$	181.9705	4.85
						135.9612	C_3H_5NSe	135.9660	35.55

The screening of experimental data for the selenohomocysteine fragment proved to be a valuable tool for the identification of various selenocompounds (Table 5.3. and Figure 5.9.). The intensity of some of these compounds was so low that they would have staid hidden in the chromatograms without the aid of in-source fragment based data-mining.

The fragments identified from the spectra of polyselenides also provided valuable information for the identification and structure elucidation of other selenohomocysteine derivatives that contain multiple Se atoms (Table 5.3.). The most abundant compounds of these SeHCy derivatives were *Se*-selenohomocysteinyl-diseleno-homocysteine (*m/z* 445) and *Se*-[(*N*-acetyl)-selenocysteyl]-diseleno-homocysteine (*m/z* 473).

Note that the search for characteristic fragments of common selenocompounds from plant origin was only partially successful. For example, the characteristic fragment *m/z* 183.9871 ($C_4H_{10}NO_2Se^+$) of selenohomolanthionine that is a typical Se species in the deeply studied *Brassica* species was not present in the samples.

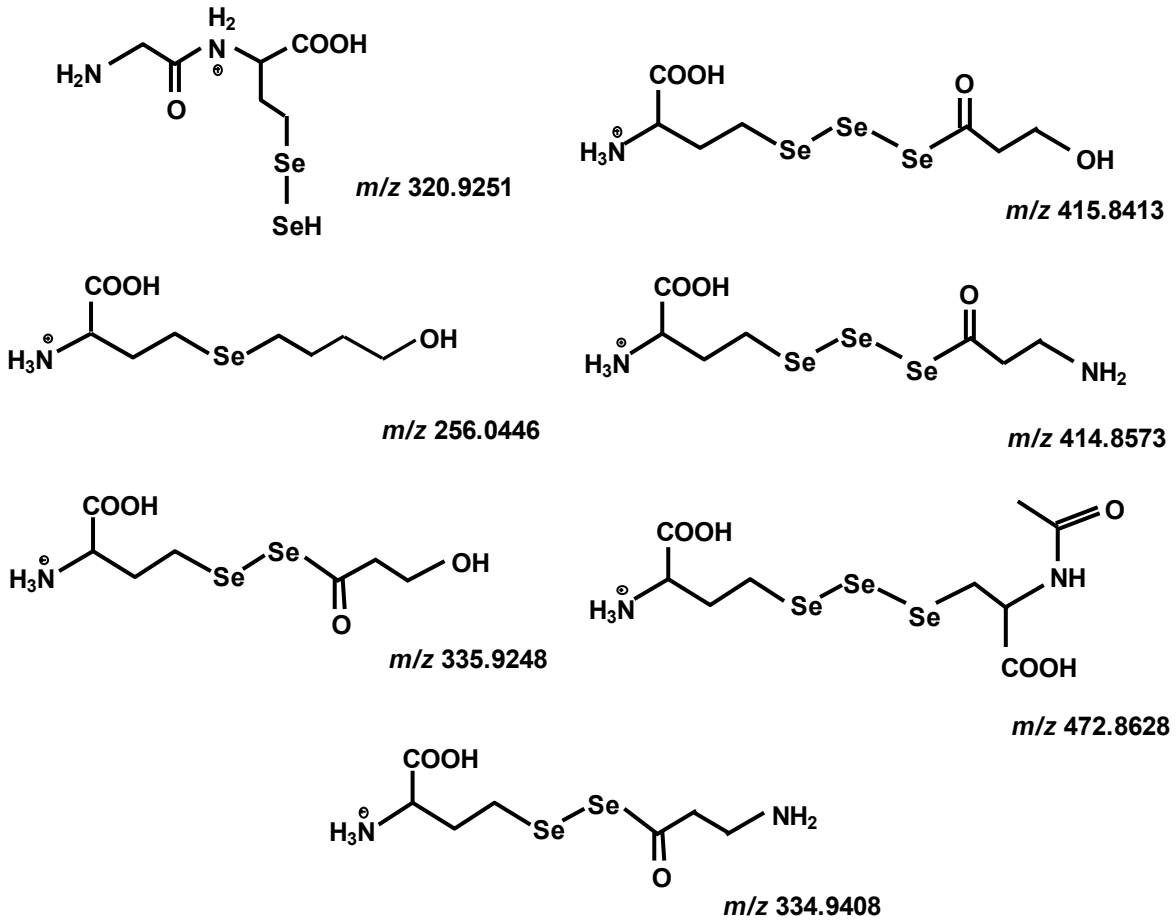

Figure 5.9. The structure of SeHCy derivatives from *L. minor*.

Altogether, the in-source fragment based search could contribute to the list of assigned selenium species of *L. minor* with 11 new compounds (additional mass spectrometric results are shown on Figure A2 and A3 in the Appendix), while none of these were found in the *B. excelsa* sample.

5.1.2.4. Synthesis of polycalchogenides

Since our results are the first representation of the *in vivo* formation of polyselenides that contain more than three Se atoms, we decided to verify the proposed structure of the molecules through their synthesis and mass spectrometric analysis. The mechanism of the *in vivo* formation of polyselenides is unknown but it is probably analogous to the formation of polysulfides. As it was mentioned before, the biological formation of polysulfides involves the reaction of hydrogen sulfide with other precursor sulfides (Westlake Jr. et al. 1950, Pickering et al. 1967).

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Therefore we decided to set up an experiment for the reaction of hydrogen selenide and homocysteine. Note that homocysteine was utilized because selenohomocysteine is not commercially available. In short, hydrogen selenide was generated from sodium-selenite in a reaction with sodium borohydride and hydrochloric acid. The gas was absorbed in the solution of homocysteine that also contained sodium iodide (as a catalyst) (Kirihara et al. 2007) and hydrogen peroxide (that provided oxidative conditions). Unfortunately, the experiment was unsuccessful probably because of the low partial pressure of H_2Se in the solution.

The second experiment for the synthesis of polycalchogenides consisted of the reaction of homocysteine and sodium selenide in the presence of sodium iodide and hydrogen peroxide. This reaction was successful and resulted in a series of polycalchogenides that contained -S-Se-S-, -S-Se-Se-S- and -S-Se-Se-Se-S- chains. This observation reinforces the theory that the formation of polyselenides is a non-enzymatic process and it is driven by the excess of SeHCy and Se^{2-}. The results of the mass spectrometric analysis of one of these compounds (*m/z* 348.97886, $C_8H_{17}N_2O_4S_2Se^+$, Δppm 0.25) are displayed on Figures 5.10. and 5.11. Additional spectra are included in the appendix (Figure A4).

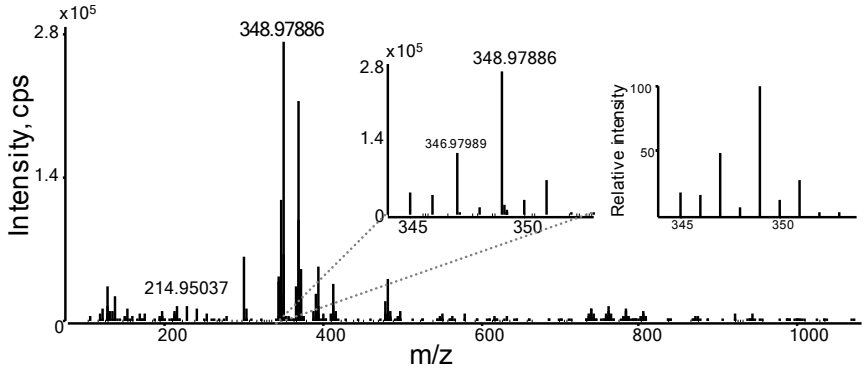

Figure 5.10. RP-HPLC-ESI- Q-TOF-MS spectrum of the synthesized compound *m/z* 348.9789 with the experimental and the theoretical isotopic patterns inserted.

The MS-MS fragmentation pattern of this compound showed a good analogy to that of *Se*-selenohomocysteinyl-diseleno-homocysteine (*m/z* 445). The mass difference between sulfur and selenium is 48 Da that means if one of the Se atoms is replaced by S, the mass of the analogous molecule or fragment should decrease by 48 Da. However, the inspection of Figure 5.11. reveals that this rule does not apply to all analogous fragments.

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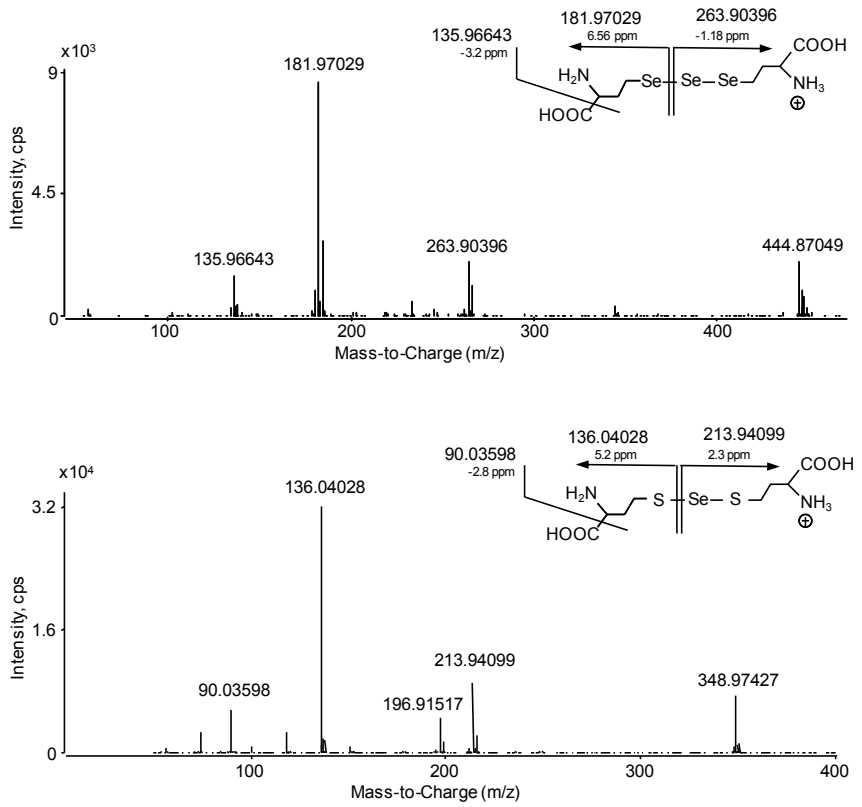

Figure 5.11. Comparison of the MS-MS fragmentation of *Se*-selenohomocysteinyl-diseleno-homocysteine and its synthesized mixed Se-S analogue.

It is due to the different fragmentation characteristics of homocysteine and SeHCys. In the case of homocysteine a –SH bond is formed (*m/z* 136) (Rubino et al. 2004), while the SeHCys residue fragmented according to the process presented on Figure 5.8.a. These results adequately confirm the structural analogy of the newly identified and the synthesized compounds. However, the unequivocal structure identification of the detected compounds could be carried out only by NMR analysis.

5.1.2.5. Manual pattern exploration

ICP-MS monitoring of the chromatographic clean-up procedures indicated several selenocompounds in the fractions that could not be assigned by targeted search. Therefore, manual searching for the characteristic selenium patterns in the ESI-MS spectra was also required. Following this approach, another distinctive group of Se species was detected. Their identification was not possible *only* on the basis of their MS-MS fragmentation pattern. The application of an integrated structure elucidation strategy that took biological aspects also into account was crucial.

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Figure 5.12. shows the MS-MS spectrum of one of the selenocompounds from this group. The fragments characteristic of a SeHCy residue and the one corresponding to the loss of this SeHCy residue were present. The elemental composition could be calculated and it showed that the only possible formula (within 75 ppm mass deviation) for the fragment was $C_{10}H_{16}^{+}$ (Δppm 12.9) that implies a monoterpene residue. The retention time (t_R=13.2 min) of the compound is also characteristic to a molecule with a hydrophobic residue.

Taking biological considerations into account, *L. minor* seeds contain up to 70 % of different kinds of lipids, therefore this tissue possesses significant lipid and also phytosterol synthetic activity (Phillips et al. 2005, Miraliakbari and Shahidi 2008). Since geranyl-pyrophosphate is one of the key metabolites of monoterpene and phytosterol biosynthesis it is probably present in the seeds. It is supposed that this selenocompound is the product of the reaction of geranyl-pyrophosphate and SeHCy that is also present and highly reactive. The structure of the molecule is proposed to be *Se*-geranyl-selenohomocysteine (*m/z* 318.0970, $C_{14}H_{24}NO_2Se^{+}$ $[M+H]^{+}$, Δppm -1.02).

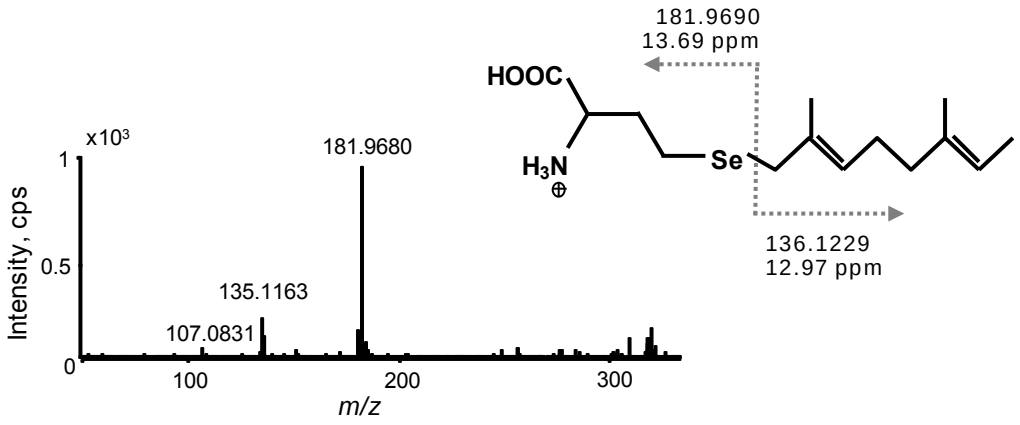

Figure 5.12. MS-MS spectrum of *Se*-geranyl-selenohomocysteine (*m/z* 318.0970)

As regards to the compound *m/z* 336.1075, its elemental composition can also be calculated from its accurate mass. This calculated formula ($C_{14}H_{26}NO_3Se^{+}$, $[M+H]^{+}$, Δppm -0.77) differs only in a hydroxyl group from geranyl-SeHCy. The addition of this hydrophilic functional group (resulting in a hydroxy-geranyl residue) contributes to the less hydrophobic character of the compound. The retention time of the compound also confirms this interpretation, since it is proportionately shorter (t_R=10.9 min) than the retention time of geranyl-SeHCy (Table 5.4, Figure 5.13.).

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Table 5.4. Mass spectrometric results of hydrophobic derivatives of SeHCy.

Name	m/z_{exp}	t_R	Elemental composition $[M+H]^+$	m/z_{theor}	Δppm	MS^2	Elemental composition [M]	m/z_{theor}	Δppm
Se-geranyl-selenohomocysteine	**318.0970**	13.3	$C_{14}H_{24}NO_2Se^+$	318.0967	-1.02	181.9690	$C_4H_7NO_2Se$	181.9705	13.69
						136.1229	$C_{10}H_{15}$	136.1246	12.97
						135.1163	$C_{10}H_{14}$	135.1168	3.93
						107.0831	C_8H_{10}	107.0855	22.88
Se-(ω-hydroxy-geranyl)-selenohomocysteine	**336.1075**	11.1	$C_{14}H_{26}NO_3Se^+$	336.1072	-0.77	318.0946	$C_{14}H_{23}NO_2Se$	318.0967	6.55
						181.9717	$C_4H_7NO_2Se$	181.9705	-1.23
						154.1356	$C_{10}H_{17}O$	154.1352	-2.5
						135.9659	C_3H_5NSe	135.9660	0.72
						135.1149	$C_{10}H_{14}$	135.1168	14.37
						107.0836	C_8H_{10}	107.0855	17.79
Se-(ω-carboxy-geranyl)-selenohomocystine	**364.1019**	11.8	$C_{15}H_{26}NO_4Se^+$	364.10216	0.76	318.0965	$C_{14}H_{23}NO_2Se$	318.0967	0.65
						181.9708	$C_4H_7NO_2Se$	181.9705	3.85
Se-(ω-hydroxy-geranyl)-selenohomocystine	**517.0708**	10.1	$C_{18}H_{33}N_2O_5Se_2^+$	517.0714	1.25	334.0940	$C_{14}H_{23}NO_3Se$	334.0923	-5.17
						316.0796	$C_{14}H_{21}NO_2Se$	316.0810	4.53
						181.9679	$C_4H_7NO_2Se$	181.9705	8.8
						135.1169	$C_{10}H_{14}$	135.1168	0.54
						89.0605	$C_4H_8O_2$	89.0597	8.7

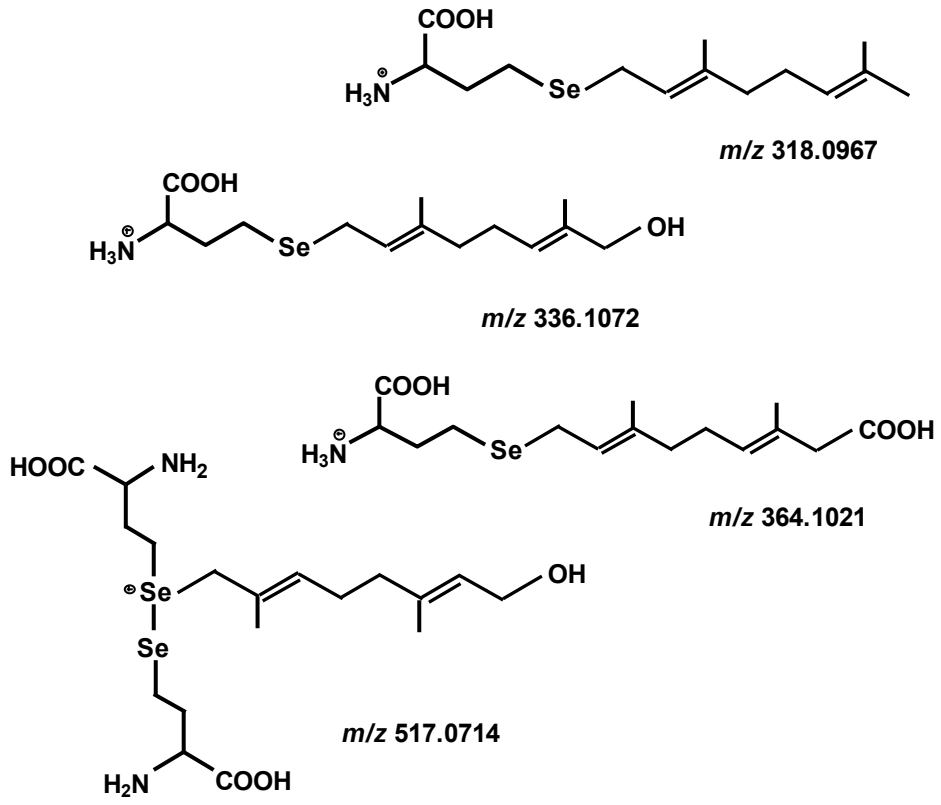

Figure 5.13. The structure of hydrophobic derivatives of SeHCy.

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Since the concentration of the compound *m/z* 364.1019 ($C_{15}H_{26}NO_4Se^+$ ($[M+H]^+$, Δppm 0.76) was not sufficient for successful fragmentation experiments, the structure of the molecule was elucidated on the basis of fragmentation-degradation relationships. This compound also contains the supposed geranyl-SeHCy fragment ($C_{14}H_{24}NO_2Se^+$, m/z 318.09647, Δppm 0.65) and its calculated molecular formula differs in a carboxyl group from geranyl-SeHCy. The retention time of the molecule (t_R=11.8 min) also supports the presence of a functional group that is less hydrophilic compared to a hydroxyl group.

The identification of the rest of the selenocompounds (Table 5.5 and Figure 5.14.) was performed on the basis of the same considerations (additional mass spectrometric results are shown on Figure A2 in the Appendix). Taking the retention times into account provided particularly useful information for the structure elucidation of unknown compounds. This consideration supported for example the discrimination between amino acid and lipid derivatives.

Table 5.5. Mass spectrometric results of other newly identified SeMet derivatives.

Name	*m/z exp*	t_R	Elemental composition $[M+H]^+$	*m/z theor*	Δppm	MS^2	Elemental composition [M]	*m/z theor*	Δppm
N-acetyl-*Se*-methyl-selenomethionine	**254.0288**	1.0	$C_8H_{16}NO_3Se^+$	254.029	0.88	144.0609	$C_6H_9NO_3$	144.0655	17.76
N-(3-hydroxy-propionyl)-selenomethionine	**270.0237**	1.3	$C_8H_{16}NO_4Se^+$	270.0239	0.77	210.0007	$C_6H_{12}NO_2Se$	210.0028	5.01
						180.9777	$C_5H_8O_2Se$	180.9762	8.18
						88.0417	$C_3H_5NO_2$	88.0393	27.12
N-(4-aminobutyryl)-selenomethionine	**283.0553**	2.1	$C_9H_{19}N_2O_3Se^+$	283.0555	0.85	220.0173	$C_8H_{13}NOSe$	220.0235	-28.4
						198.0069	$C_5H_{11}NO_2Se$	198.0028	20.93
						180.9746	$C_5H_8O_2Se$	180.9762	8.18
						151.9949	C_4H_9NSe	151.9973	-15.9
						108.9554	C_2H_4Se	108.9551	-2.76
						56.0516	C_3H_5N	56.0495	-38.9
γ-glutamyl-*Se*-methyl-selenomethionine	**341.0606**	6.3	$C_{11}H_{21}N_2O_5Se^+$	341.061	1.24	212.0195	$C_6H_{13}NO_2Se$	212.0184	-5.08
						194.9959	$C_6H_{10}O_2Se$	194.9919	4.53
						151.9975	C_4H_9NSe	151.9973	1.34
						130.0494	$C_5H_7NO_3$	130.0499	3.64
						84.0425	C_4H_5NO	84.0444	22.76
						56.0502	C_3H_5N	56.0495	-13.2

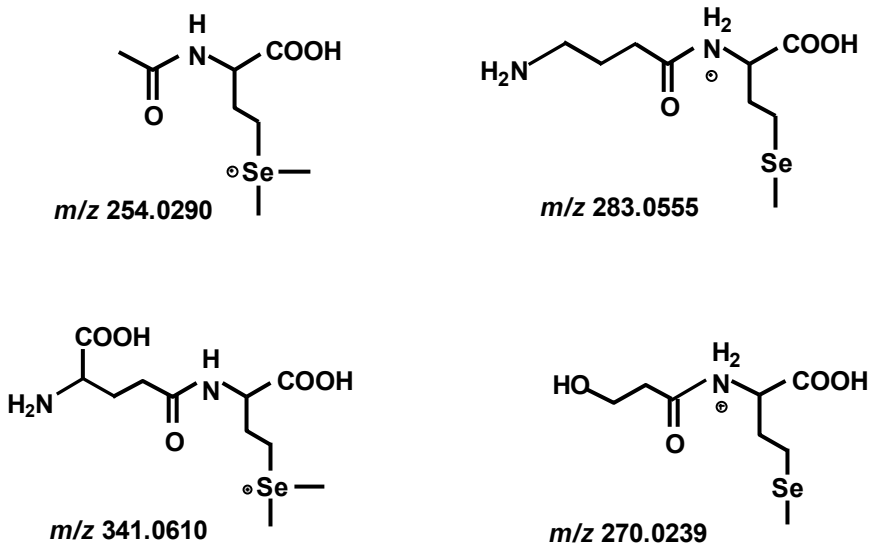

Figure 5.14. The structure of SeMet derivatives.

I would like to introduce one of the most relevant members of the newly identified selenomethionine derivatives (Figure 5.14.). This compound (*m/z* 341) was the only new selenocompound found in Brazil nut. Figure 5.15.a shows its fullscan spectrum and isotopic distribution. Figure 5.15.b and c show the fragmentation of this selenometabolite with two different collision energies (CE 20 eV and 10 eV). With regards to fragmentation, an MS/MS experiment is usually considered successful, if the MS/MS spectrum contains as many fragments as possible and enables the identification of the molecule. In general, the collision energies that are required for the successful fragmentation of a molecule can be estimated on the basis of its molecular mass. Nonetheless, the structure of the molecule can remarkably influence its fragmentation, therefore the individual optimization of the applied collision energy was usually required.

It can be seen that a collision energy of 20 eV was too high for the fragmentation of this molecule. The MS/MS spectrum (Figure 5.15.b) is dominated by low molecular weight fragments that carry little information about the structure of the molecule. Decreasing the collision energy to 10 eV resulted in a richer spectrum (Figure 5.15.c) with many characteristic fragments that aided the identification of the compound. The fragments *m/z* 212, 195 and 152 are characteristic to *Se*-Met-SeMet, while *m/z* 130 and 84 are the characteristic fragments of γ-Glu containing molecules. Consequently, this compound was identified as γ-glutamyl-*Se*-methyl-selenomethionine (Figure 5.15.d).

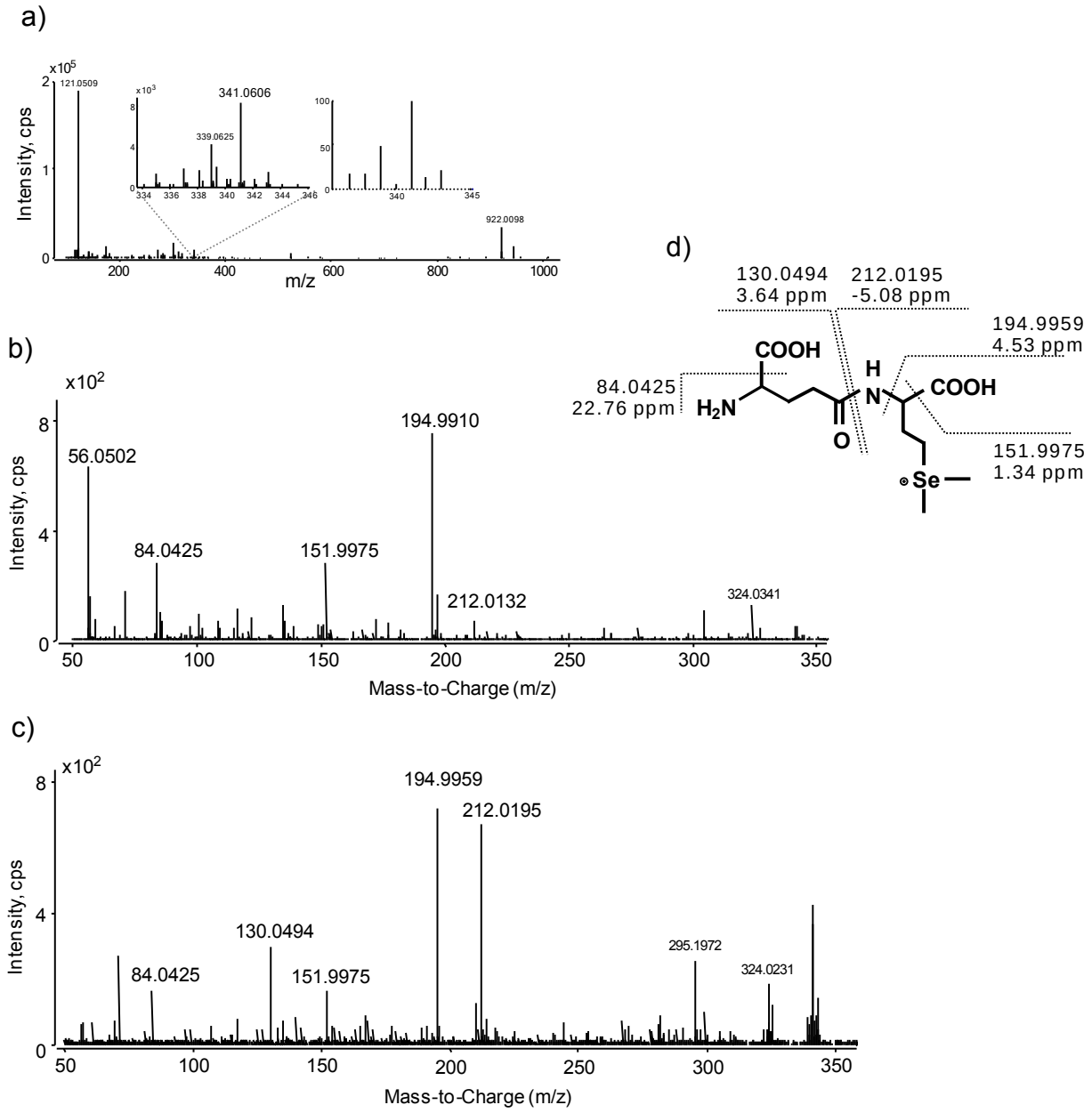

Figure 5.15. The results of the mass spectrometric analysis of γ-Glu-*Se*-Met-SeMet. a) Fullscan spectrum with the experimental and theoretical isotopic distribution. b) Ms-MS spectrum with CE 20 eV. c) Ms-MS spectrum with CE 10 eV. d) Structure of the molecule with the characteristic fragments.

Altogether, the manual pattern exploration could contribute to the list of assigned selenium species of *L. minor* with eight new compounds. I would like to note here that when it was feasible, previously described, characteristic fragments (such as the SeHCy residue or the *m/z* 130.05 – 84.04 characteristic couple of fragments of γ-Glu containing molecules) were utilized to facilitate the identification of new compounds. In addition, many possible chemical

and biological aspects were also taken into account. However, the ultimate assignment of the structures can only be achieved by a synthetic approach assisted by NMR experiments.

5.1.2.6. Comparing the Se speciation of the two plants

There was only one previously unreported compound that could be assigned from Brazil nut: γ-glutamyl-*Se*-methyl-selenomethionine. This compound was also found in *L. minor* by targeted search. All the 18 newly identified compounds from *L. minor* were retrospectively screened in the spectra of the *B. excelsa* as well, in order not to overlook any of them because of their lower concentration. Finally, none of these compounds were detected in Brazil nut.

As a result of this approach we found that in spite of their taxonomic proximity the two plants accumulate Se in their seeds in remarkably different organic forms. It confirms that the speciation of Se in different plants is basically determined by the amount of Se they accumulate and it is not closely related to taxonomy. This concept was also suggested by Freeman and coworkers (2006) in the case of *Brassicaceae* species.

On the other hand, a common feature of selenocompound distribution could also be observed in the two *Lecythidaceae* species. MetSeCys that plays an important role in the Se tolerance of various plant species was not detected in the samples. At the same time, the γ-Glu derivative of MetSeCys was an abundant selenium species in *L. minor* and the γ-Glu derivative of SeMet was present in the seeds of both plants. Since the formation of γ-Glu derivatives is a consequence of high γ-Glu-transpeptidase activity observed in the case of oxidative stress, our findings highlight the relevance of this mechanism in the adaptation of the plants to Se accumulation.

Since many plants accumulate Se in the form of the Se analogues of their S-containing metabolites, we intended to determine if this feature is also characteristic for *B. excelsa* and *L. minor*. For this reason, the sulfur containing fractions of the extracts were also collected throughout the multidimensional clean-up procedure (data not shown). Finally, the sulfur analogues of all the newly identified selenocompounds were screened in the S-containing fractions, but none of them could be detected. These results suggest the existence of Se-specific metabolic pathways in these Se-accumulator plants.

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5.1.3. Screening for selenocompounds in negative ion mode

Although most Se speciation studies rely on positive ion mode analysis our samples were also analyzed in negative ion mode in order to cover the highest variety of previously unidentified Se species. SeCysta and $SeHCy_2$ were also detected in negative ion mode, but had an in-source fragmentation pattern different from the positive ion mode fragmentation. There was only one unknown compound (m/z 479.24101) detected in negative ion mode that had analogous retention time and ionization characteristics as one of the compounds detected in positive ion mode. All the Se species detected in the negative ion mode experiments are displayed on Table A2 in the Appendix. Unfortunately the intensities of these compounds were not sufficient to carry out successful MS/MS experiments. Nevertheless these results highlight the importance of the application of negative ion mode analyses in comprehensive Se speciation studies.

The MS spectrum of the most noticeable compound (*m/z* 428.2) is displayed on Figure 5.16. The fact that this compound cannot be ionized in positive ion mode suggests that it is not an amino acid derivative (since amine groups are always subjected to proton adduct formation). The mass defect of the compound (+ 0.2) suggests that it contains unusually high number of A+1 type elements, such as carbon and hydrogen. One of the possible elemental compositions that can be calculated for this compound is $C_{22}H_{39}NO_2Se^-$ $[M-H]^-$, Δppm 3.34.

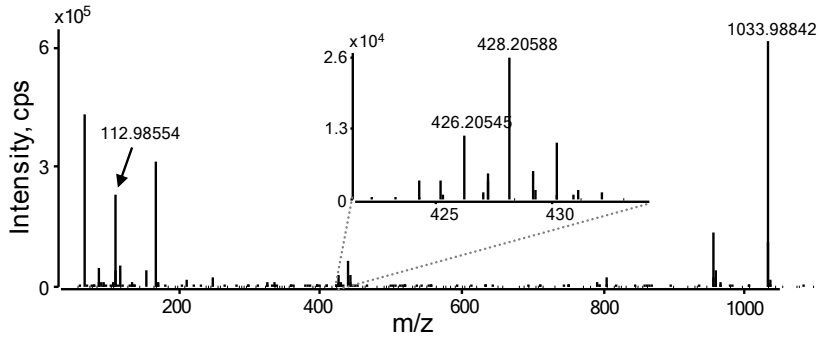

Figure 5.16. Fullscan spectrum of one of the compounds detected form *L. minor* in negative ion mode (*m/z* 428.2059).

5.2. Proteomic analysis of monkeypot nut seeds

Proteomic studies carried out on *Lecythidaceae* species have mainly focused on the analysis of *B. excelsa* proteins. The defatted seeds of Brazil nuts contain around 50 % of proteins that are remarkably rich in sulfur-containing amino acids. Despite that Brazil nut seeds contain a relatively large amount of protein, this protein fraction consists of only three main types of seed storage proteins, namely 2S, 7S and 11S proteins. One of its major protein fractions, 2S albumin, contains around 30% of methionine (Met) and cysteine (Cys) (Ampe et al. 1986, Altenbach et al. 1987). These sulfur containing amino acids are prone to be replaced by their Se analogues to form selenium containing proteins (Chunhieng et al. 2004, Moreno et al. 2004, Jayasinghe and Caruso 2011). Selenium accumulation in seed proteins is a suitable way for Se storage and it also represents an elemental defense mechanism that provides reproductive advantages to Se accumulator plants (Prins et al. 2011). For instance, the seeds of *Lecythis* species contain such a high amount of Se that can cause acute toxicity in animals (Ferri et al. 2004).

The main challenges in selenoproteomic analysis of plants are the results of the relatively low amount of Se and its non-specific incorporation into proteins (Bianga et al. 2013, Cheajesadagul et al. 2014). Therefore, the screening and preconcentration of the Se-containing protein fractions is crucial (Bianga and Szpunar 2013, Dernovics et al. 2012). For instance, previous studies demonstrate that the application of sophisticated multi-step fractionation, nano-HPLC separation and state-of-the-art MS detection was necessary to ensure successful mass spectrometric analysis of Se containing proteins of Brazil nuts (Dernovics et al. 2007b, Jayasinghe and Caruso 2011).

In the current study, however, the remarkably high Se content of the defatted monkeypot nut seeds (4480 ± 22 mg Se kg^{-1} DW) made the application of a shotgun proteomics approach suitable for the characterization of Se containing proteins and selenopeptides of the sample. The main advantage of a shotgun proteomic approach is that it allows the analysis of a complex mixture of proteins (McSheehy et al. 2005, Fercha et al. 2013). The combination of a straightforward sample preparation procedure (*i.e.*, water extraction followed by acetone precipitation and reversed phase SPE), the separation of the tryptic peptides by RP-HPLC and the application of high resolution mass spectrometric detection provided adequate information for the proteomic analysis. The analysis of a complex protein fraction resulted in a relatively high coverage regarding the whole seed protein fraction. Our objective was to compare the protein fraction of *L. minor* seeds to Brazil nut proteins and peptides and determine the differences. We also intended to extend the current knowledge available on the proteomics of *Lecythidaceae* by determining the sequence of previously unknown selenopeptides.

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5.2.1. Database dependent protein identification

Identification of monkeypot nut seed proteins was based on the analysis of the MS-MS spectra of tryptic peptides. Our major objective was to identify Se containing peptides, but the automatic fragmentation of the most intensive peptides of the sample was also carried out in order to achieve a more complete characterization of the protein fraction and identify as many proteins as possible. This approach resulted in the identification of several peptides that contributed to the reliable identification of proteins.

After the mass spectrometric experiments, the results were compared to a database of selected proteins. The ideal protein database would consist of the reviewed proteins (confirmed by an expert group) of the actual organism or its close taxonomical relative. However, Uniprot database contains only one protein known from *L. minor* and 441 proteins from *Lecythidaceae* (only five of which are reviewed). The attempt to use a database that contained only the reviewed proteins of *Lecythidaceae* and the well-characterized model plant *Arabidopsis* (14142 reviewed proteins) did not provide reasonable results.

Finally the protein database for the identification was created from the known proteins (both reviewed and unreviewed) of the order *Ericales*. On the whole, five different proteins were identified from the *L. minor* sample, both of which had been detected in *B. excelsa* before (Dernovics et al. 2007b, Jayasinghe and Caruso 2011). This result is possibly due to the taxonomical proximity of the two species.

For the validation of the protein identification results, a concatenated database was applied and the default settings were changed in order to avoid false positive results. Both false discovery rate (FDR) and false negative rate (FNR) was set to 1% that resulted in the decrease of the number of validated proteins to eight. The spectra of the assigned peptides were also manually checked in order to overcome the frequently overlooked weaknesses of automated protein identification (Nilsson et al. 2010). We have found that the spectra of several assigned peptides did not match the proposed sequences. As a result of this approach, the false positive hits were excluded and the number of validated proteins was finally reduced to five (Table 5.6).

Most of the identified peptides belonged to 11S globulin (Q84ND2), therefore, a relatively high coverage (31 %) was obtained for this protein. In previous studies 11S globulin was found to be one of the major proteins of *B. excelsa* seeds too (Dernovics et al. 2007b, Jayasinghe and Caruso 2011). An 11S globulin-like protein was also identified (A0EM48) with a relatively low coverage (2%) but with 100% confidence. Moreover, we identified 2S albumin (B6EU54) and 2S sulfur-rich seed storage proteins (P04403 and P0C8Y8) from the *L. minor* protein extract.

Table 5.6. Protein database search results. Se-containing peptides are highlighted. 'B' denotes to selenomethionine.

Accession number	Description	Organism	Software coverage	Calculated coverage	Methionine coverage	Peptides						
						Sequence	m/z	z	Mass	Mass error	Start	End
Q84ND2	11S globulin	*B. excelsa*	31	32	25	VQIVDHR	289.499	3	866.4822	2.24	360	366
						VIRPP	291.1921	2	581.3768	0.03	260	264
						HFFLAGNIQR	401.5506	3	1202.637	4.78	185	194
						CAGVAALR	409.2206	2	817.4338	1.15	64	71
						LYYVTGR	500.2654	2	999.5234	2.27	90	97
						NGETVFDDNLR	583.2778	2	1165.5482	0	367	376
						VQHTASDLNQLDQNPR	612.6377	3	1835.8983	0.47	169	184
						NTIRPQGLLLPVYTNAPK	665.7175	3	1995.1377	0.27	72	89
						KGDIIAIPAGVALW	712.4186	2	1423.8298	0.62	142	155
						GILGVLMPGCPETF	745.8737	2	1490.74	1.32	98	111
						GILGVLBPGCPETF	769.8457	2	1538.684	0.97	98	111
						LTTVNSLKVPILTF	773.4643	2	1545.9212	2.46	310	323
						FIQNIDNPAEDFYNPR	1012.4823	2	2023.9572	3.18	290	306
						VPILTFLQL	1043.6495	2	2086.2916	0.39	318	326
B6EU54	2S albumin	*B. excelsa*	18	29	29	GEQMR	310.6446	2	620.2818	4.78	112	116
						CEGLR	317.6519	2	634.2964	1.83	93	97
						EMQPR	330.6594	2	660.3114	2.77	107	111
						GEQBR	334.615	2	668.2226	5.61	112	116
						EBQPR	354.6315	2	708.2556	2.96	107	111
						QQMLSH	372.1772	2	743.347	4.53	46	51
						pyro-QQBLSH	396.1521	2	791.2968	2.43	46	51
						CNLSPQR	437.7131	2	874.4188	1.18	129	135
						MAENLPSR	459.229	2	917.4506	0.15	121	128
						BAENLPSR	483.2005	2	965.3936	1.73	121	128
P04403	2S sulfur-rich storage protein 1	*B. excelsa*	15	32	32	GEQMR	310.6446	2	620.2818	0.26	113	117
						CEGLR	317.6519	2	634.2964	1.83	94	98
						EMQPR	330.6594	2	660.3114	2.77	108	112
						GEQBR	334.615	2	668.2226	5.61	113	117
						EBQPR	354.6315	2	708.2556	2.96	108	112
						QQMLSH	372.1772	2	743.347	4.53	47	52
						AENLPSR	393.7075	2	786.4076	3.04	123	129
						pyro-QQBLSH	396.1521	2	791.2968	2.43	47	52
						MQQEEMQPR	588.7606	2	1176.5138	0.3	104	112
						BQQEEMQPR	612.7319	2	1224.4564	1.29	104	112
						BQQEEBQPR	636.7047	2	1272.402	0.38	104	112
						QQBLSH	774.2685	1	774.2685	0.14	47	52
P0C8Y8	2S sulfur-rich storage protein 2	*B. excelsa*	5	15	19	MYLR	291.6574	2	582.3074	3.11	62	65
						BYLR	315.6289	2	630.2504	1.03	62	65
						CEGLR	317.6519	2	634.2964	4.75	103	107
						CNLSPQR	437.7121	2	874.4168	1.18	138	144
A0EM48	11S globulin-like protein	*Actinidia chinensis*	2	2	0	GVLYK	290.1775	2	579.3472	4.04	139	143

The 2S sulfur-rich seed proteins are the most well characterized proteins of the *Lecythidaceae* family (Sun et al. 1987). They gained special attention primarily due to their nutritional value but also because of their allergen properties (Miernyk and Hajduch 2011, Nakamura and Teshima 2013). The sequences of these proteins are quite similar, but both of them contain some unique peptides that facilitate their identification. The results of the protein identification are summarized in Table 5.6.

The coverage was calculated by the software based on the sequence of the precursor protein. In the case of 2S albumin and 2S sulfur-rich proteins, however, the signal- and pro-peptides are removed during the maturation of the proteins. The revised coverage was calculated based on the sequence of the mature proteins. Methionine coverage is the coverage of methionine containing peptides.

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5.2.2. Searching for selenocysteine containing peptides

As it was mentioned before, Se can incorporate into proteins in the form of SeMet or SeC. Several SeMet-containing peptides were detected in the sample, but only one selenopeptide was identified through the automated protein database search as a Sec-containing peptide. The analysis of the MS-MS spectrum of the peptide, however, revealed that the putative Sec-containing fragments ($y_{5'}$, $y_{6'}$ and $y_{7'}$) are not present (Figure 5.17.). Further inspection of the spectrum confirmed that this selenopeptide contains a SeMet instead of a Sec.

Nonetheless, the MS-MS spectra were also searched for possible Sec-containing peptides on the basis of (i) the characteristic SeC immonium ions (Sec: *m/z* 124 and carbamidomethylated-Sec: *m/z* 181) and (ii) by targeting the Se-analogues of Cys-containing peptides. Finally, no Sec-containing peptides were detected in the protein fraction of *L. minor*. Our results confirm the existence of specific detoxification pathways that protect *L. minor* from the toxic effects of Se.

The possible explanations of the exclusion of Sec from proteins include the (i) depletion of SeC by the formation of non-protein amino acids (Terry et al. 2000), (ii) the inability of $tRNA^{Cys}$ to be charged by SeC (Burnell and Shrift 1979) and (iii) the removal of SeC containing proteins through enzymatic processes or incorrect protein folding (Goldenberg 1992, Dimkovikj et al. 2015). I would like to note here, that despite of all these possible mechanisms, SeC was recently found to be present in the proteins of non-accumulator plants (Bianga et al. 2013, Cheajesadagul et al. 2014). Our results suggest that the exclusion of SeC is probably a complex process that is more efficient in Se accumulator plants than in non-accumulator species.

5.2.1. Trends in Se/S substitution

Theoretically, the tryptic digestion of the identified proteins would produce numerous methionine-containing peptides. Actually, many of these peptides could be detected in the monkeypot nut sample and the calculated coverage for methionine-containing peptides was in the range of 19-32% (Table 5.6.). In order to extend the identification of selenopeptides from *L. minor* we created an exact mass database of the putative Se analogues of all Met-containing tryptic peptides of the identified proteins. We also included in the database the previously identified selenium containing peptides from Brazil nuts (Moreno et al. 2004, Dernovics et al. 2007b).

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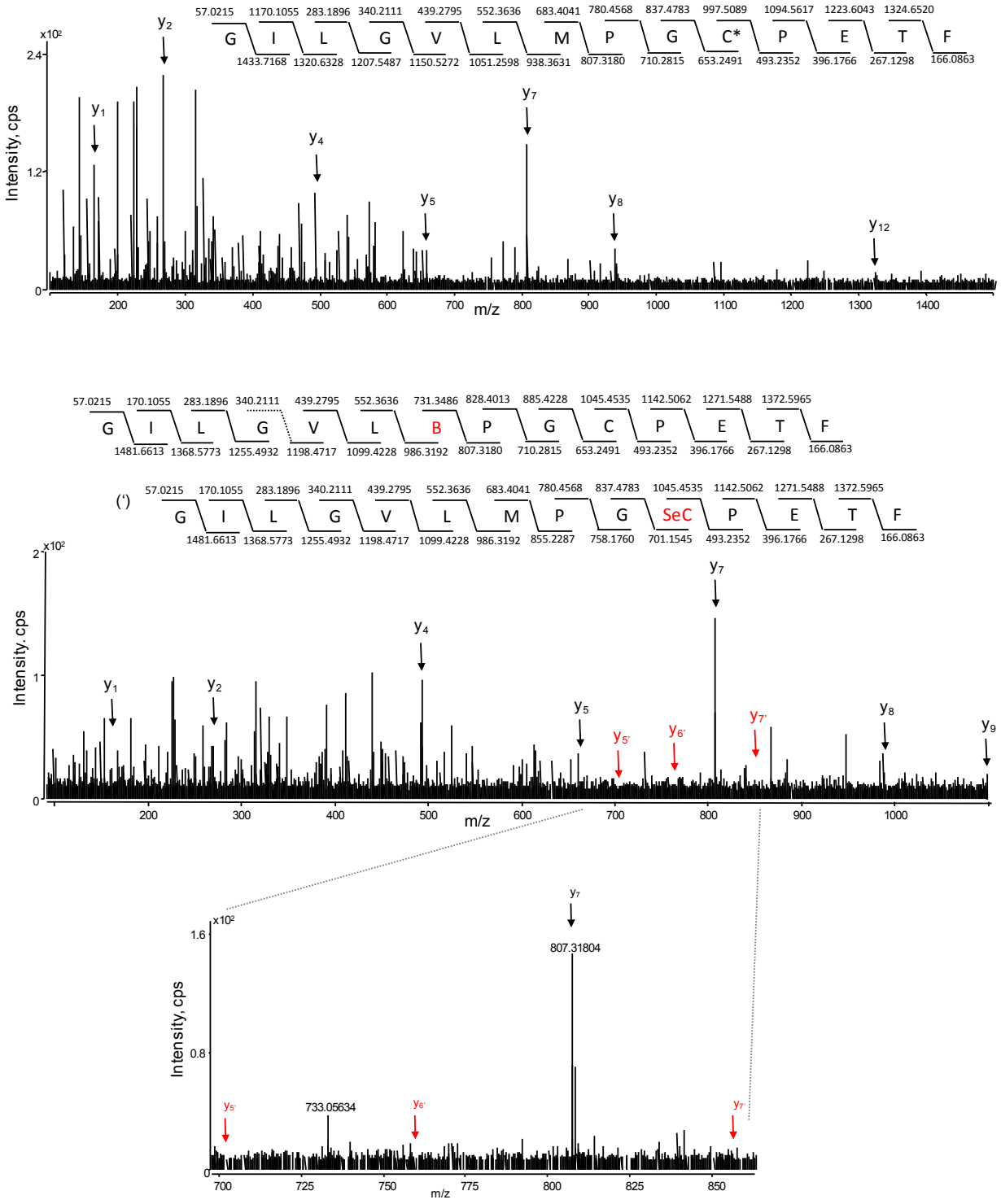

Figure 5.17. The MS-MS spectra and the identification details of the peptide GILGVLMPGCPETF and its selenium analogue. In the peptide sequence C* corresponds to carbamydomethyl-cysteine, B denotes to selenomethionine and SeC represents selenocysteine. Red arrows show the missing y-ions of the putative SeC containing peptide.

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The searching of our experimental data against this peptide database contributed to the identification of five selenopeptides. Table 5.7. summarizes all selenopeptides that were identified through the previously mentioned database dependent protein identification and peptide database search. This kind of complex data-mining strategy contributed to the identification of seven previously unreported selenopeptides (highlighted in Table 5.7.).

Table 5.7. The list of identified selenopeptides. The previously unreported peptides are highlighted.

Sequence	Theoretical $[M+H]^+$	*m/z*		Mass difference (ppm)
		Theoretical	Experimental	
BYLR	**630.2514**	**315.6296**	**315.62922**	**1.20**
BYMR	648.20782	324.60781	324.60719	1.91
GEQBR	**668.2267**	**334.6172**	**334.6150**	**6.64**
EBQPR	**708.25795**	**354.6329**	**354.6315**	**3.87**
pyro-QQBLSH	791.29505	396.15143	396.1521	-1.69
MMMBR, MMBMR, MBMMR, BMMMR	747.22547	374.11664	374.11594	1.87
MMBBR, MBMBR, MBBMR, BBMMR, BMBMR	795.12596	398.06688	398.08775	- 5.24
MBBBR, BMBBR, BBMBR, BBBBMR	**843.11446**	**422.06113**	**422.06030**	**1.96**
BBBBR	**891.05896**	**446.03338**	**446.03268**	**1.57**
BAENLPSR	965.39555	483.20168	483.2005	2.43
BQQEEMQPR, MQQEEMQBPR	1224.4312	612.7195	612.7319	-2.02
BQQEEBQPR	**1272.4027**	**636.7052**	**636.7051**	**0.24**
GILGVLBPGCPETF	**1538.6828**	**769.8453**	**769.8457**	**-0.51**

These results prove the relationship between the *B. excelsa* and *L. minor* on the protein level but also demonstrate the differences resulting from the different level of Se accumulation. For instance, some Se analogues of the peptide MMMMR were previously detected in *B. excelsa* (the large subunit of 2S albumin, shown at Figure 5.18.) but only those that contain one or two selenomethionines (*m/z* 374 and *m/z* 398). In our study, the peptides that contain three or four selenomethionines (*m/z* 422 and *m/z* 446) could also be detected in the *L. minor* sample (Figure 5.18.).

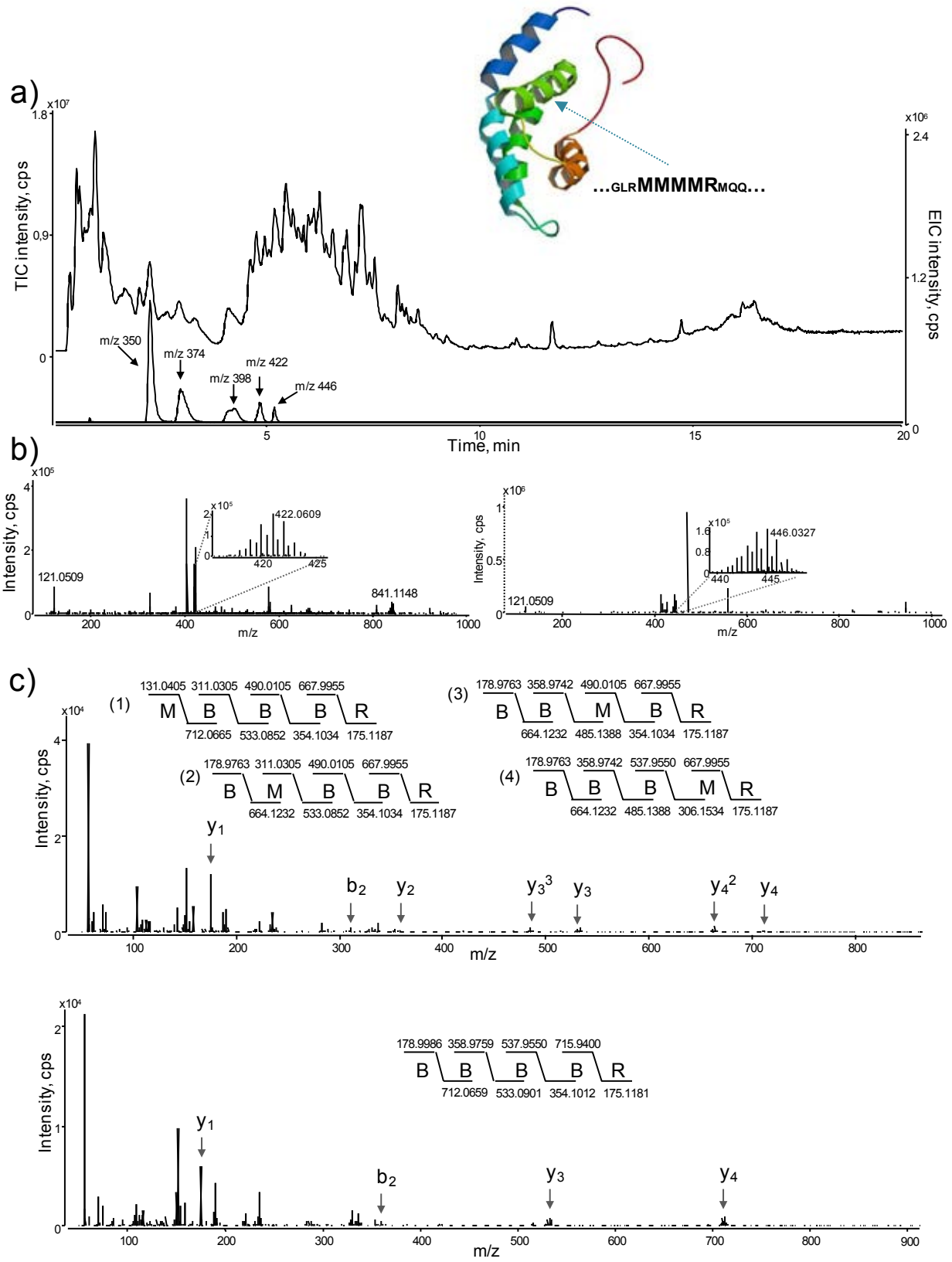

Figure 5.18. The 3D structure of the large subunit of 2S albumin (Alcocer et al. 2002, Rundqvist et al. 2012) and the results of the mass spectrometric analyses of the sulfur and selenium analogues of the peptide MMMMR. a) The TIC and the EIC of the analogues: *m/z* 350, *m/z* 374, *m/z* 398, *m/z* 422 and *m/z* 446. b) Full scan spectra of the newly identified selenopeptides. c) MS-MS spectra and identification details of the newly identified selenopeptides.

Figure 5.18.a shows the total ion chromatogram (TIC) of the tryptic digest with the extracted ion chromatograms (EIC) of the five analogues of the peptide MMMMR. It can be observed that the retention time of the peptides increase according to their Se content (comparable with the retention times of polyselenides in Table 5.2.). As regards to the uneven shape of the peaks it is worth noting that except for the peptides MMMMR and BBBBR, these peaks contain a mixture of isomers (Table 5.7.). The number of isomers is the highest in the case of the peptide *m/z* 398 (BBMMR, BMBMR, BMMBR, MMBBR and MBMBR) that results in a wide chromatographic peak (almost 1 min). Nonetheless, the co-elution of isomers does not only degrade the chromatographic profile of selenopeptides, but also hinders their individual identification.

The MS/MS spectrum of the peptide *m/z* 422 also contains fragments from four different isomers (the fragments that are different from the first isomer are indicated by superscripts, see also the *m/z* of y and b ions on Figure 5.18.c). This phenomenon is frequently observed in selenoproteomic studies due to the non-selective incorporation of SeMet into selenium containing proteins. The simultaneous occurrence of different fragments usually complicates or even hinders the automated identification or *de novo* sequencing of selenopeptides.

The relative concentration of the peptides can be estimated from their extracted ion chromatograms with some considerations. The peptides are separated from the void, where ion suppression is the most serious but some degree of suppression still might be possible. Moreover, the efficiency of tryptic digestion might be different for each peptide analogue and the gradient also slightly increases during their retention. Therefore, the accurate quantification process would require isotope dilution based HPLC-ICP-MS coupling (Giusti et al. 2005) but the integrated EIC data can serve for semi-quantitative analysis.

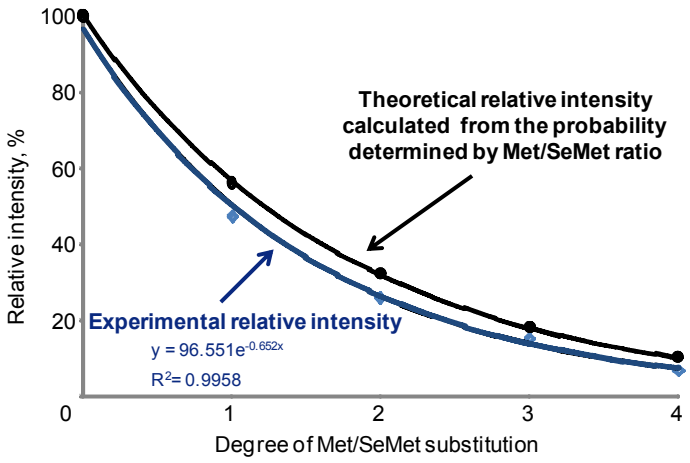

Figure 5.19. Theoretical and experimental relative intensities of SeMet substituted peptides.

If the relative abundance of the sulfur containing peptide (MMMMR) is considered to be 100 %, then the relative intensities of the Se-containing peptides account for 47 %, 25 %, 15 % and 7 %, respectively, as their Se content increases from one to four selenomethionines (Figure 5.19.). These ratios show an exponential correlation with a high coefficient of determination ($R^2 > 0.99$).

Due to the lack of a SeMet-specific tRNA, the non-selective incorporation of SeMet into proteins occurs through methionine tRNAs and its extent is usually considered to depend on the Met/SeMet ratio in the plant tissue. Considering the molar S/Se ratio (1.4 : 1) calculated from total sulfur (2560 mg kg^{-1}, molar weight: 32.07 g mol^{-1}) and Se concentration (4480 mg kg^{-1}, molar weight: 78.96 g mol^{-1}) should have resulted in a much lower level of selenopeptides. However, calculating the probability of the incorporation of SeMet into proteins on the basis of the direct quantification of Met and SeMet in the *L. minor* sample provided a better approximation to our experimental results.

5.8. Concentrations and ratios of different forms of S and Se in the *L. minor* sample.

	Sulfur	**Selenium**	**Ratio**
Total elemental concentration, g kg^{-1}	2.56	4.48	~ 0.6
Molar concentration, mmol kg^{-1}	80	57	~ 1.4
Cys and SeCys conc., mmol kg^{-1}	27	0.01	~ 2700
Met and SeMet conc., mmol kg^{-1}	39	51	~ 0.7

The ratio of Met/SeMet was determined to be 1:1.3. In practice, it means if the incorporation of SeMet solely depends on the ratio of Met/SeMet, in each elongation step the probability of SeMet incorporation would be 57 %. In this case, the theoretical relative intensities of S/Se peptide analogues will be as shown on Figure 5.19. This calculation correlates well with our experimental results that suggest that the level of substitution of Met by SeMet is closely related to the ratio of Met/SeMet in the tissue.

However, it can be also seen that the experimental data stay below the theoretical intensities. It could be the result of the inhibition of protein elongation or protein folding by SeMet incorporation (like in the case of SeC), but the extent of this mechanism is not significant. Note, that since the quantified peaks usually contain a mixture of isomers (Table 5.7), this correlation also implies that SeMet is probably randomly incorporated and does not substantially inhibit protein elongation and protein folding. Such a correlation could not have been reported for

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seleno-amino acids before because this kind of methionine-rich tryptic peptides that contain four methionine residues in a successive order have never been found in selenoproteomic studies.

5.2.2. Manual screening and sequencing of unknown selenopeptides

The MS spectra were also manually screened for Se-containing peptides based on their characteristic Se isotopic distribution pattern. The manual search for selenopeptides resulted in the detection of over 60 peptides, approximately half of which were present in a concentration that made successful fragmentation possible. A database search was performed to find the sulfur analogues of these peptides as well. The sulfur analogues were usually present in a higher concentration that provided the opportunity to carry out successful MS-MS experiments to facilitate their identification. These results supported the identification of the corresponding selenopeptides too. For example, the MS-MS spectra of the three S/Se analogues of the peptide FMDMRTK contained complementary information that made the complete *de novo* sequencing possible (Figure 5.20.).

Table 5.9. shows the list of selenopeptides, that were sequenced by manual *de novo* sequencing (see MS/MS spectra and the details of the identification in the Appendix, Figure A5). The application of manual *de novo* sequencing was inevitable because automated *de novo* sequencing of the selenopeptides was unsuccessful. The most possible reason for this fact is the inadequacy of the currently available software to handle selenopeptides because of the relative complexity of spectra due to (i) the broad isotopic pattern of Se and (ii) the presence of several isomers. The Blast analysis of the manually sequenced peptides was also carried out. The peptide RIYENABMAPR could be assigned to 11S globulin from *B. excelsa* (Q84ND2).

Table 5.9. The list of selenopeptides identified by manual *de novo* sequencing.

Sequence	Theoretical $[M+H]^+$	Experimental $[M+H]^+$	Experimental m/z	Mass difference ppm
FMDBRTK	967.3827	976.3725	488.6891	10.5
FBDBRTK	1024.327	1024.3177	512.6616	9.08
BGENLPSR	951.3806	951.3806	476.1927	0.01
MMBSK, MBMSK, BMMSK	675.2110	675.2096	338.1076	2.07
BBMSK, BMBSK, MBBSK	723.1555	723.1546	362.0810	0.97
ESFBSGSR	948.3326	948.3329	474.6681	-0.32
FGKNVC*ABR	1130.4680	1130.4293	565.7198	34.23
MBGMAENPESR	1300.456	1300.5286	650.7686	-55.82
RIYENABMAPR	1399.6060	1399.5903	700.3002	11.2

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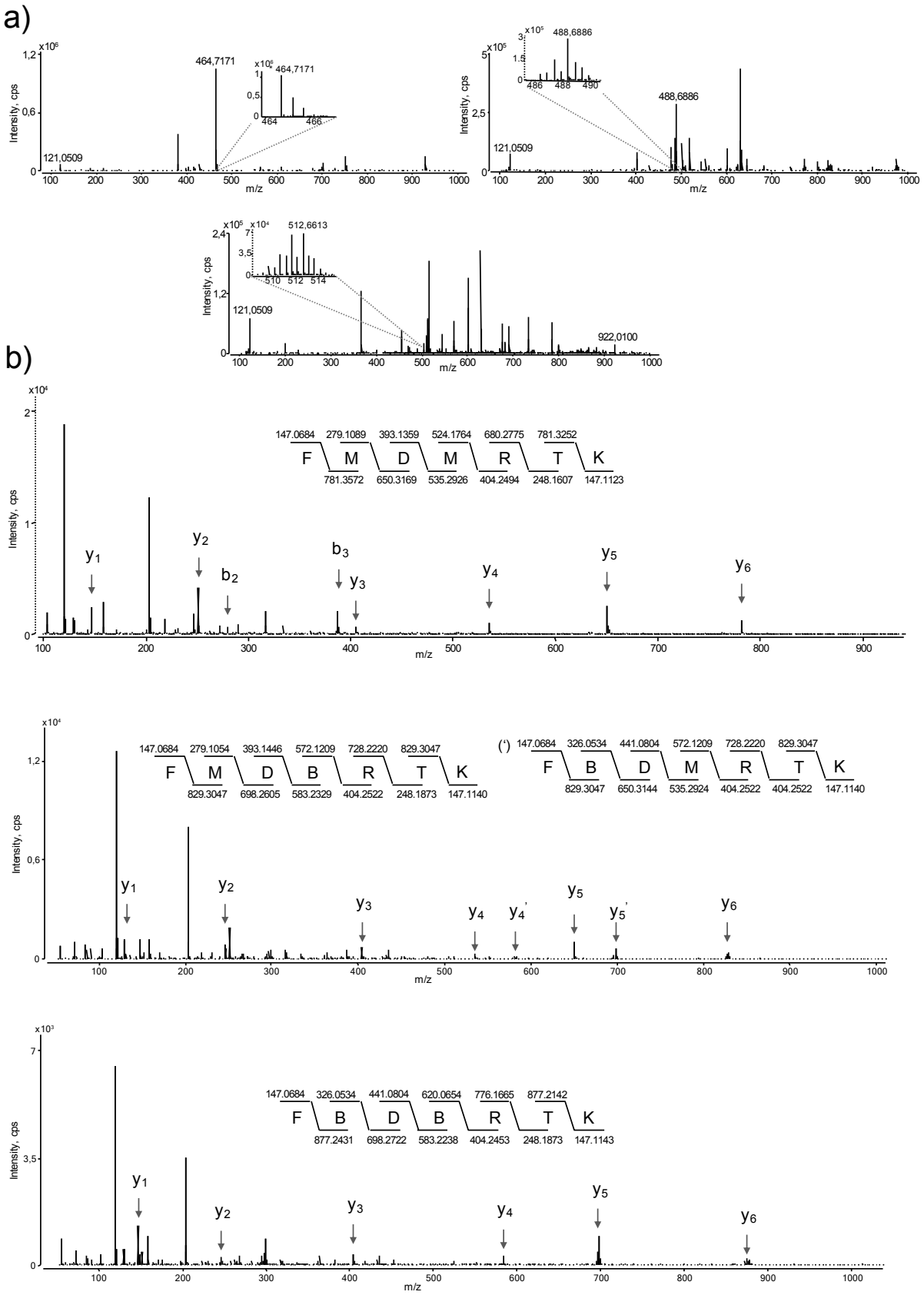

Figure 5.20. The results of the mass spectrometric measurements of the sulfur and selenium analogues of the peptide FMDMRTK. a) The full scan spectra of the three peptides *m/z* 465 (FMDMRTK), *m/z* 489 (FBDMRTK and FMDBRTK) and *m/z* 513 (FBDBRTK). b) The MS-MS spectra and the identification details of the identified peptides.

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6. CONCLUSIONS

6.1. Selenometabolomics

In our study, the application of sophisticated coupled analytical techniques allowed the identification of several new selenocompounds that remained undetected before. Our results confirm that the high concentration of Se in hyperaccumulator species induces the formation of unusual organic selenocompounds. We identified a considerable number of SeHCy, SeMet and Se-Met-SeMet derivatives. The most relevant of these belong to the group of SeHCy derived polyselenides. As the result of an effective data mining strategy and the complementary use of various analytical information such as accurate mass, isotopic pattern and retention times of the compounds, we were also able to detect and identify Se compounds that are linked to lipid metabolism (and show hydrophobic properties in reversed phase chromatography).

The presence of various SeHCy derivatives reveals the importance of this compound that has probably been underestimated in plant metabolic pathways. Our results show that the highly reactive –SeH residue of SeHCy is able to react (supposedly in a non-enzymatic way) with other selenium containing amino acids and their derivatives and various secondary metabolites. Considering these findings we propose to add two new pathways to the currently recognized Se metabolic pathways of plants (Figure 6.1).

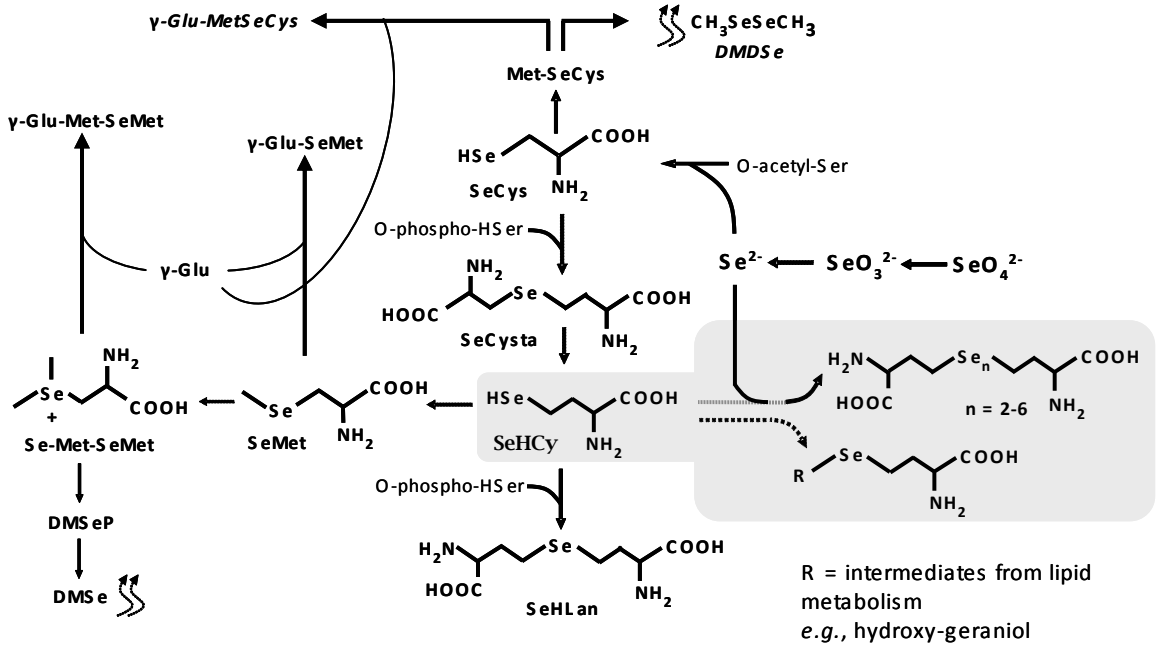

Figure 6.1. Modified Se metabolic pathway of plants, based on the scheme published by Ogra and Anan (Ogra and Anan 2012). The highlighted section shows the proposed new metabolic pathways. The dotted line represents a reaction that might include more than one steps.

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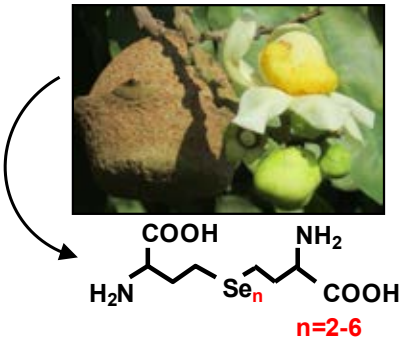
One of the main results of the investigation of *Lecythidaceae* seeds was the detection of polyselenides. These compounds are the proof of a substantially new Se storage and sequestration mechanisms in *L. minor*. Diselenides, selenotrisulfides and diseleno-trisulfides with analogous structures have been found in higher plants mostly in the form of volatile species (Meija et al. 2002, Kubachka et al. 2007). Triselenides have been recently identified from bacterial origin (Swearingen Jr. et al. 2006) but compounds containing more than three selenium atoms have never been reported before.



The accumulation of multiple Se atoms in the center of the molecules is supposedly an effective way of detoxification in plants, while these compounds are still able to play an important role in defense mechanisms. Considering the structure of polyselenides, they are probably highly toxic because their metabolism might lead to the depletion of glutathione (with the analogy of $SeCys_2$ metabolism through the formation of selenodiglutathiones). Further toxicological studies would be necessary to determine if monkeypot nut is safely applicable as a feed supplement or its utilization should be avoided. It must be highlighted that *B. excelsa* seeds did not contain any of these polyselenides that means our results do not raise any new toxicological concerns about the consumption of Brazil nuts.

Finally, the remarkable difference between the Se speciation patterns of the two *Lecythidaceae* plants should also be underlined. Altogether, from the 26 compounds assigned in the study only three are present in both plants, namely, SeMet, γ-Glu-SeMet and γ-Glu-*Se*-methyl-SeMet. *B. excelsa* was shown in several studies to contain up to 96% of its total selenium content in the form of either free or protein bound SeMet. Our current observation also suggests that the metabolism of selenium in Brazil nut strongly focuses on the allocation of SeMet, while the metabolism of selenium in *L. minor* involves far more diverse metabolic pathways.

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6.2. Selenoproteomics

As a result of a shotgun proteomic study carried out on the protein fraction of *L. minor* seeds we have identified five different proteins. While most of the peptides found in the sample belonged to 11S globulin, three sulfur-rich seed proteins and an 11S globulin-like protein have also been identified. Our results demonstrate the close relationship between *L. minor* and *B. excelsa* in the proteome level since most of the identified proteins have been previously identified in *B. excelsa*.

On the other hand the difference between the two plants could also be confirmed by the selenopeptide mapping of *L. minor*. The two orders of magnitude higher Se concentration of *L. minor* seeds led to a higher level of Met/SeMet substitution in proteins and allowed the identification of several new selenopeptides that could not be detected in *B. excelsa*. On the whole, 13 different selenopeptides were identified by database search and the amino acid sequences of an additional nine selenopeptides were determined by *de novo* sequencing.

The high methionine content of the identified proteins provided the opportunity to detect various selenopeptides in case if methionine is substituted by selenomethionine. One of the most noticeable results of our investigation was the detection of multiple Se containing peptides that contain up to four selenomethionine residues. Some of the analogues of these peptides have previously been identified in *B. excelsa* but they contained only one or two SeMet residues.

The non-selective incorporation of SeMet into proteins occurs through methionyl- $tRNA^{Met}$. The extent of this process is usually considered to depend on the Met/SeMet ratio in the plant tissue. In our study, the *in vivo* substitution of multiple methionine residues offered a unique opportunity to investigate this phenomenon. We have determined the Met/SeMet ratio and found that our experimental data basically follow the trend of theoretically expected selenopeptide intensities. Therefore, our results confirm that the level of substitution of Met by SeMet depends on the Met/SeMet ratio in the tissue.

Despite of the extremely high Se content of *L. minor* seeds and the considerable number of selenopeptides identified we could not detect any SeC containing peptides. It is probably due to the very effective detoxification mechanisms that prevent the non-specific incorporation of SeC by the formation of non-protein amino acids and amino acid derivatives. The depletion of SeC by the production of selenomethionine through selenocystathionine is also part of this defense mechanism. Our results confirm the existence of specific detoxification pathways that protect the *L. minor* plant from the toxic effects of Se.

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7. THESIS STATEMENTS

1. I extended the selenium speciation profile of *B. excelsa* and *L. minor* by identifying seven previously known and 19 newly assigned low molecular weight selenium compounds. I confirmed that the selenium metabolomes of the secondary accumulator *B. excelsa* and the hyperaccumulator *L. minor* are considerably different. From the 26 compounds assigned in the study only three were present in both plants, namely, SeMet, γ-Glu-SeMet and γ-Glu-*Se*-methyl-SeMet.

2. I identified a homologous series of polyselenides that contain two to six selenium atoms between two selenohomocysteine residues. The fragmentation-based identification of these compounds was confirmed by the synthesis of their mixed Se-S analogues. This is the first *in vivo* representation of the accumulation of Se into long polyselenide chains ($n_{(Se)}$ = 2-6).

3. We have contributed to the broadening of the available knowledge about the selenoproteome of *L. minor* by the mass spectrometry based, database-dependent identification of 13 different selenopeptides and four selenium-containing proteins. The amino acid sequences of an additional nine selenopeptides were also determined by manual *de novo* sequencing.

4. I accomplished a selenoproteomic study on the seed proteins of *L. minor* that demonstrated the exceptionally high level of Met/SeMet substitution. The *in vivo* substitution of up to four methionine residues by selenomethionine has never been reported before. This process is one of the effective detoxification mechanisms that are responsible for the extreme Se tolerance of *L. minor*.

5. I confirmed the complete lack of selenocysteine residues in the protein fraction of *L. minor.* The results prove that the exclusion of selenocysteine from proteins – that is a key process in the Se tolerance of plants - is remarkably efficient in the hyperaccumulator *L. minor*.

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8. SUMMARY

Comparative study of selenium (Se) speciation in hyperaccumulator plants offers an interesting challenge from the analytical point of view. In our study the application of a sophisticated sample clean-up procedure and the combination of elemental and molecular mass spectrometric methods led to the identification of several new selenocompounds. The difference between the Se speciation of the primary accumulator *Lecythis minor* and the secondary accumulator *Bertholletia excelsa* confirmed the current opinion that the speciation pattern in hyperaccumulator plants is principally related to the mechanism of accumulation and not to taxonomy. The most abundant new selenocompounds were found to be the derivatives of selenohomocysteine (SeHCy) and selenomethionine (SeMet), including fatty acid metabolism related compounds. A series of SeHCy derived species containing multiple Se atoms (>2) was also detected and their structure was validated by the synthesis of their S-Se analogues.

A shotgun proteomic approach was applied to characterize the selenium (Se) containing proteins of the selenium hyperaccumulator monkeypot nut (*Lecythis minor*) seeds. The exceptionally high Se content (> 4000 mg kg^{-1}) of the sample enabled a straightforward procedure without the need for multiple preconcentration and fractionation steps. The proteins identified were sulfur-rich seed proteins, namely 11S globulin (Q84ND2), 2S albumin (B6EU54), 2S sulfur-rich seed storage proteins (P04403 and P0C8Y8) and a 11S globulin-like protein (A0EM48). Database directed search for theoretically selenium containing peptides was assisted by manual spectrum evaluation to achieve around 25 % coverage on sulfur analogues. Remarkable detoxification mechanisms on the proteome level were revealed in the form of multiple selenomethionine-methionine substitution and the lack of selenocysteine residues. Our results contribute to the deeper understanding of selenium detoxification procedures in hyperaccumulator plants.

9. REFERENCES

Adams, M.L., Lombi, E., Zhao, F.-J., and McGrath, S.P. 2002. Evidence of low selenium concentrations in UK bread-making wheat grain. J. Sci. Food Agric. **82**(10): 1160–1165. doi: 10.1002/jsfa.1167.

Alcocer, M.J.C., Murtagh, G.J., Bailey, K., Dumoulin, M., Sarabia Meseguer, A., Parker, M.J., and Archer, D.B. 2002. The disulphide mapping, folding and characterisation of recombinant Ber e 1, an allergenic protein, and SFA8, two sulphur-rich 2 S plant albumins. J. Mol. Biol. **324**(1): 165–175. doi: 10.1016/S0022-2836(02)01061-6.

Alexander, A.R., Whanger, P.D., and Miller, L.T. 1983. Bioavailability to rats of selenium in various tuna and wheat products. J. Nutr. **113**(1): 196–204.

Alfthan, G., Eurola, M., Ekholm, P., Venäläinen, E.-R., Root, T., Korkalainen, K., Hartikainen, H., Salminen, P., Hietaniemi, V., Aspila, P., and Aro, A. 2014. Effects of nationwide addition of selenium to fertilizers on foods, and animal and human health in Finland: From deficiency to optimal selenium status of the population. J. Trace Elem. Med. Biol. doi: 10.1016/j.jtemb.2014.04.009.

Altenbach, S.B., Pearson, K.W., Leung, F.W., and Sun, S.S.M. 1987. Cloning and sequence analysis of a cDNA encoding a Brazil nut protein exceptionally rich in methionine. Plant Mol. Biol. **8**(3): 239–250. doi: 10.1007/BF00015032.

Ampe, C., Van Damme, J., de Castro, L.A., Sampaio, M.J., Van Montagu, M., and Vandekerckhove, J. 1986. The amino-acid sequence of the 2S sulphur-rich proteins from seeds of Brazil nut (Bertholletia excelsa H.B.K.). Eur. J. Biochem. FEBS **159**(3): 597–604.

Anan, Y., Ishiwata, K., Suzuki, N., Tanabe, S., and Ogra, Y. 2011. Speciation and identification of low molecular weight selenium compounds in the liver of sea turtles. J. Anal. At. Spectrom. **26**(1): 80–85. doi: 10.1039/c0ja00031k.

Arnaudguilhem, C., Bierla, K., Ouerdane, L., Preud'homme, H., Yiannikouris, A., and Lobinski, R. 2012. Selenium metabolomics in yeast using complementary reversed-phase/hydrophilic ion interaction (HILIC) liquid chromatography-electrospray hybrid quadrupole trap/Orbitrap mass spectrometry. Anal. Chim. Acta **757**: 26–38. doi: 10.1016/j.aca.2012.10.029.

Arnér, E.S., and Holmgren, A. 2000. Physiological functions of thioredoxin and thioredoxin reductase. Eur. J. Biochem. **267**(20): 6102–6109.

Arpino, P.J. 1982. On-line liquid chromatography/mass spectrometry? An odd couple! TrAC Trends Anal. Chem. **1**(7): 154–158. doi: 10.1016/0165-9936(82)80049-6.

Aureli, F., Ouerdane, L., Bierla, K., Szpunar, J., Prakash, N.T., and Cubadda, F. 2012. Identification of selenosugars and other low-molecular weight selenium metabolites in high-selenium cereal crops. Metallomics **4**(9): 968–978. doi: 10.1039/c2mt20085f.

D'Autréaux, B., and Toledano, M.B. 2007. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. Nat. Rev. Mol. Cell Biol. **8**(10): 813–824.

Baker, A.J.M., and Brooks, R. 1989. Terrestrial higher plants which hyperaccumulate metallic elements. A review of their distribution, ecology and phytochemistry. Biorecovery. **1**(2): 81–126.

Barillas, J.R.V., Quinn, C.F., and Pilon-Smits, E.A.H. 2011. Selenium accumulation in plants-phytotechnological applications and ecological implications. Int. J. Phytoremediation **13**(SUPPL.1): 166–178. doi: 10.1080/15226514.2011.568542.

Barsnes, H., Vaudel, M., Colaert, N., Helsens, K., Sickmann, A., Berven, F.S., and Martens, L. 2011. Compomics-utilities: An open-source Java library for computational proteomics. BMC Bioinformatics **12**. doi: 10.1186/1471-2105-12-70.

Beath, O.A., Draize, J.H., Eppson, H.F., Gilbert, C.S., and McCreary, O.C. 1934. Certain poisonous plants of wyoming activated by selenium and their association with respect to soil types. J. Am. Pharm. Assoc. **23**(2): 94–97. doi: 10.1002/jps.3080230204.

Becker, J.S., Becker, J.S., Zoriy, M.V., Dobrowolska, J., and Matusch, A. 2007. Imaging mass spectrometry in biological tissues by laser ablation inductively coupled plasma mass spectrometry. Eur. J. Mass Spectrom. **13**(1): 1–6. doi: 10.1255/ejms.833.

Becker, J.S., and Jakubowski, N. 2009. The synergy of elemental and biomolecular mass spectrometry: New analytical strategies in life sciences. Chem. Soc. Rev. **38**(7): 1969–1983. doi: 10.1039/b618635c.

Becker, J.S., Matusch, A., and Wu, B. 2014. Bioimaging mass spectrometry of trace elements – recent advance and applications of LA-ICP-MS: A review. Anal. Chim. Acta **835**: 1–18. doi: 10.1016/j.aca.2014.04.048.

Becker, S. 2008. Inorganic Mass Spectrometry: Principles and Applications. John Wiley & Sons.

Bednar, A.J., Kirgan, R.A., and Jones, W.T. 2009. Comparison of standard and reaction cell inductively coupled plasma mass spectrometry in the determination of chromium and selenium species by HPLC-ICP-MS. Anal. Chim. Acta **632**(1): 27–34. doi: 10.1016/j.aca.2008.10.050.

Bence, N.F., Sampat, R.M., and Kopito, R.R. 2001. Impairment of the Ubiquitin-Proteasome System by Protein Aggregation. Science **292**(5521): 1552–1555. doi: 10.1126/science.292.5521.1552.

Bianga, J., Govasmark, E., and Szpunar, J. 2013. Characterization of selenium incorporation into wheat proteins by two-dimensional gel electrophoresis-laser ablation ICP MS followed by capillary HPLC-ICP MS and electrospray linear trap quadrupole orbitrap MS. Anal. Chem. **85**(4): 2037–2043.

Bianga, J., and Szpunar, J. 2013. ICP-MS-assisted identification of selenium-containing proteins in 2D gels using a new capillary HPLC-ICP MS interface and Orbitrap tandem mass spectrometry. J. Anal. At. Spectrom. **28**(2): 288–292. doi: 10.1039/c2ja30273j.

Bierla, K., Bianga, J., Ouerdane, L., Szpunar, J., Yiannikouris, A., and Lobinski, R. 2013. A comparative study of the Se/S substitution in methionine and cysteine in Se-enriched yeast using an inductively coupled plasma mass spectrometry (ICP MS)-assisted proteomics approach. J. Proteomics **87**: 26–39. doi: 10.1016/j.jprot.2013.05.010.

Bierla, K., Dernovics, M., Vacchina, V., Szpunar, J., Bertin, G., and Lobinski, R. 2008. Determination of selenocysteine and selenomethionine in edible animal tissues by 2D size-exclusion reversed-phase HPLC-ICP MS following carbamidomethylation and proteolytic extraction. Anal. Bioanal. Chem. **390**(7): 1789–1798. doi: 10.1007/s00216-008-1883-5.

Bird, S.M., Ge, H., Uden, P.C., Tyson, J.F., Block, E., and Denoyer, E. 1997a. High-performance liquid chromatography of selenoamino acids and organo selenium compounds. Speciation by inductively coupled plasma mass spectrometry. J. Chromatogr. A **789**(1-2): 349–359. doi: 10.1016/S0021-9673(97)00657-2.

Bird, S.M., Uden, P.C., Tyson, J.F., Block, E., and Denoyer, E. 1997b. Speciation of selenoamino acids and organoselenium compounds in selenium-enriched yeast using high-performance liquid chromatography-inductively coupled plasma mass spectrometry. J. Anal. At. Spectrom. **12**(7): 785–788.

Birringer, M., Pilawa, S., and Flohé, L. 2002. Trends in selenium biochemistry. Nat. Prod. Rep. **19**(6): 693–718. doi: 10.1039/b205802m.

Böck, A. 2007. Stories of Success: Personal Recollections. in Comprehensive biochemistry. (edited by G. Semenza). Elsevier, ISBN: 9780080553573.

Bodó, E.T., Stefánka, Z., Ipolyi, I., Sörös, C., Dernovics, M., and Fodor, P. 2003. Preparation, homogeneity and stability studies of a candidate LRM for Se speciation. Anal. Bioanal. Chem. **377**(1): 32–38. doi: 10.1007/s00216-003-1941-y.

Boyd, R.S. 2007. The defense hypothesis of elemental hyperaccumulation: Status, challenges and new directions. Plant Soil **293**(1-2): 153–176. doi: 10.1007/s11104-007-9240-6.

Broadley, M.R., White, P.J., Bryson, R.J., Meacham, M.C., Bowen, H.C., Johnson, S.E., Hawkesford, M.J., McGrath, S.P., Zhao, F.-J., Breward, N., Harriman, M., and Tucker, M. 2006. Biofortification of UK food crops with selenium. Proc. Nutr. Soc. **65**(2): 169–181. doi: 10.1079/PNS2006490.

Brown, T.A., and Shrift, A. 1981. Exclusion of selenium from proteins of selenium-tolerant Astragalus species. Plant Physiol. **67**(5): 1051–1053. doi: 10.1104/pp.67.5.1051.

Bueno, M., and Pannier, F. 2009. Quantitative analysis of volatile selenium metabolites in normal urine by headspace solid phase microextraction gas chromatography-inductively coupled plasma mass spectrometry. Talanta **78**(3): 759–763. doi: 10.1016/j.talanta.2008.12.044.

Bueno, M., and Potin-Gautier, M. 2002. Solid-phase extraction for the simultaneous preconcentration of organic (selenocystine) and inorganic [Se(IV), Se(VI)] selenium in natural waters. J. Chromatogr. A **963**(1–2): 185–193. doi: 10.1016/S0021-9673(02)00125-5.

Bulska, E., Wysocka, I.A., Wierzbicka, M.H., Proost, K., Janssens, K., and Falkenberg, G. 2006. In vivo investigation of the distribution and the local speciation of selenium in Allium cepa L. by means of microscopic X-ray absorption near-edge structure spectroscopy and confocal microscopic X-ray fluorescence analysis. Anal. Chem. **78**(22): 7616–7624. doi: 10.1021/ac060380s.

Burnell, J.N., and Shrift, A. 1979. Cysteinyl-tRNA synthetase from Astragalus species. Plant Physiol. **63**(6): 1095–1097. doi: 10.1104/pp.63.6.1095.

Burra, R., Pradenas, G.A., Montes, R.A., Vásquez, C.C., and Chasteen, T.G. 2010. Production of dimethyl triselenide and dimethyl diselenenyl sulfide in the headspace of metalloid-resistant Bacillus species grown in the presence of selenium oxyanions. Anal. Biochem. **396**(2): 217–222. doi: 10.1016/j.ab.2009.09.026.

Cabañero, A.I., Madrid, Y., and Cámara, C. 2005. Enzymatic probe sonication extraction of Se in animal-based food samples: A new perspective on sample preparation for total and Se speciation analysis. Anal. Bioanal. Chem. **381**(2): 373–379. doi: 10.1007/s00216-004-2798-4.

Callahan, D.L., Baker, A.J.M., Kolev, S.D., and Wedd, A.G. 2006. Metal ion ligands in hyperaccumulating plants. J. Biol. Inorg. Chem. **11**(1): 2–12. doi: 10.1007/s00775-005-0056-7.

Campbell, M.B., and Kanert, G.A. 1992. High-pressure microwave digestion for the determination of arsenic, antimony, selenium and mercury in oily wastes. The Analyst **117**(2): 121–124. doi: 10.1039/AN9921700121.

Canelas, A.B., Pierick, A. Ten, Ras, C., Seifar, R.M., Van Dam, J.C., Van Gulik, W.M., and Heijnen, J.J. 2009. Quantitative evaluation of intracellular metabolite extraction techniques for yeast metabolomics. Anal. Chem. **81**(17): 7379–7389. doi: 10.1021/ac900999t.

Capelo, J.L., Ximénez-Embún, P., Madrid-Albarrán, Y., and Cámara, C. 2004. Enzymatic Probe Sonication: Enhancement of Protease-Catalyzed Hydrolysis of Selenium Bound to Proteins in Yeast. Anal. Chem. **76**(1): 233–237. doi: 10.1021/ac034871d.

Casal, S.G., Far, J., Bierla, K., Ouerdane, L., and Szpunar, J. 2010. Study of the Se-containing metabolomes in Se-rich yeast by size-exclusion - Cation-exchange HPLC with the parallel ICP MS and electrospray orbital ion trap detection. Metallomics **2**(8): 535–548. doi: 10.1039/c0mt00002g.

Casiot, C., Vacchina, V., Chassaigne, H., Szpunar, J., Potin-Gautier, M., and Łobiński, R. 1999. An approach to the identification of selenium species in yeast extracts using pneumatically-assisted electrospray tandem mass spectrometry. Anal. Commun. **36**(3): 77–80. doi: 10.1039/A900319C.

Chang, J.C., Gutemann, W.B., Reid, C.M., and Lisk, D.J. 1995. Selenium content of brazil nuts from two geographic locations in Brazil. Chemosphere **30**(4): 801–802. doi: 10.1016/0045-6535(94)00409-N.

Cheajesadagul, P., Bianga, J., Arnaudguilhem, C., Lobinski, R., and Szpunar, J. 2014. Large-scale speciation of selenium in rice proteins using ICP-MS assisted electrospray MS/MS proteomics. Metallomics **6**(3): 646–653. doi: 10.1039/c3mt00299c.

Chen, Y., and Maret, W. 2001. Catalytic selenols couple the redox cycles of metallothionein and glutathione. Eur. J. Biochem. **268**(11): 3346–3353. doi: 10.1046/j.1432-1327.2001.02250.x.

Chernushevich, I.V., Loboda, A.V., and Thomson, B.A. 2001. An introduction to quadrupole-time-of-flight mass spectrometry. J. Mass Spectrom. JMS **36**(8): 849–865. doi: 10.1002/jms.207.

Chunhieng, T., Pétritis, K., Elfakir, C., Brochier, J., Goli, T., and Montet, D. 2004. Study of selenium distribution in the protein fractions of the Brazil nut, Bertholletia excelsa. J. Agric. Food Chem. **52**(13): 4318–4322. doi: 10.1021/jf049643e.

Cox, J., and Mann, M. 2011. Quantitative, High-Resolution Proteomics for Data-Driven Systems Biology. Annu. Rev. Biochem. **80**(1): 273–299. doi: 10.1146/annurev-biochem-061308-093216.

Cravatt, B.F., Simon, G.M., and Yates, J.R. 2007. The biological impact of mass-spectrometry-based proteomics. Nature **450**(7172): 991–1000. doi: 10.1038/nature06525.

Cubadda, F., Aureli, F., Ciardullo, S., D'Amato, M., Raggi, A., Acharya, R., Reddy, R.A.V., and Prakash, N.T. 2010. Changes in selenium speciation associated with increasing tissue concentrations of selenium in wheat grain. J. Agric. Food Chem. **58**(4): 2295–2301. doi: 10.1021/jf903004a.

Date, A.R., and Gray, A.L. 1985. Determination of trace elements in geological samples by inductively coupled plasma source mass spectrometry. Spectrochim. Acta Part B At. Spectrosc. **40**(1–2): 115–122. doi: 10.1016/0584-8547(85)80015-X.

Dawkins, B.G., Arpino, P.J., and McLafferty, F.W. 1978. Polypeptide sequencing by liquid chromatography mass spectrometry. Biomed. Mass Spectrom. **5**(1): 1–6.

Day, J.A., Kannamkumarath, S.S., Yanes, E.G., Montes-Bayón, M., and Caruso, J.A. 2002. Chiral speciation of Marfey's derivatized DL-selenomethionine using capillary electrophoresis with UV and ICP-MS detection. J. Anal. At. Spectrom. **17**(1): 27–31. doi: 10.1039/B104679A.

Dean, J.R., Munro, S., Ebdon, L., Crews, H.M., and Massey, R.C. 1987. Studies of metalloprotein species by directly coupled high-performance liquid chromatography inductively coupled plasma mass spectrometry. J. Anal. At. Spectrom. **2**(6): 607–610. doi: 10.1039/JA9870200607.

Dear, T.N., Campbell, K., and Rabbitts, T.H. 1991. Molecular cloning of putative odorant-binding and odorant-metabolizing proteins. Biochemistry (Mosc.) **30**(43): 10376–10382.

Dernovics, M., García-Barrera, T., Bierla, K., Preud'homme, H., and Lobinski, R. 2007a. Standardless identification of selenocystathionine and its γ-glutamyl derivatives in monkeypot nuts by 3D liquid chromatography with ICP-MS detection followed by nanoHPLC-Q-TOF-MS/MS. Analyst **132**(5): 439–449. doi: 10.1039/b618637h.

Dernovics, M., Giusti, P., and Lobinski, R. 2007b. ICP-MS-assisted nanoHPLC-electrospray Q/time-of-flight MS/MS selenopeptide mapping in Brazil nuts. J. Anal. At. Spectrom. **22**(1): 41–50. doi: 10.1039/b608041c.

Dernovics, M., and Lobinski, R. 2008. Speciation analysis of selenium metabolites in yeast-based food supplements by ICPMS-assisted hydrophilic interaction HPLC-hybrid linear ion trap/orbitrap MSn. Anal. Chem. **80**(11): 3975–3984. doi: 10.1021/ac8002038.

Dernovics, M., Ouerdane, L., Tastet, L., Giusti, P., Preud'Homme, H., and Lobinski, R. 2006. Detection and characterization of artefact compounds during selenium speciation analysis

in yeast by ICP-MS-assisted MALDI MS, oMALDI MS/MS and LC-ES-MS/MS. J. Anal. At. Spectrom. **21**(7): 703–707. doi: 10.1039/b604885d.

Dernovics, M., Stefánka, Z., and Fodor, P. 2002. Improving selenium extraction by sequential enzymatic processes for Se-speciation of selenium-enriched Agaricus bisporus. Fresenius J. Anal. Chem. **372**(3): 473–480. doi: 10.1007/s00216-001-1215-5.

Dernovics, M., Vass, A., Németh, A., and Magyar, A. 2012. Synthesis and application of a Sec2-containing oligopeptide for method evaluation purposes in selenium speciation. Talanta **99**: 186–193. doi: 10.1016/j.talanta.2012.05.038.

Dietz, C., Sanz Landaluze, J., Ximénez-Embún, P., Madrid-Albarrán, Y., and Cámara, C. 2004. Volatile organo-selenium speciation in biological matter by solid phase microextraction-moderate temperature multicapillary gas chromatography with microwave induced plasma atomic emission spectrometry detection. Anal. Chim. Acta **501**(2): 157–167. doi: 10.1016/j.aca.2003.09.027.

Dimkovikj, A., Fisher, B., Hutchison, K., and Van Hoewyk, D. 2015. Stuck between a ROS and a hard place: Analysis of the ubiquitin proteasome pathway in selenocysteine treated Brassica napus reveals different toxicities during selenium assimilation. J. Plant Physiol. **181**: 50–54. doi: 10.1016/j.jplph.2015.04.003.

Djanaguiraman, M., Devi, D.D., Shanker, A.K., Sheeba, J.A., and Bangarusamy, U. 2005. Selenium - An antioxidative protectant in soybean during senescence. Plant Soil **272**(1-2): 77–86. doi: 10.1007/s11104-004-4039-1.

Djanaguiraman, M., Prasad, P.V.V., and Seppanen, M. 2010. Selenium protects sorghum leaves from oxidative damage under high temperature stress by enhancing antioxidant defense system. Plant Physiol. Biochem. **48**(12): 999–1007. doi: 10.1016/j.plaphy.2010.09.009.

Draize, J.H., and Beath, O.A. 1935. Observations on the pathology of blind staggers and alkali disease. J. Am. Vet. Med. Assoc. **86**: 753–763.

Dressler, V.L., Antes, F.G., Moreira, C.M., Pozebon, D., and Duarte, F.A. 2011. As, Hg, I, Sb, Se and Sn speciation in body fluids and biological tissues using hyphenated-ICP-MS techniques: A review. Int. J. Mass Spectrom. **307**(1-3): 149–162. doi: 10.1016/j.ijms.2011.01.026.

Dumont, E., Ogra, Y., Vanhaecke, F., Suzuki, K.T., and Cornelis, R. 2006a. Liquid chromatography-mass spectrometry (LC-MS): A powerful combination for selenium speciation in garlic (Allium sativum). Anal. Bioanal. Chem. **384**(5): 1196–1206. doi: 10.1007/s00216-005-0272-6.

Dumont, E., De Pauw, L., Vanhaecke, F., and Cornelis, R. 2006b. Speciation of Se in Bertholletia excelsa (Brazil nut): A hard nut to crack? Food Chem. **95**(4): 684–692. doi: 10.1016/j.foodchem.2005.04.004.

Egressy-Molnár, O., Vass, A., Németh, A., García-Reyes, J.F., and Dernovics, M. 2011. Effect of sample preparation methods on the d,l-enantiomer ratio of extracted selenomethionine. Anal. Bioanal. Chem. **401**(1): 373–380. doi: 10.1007/s00216-011-5077-1.

Ellis, D.R., Sors, T.G., Brunk, D.G., Albrecht, C., Orser, C., Lahner, B., Wood, K.V., Harris, H.H., Pickering, I.J., and Salt, D.E. 2004. Production of Se-methylselenocysteine in transgenic plants expressing selenocysteine methyltransferase. BMC Plant Biol. **4**. doi: 10.1186/1471-2229-4-1.

Encinar, J.R., Ouerdane, L., Buchmann, W., Tortajada, J., Lobinski, R., and Szpunar, J. 2003. Identification of water-soluble selenium-containing proteins in selenized yeast by size-exclusion-reversed-phase HPLC/ICPMS followed by MALDI-TOF and electrospray Q-TOF mass spectrometry. Anal. Chem. **75**(15): 3765–3774. doi: 10.1021/ac034103m.

Far, J., Preud'homme, H., and Lobinski, R. 2010. Detection and identification of hydrophilic selenium compounds in selenium-rich yeast by size exclusion-microbore normal-phase HPLC with the on-line ICP-MS and electrospray Q-TOF-MS detection. Anal. Chim. Acta **657**(2): 175–190. doi: 10.1016/j.aca.2009.10.040.

Feldmann, J., Salaün, P., and Lombi, E. 2009. Critical review perspective: Elemental speciation analysis methods in environmental chemistry-moving towards methodological integration. Environ. Chem. **6**(4): 275–289. doi: 10.1071/EN09018.

Feng, R., Wei, C., and Tu, S. 2013. The roles of selenium in protecting plants against abiotic stresses. Environ. Exp. Bot. **87**: 58–68. doi: 10.1016/j.envexpbot.2012.09.002.

Fercha, A., Capriotti, A.L., Caruso, G., Cavaliere, C., Gherroucha, H., Samperi, R., Stampachiacchiere, S., and Lagana, A. 2013. Gel-free proteomics reveal potential biomarkers of priming-induced salt tolerance in durum wheat. J. Proteomics **91**: 486–499. doi: 10.1016/j.jprot.2013.08.010.

Ferri, T., Coccioli, F., De Luca, C., Callegari, C.V., and Morabito, R. 2004. Distribution and speciation of selenium in Lecythis ollaria plant. Microchem. J. **78**(2): 195–203. doi: 10.1016/j.microc.2004.06.001.

Flohe, L., Günzler, W.A., and Schock, H.H. 1973. Glutathione peroxidase: a selenoenzyme. FEBS Lett. **32**(1): 132–134.

Freeman, J.L., Lindblom, S.D., Quinn, C.F., Fakra, S., Marcus, M.A., and Pilon-Smits, E.A.H. 2007. Selenium accumulation protects plants from herbivory by Orthoptera via toxicity and deterrence. New Phytol. **175**(3): 490–500. doi: 10.1111/j.1469-8137.2007.02119.x.

Freeman, J.L., Tamaoki, M., Stushnoff, C., Quinn, C.F., Cappa, J.J., Devonshire, J., Fakra, S.C., Marcus, M.A., McGrath, S.P., van Hoewyk, D., and Pilon-Smits, E.A.H. 2010. Molecular mechanisms of selenium tolerance and hyperaccumulation in Stanleya pinnata. Plant Physiol. **153**(4): 1630–1652. doi: 10.1104/pp.110.156570.

Freeman, J.L., Zhang, L.H., Marcus, M.A., Fakra, S., McGrath, S.P., and Pilon-Smits, E.A.H. 2006. Spatial imaging, speciation, and quantification of selenium in the hyperaccumulator plants Astragalus bisulcatus and Stanleya pinnata. Plant Physiol. **142**(1): 124–134. doi: 10.1104/pp.106.081158.

Gabos, M.B., Alleoni, L.R.F., and Abreu, C.A. 2014. Background levels of selenium in some selected Brazilian tropical soils. J. Geochem. Explor. **145**: 35–39. doi: 10.1016/j.gexplo.2014.05.007.

Gammelgaard, B., Gabel-Jensen, C., Stürup, S., and Hansen, H.R. 2008. Complementary use of molecular and element-specific mass spectrometry for identification of selenium compounds related to human selenium metabolism. Anal. Bioanal. Chem. **390**(7): 1691–1706. doi: 10.1007/s00216-007-1788-8.

Gammelgaard, B., Madsen, K.G., Bjerrum, J., Bendahl, L., Jøns, O., Olsen, J., and Sidenius, U. 2003. Separation, purification and identification of the major selenium metabolite from human urine by multi-dimensional HPLC-ICP-MS and APCI-MS. J. Anal. At. Spectrom. **18**(1): 65–70. doi: 10.1039/b209832f.

Gilon, N., Potin-Gautier, M., and Astruc, M. 1996. Optimization of the determination of inorganic and organic selenium species using high-performance liquid chromatography-electrothermal atomic absorption spectrometry. J. Chromatogr. A **750**(1–2): 327–334. doi: 10.1016/0021-9673(96)00484-0.

Giusti, P., Schaumlöffel, D., Encinar, J.R., and Szpunar, J. 2005. Interfacing reversed-phase nanoHPLC with ICP-MS and on-line isotope dilution analysis for the accurate quantification of selenium-containing peptides in protein tryptic digests. J. Anal. At. Spectrom. **20**(10): 1101–1107. doi: 10.1039/B506620D.

Giusti, P., Schaumlöffel, D., Preud'homme, H., Szpunar, J., and Lobinski, R. 2006. Selenopeptide mapping in a selenium-yeast protein digest by parallel nanoHPLC-ICP-MS and nanoHPLC-electrospray-MS/MS after on-line preconcentration. J. Anal. At. Spectrom. **21**(1): 26–32. doi: 10.1039/b511288e.

Goenaga-Infante, H., Sturgeon, R., Turner, J., Hearn, R., Sargent, M., Maxwell, P., Yang, L., Barzev, A., Pedrero, Z., Cámara, C., Díaz Huerta, V., Fernández Sánchez, M.L., Sanz-Medel, A., Emese, K., Fodor, P., Wolf, W., Goldschmidt, R., Vacchina, V., Szpunar, J.,

Valiente, L., Huertas, R., Labarraque, G., Davis, C., Zeisler, R., Turk, G., Rizzio, E., MacKay, L.G., Myors, R.B., Saxby, D.L., Askew, S., Chao, W., and Jun, W. 2008. Total selenium and selenomethionine in pharmaceutical yeast tablets: Assessment of the state of the art of measurement capabilities through international intercomparison CCQM-P86. Anal. Bioanal. Chem. **390**(2): 629–642. doi: 10.1007/s00216-007-1654-8.

Goldenberg, D.P. 1992. Native and non-native intermediates in the BPTI folding pathway. Trends Biochem. Sci. **17**(7): 257–261. doi: 10.1016/0968-0004(92)90405-X.

Gómez-Ariza, J.L., Pozas, J.A., Giráldez, I., and Morales, E. 1998. Speciation of volatile forms of selenium and inorganic selenium in sediments by gas chromatography-mass spectrometry. J. Chromatogr. A **823**(1-2): 259–277. doi: 10.1016/S0021-9673(98)00581-0.

Goodson, C.C., Parker, D.R., Amrhein, C., and Zhang, Y. 2003. Soil selenium uptake and root system development in plant taxa differing in Se-accumulating capability. New Phytol. **159**(2): 391–401.

Grant, T.D., Montes-Bayón, M., Leduc, D., Fricke, M.W., Terry, N., and Caruso, J.A. 2004. Identification and characterization of Se-methyl selenomethionine in Brassica juncea roots. J. Chromatogr. A **1026**(1-2): 159–166. doi: 10.1016/j.chroma.2003.11.039.

Gray, A.L. 1985a. Solid sample introduction by laser ablation for inductively coupled plasma source mass spectrometry. The Analyst **110**(5): 551–556. doi: 10.1039/AN9851000551.

Gray, A.L. 1985b. The ICP as an ion source - origins, achievements and prospects. Spectrochim. Acta Part B At. Spectrosc. **40**(10–12): 1525–1537. doi: 10.1016/0584-8547(85)80176-2.

Gustavsson, N., Bølviken, B., Smith, D.B., and Severson, R.C. 2001. Geochemical Landscapes of the Conterminous United States -- New Map Presentations for 22 Elements. US Geol. Surv. Prof. Pap. 1648 (4): 38.

Hafeman, D.G., Sunde, R.A., and Hoekstra, W.G. 1974. Effect of dietary selenium on erythrocyte and liver glutathione peroxidase in the rat. J. Nutr. **104**(5): 580–587.

Hanson, B., Lindblom, S.D., Loeffler, M.L., and Pilon-Smits, E.A.H. 2004. Selenium protects plants from phloem-feeding aphids due to both deterrence and toxicity. New Phytol. **162**(3): 655–662. doi: 10.1111/j.1469-8137.2004.01067.x.

Han, X., Aslanian, A., and Yates III, J.R. 2008. Mass spectrometry for proteomics. Curr. Opin. Chem. Biol. **12**(5): 483–490. doi: 10.1016/j.cbpa.2008.07.024.

Hartikainen, H. 2005. Biogeochemistry of selenium and its impact on food chain quality and human health. J. Trace Elem. Med. Biol. **18**(4): 309–318. doi: 10.1016/j.jtemb.2005.02.009.

Hartikainen, H., Xue, T., and Piironen, V. 2000. Selenium as an anti-oxidant and pro-oxidant in ryegrass. Plant Soil **225**(1-2): 193–200. doi: 10.1023/A:1026512921026.

Hasanuzzaman, M., Hossain, M.A., and Fujita, M. 2010. Selenium in Higher Plants: Physiological Role, Antioxidant Metabolism and Abiotic Stress Tolerance. J. Plant Sci. **5**(4): 354–375. doi: 10.3923/jps.2010.354.375.

Hasanuzzaman, M., Nahar, K., Alam, M.M., Roychowdhury, R., and Fujita, M. 2013. Physiological, Biochemical, and Molecular Mechanisms of Heat Stress Tolerance in Plants. Int. J. Mol. Sci. **14**(5): 9643–9684. doi: 10.3390/ijms14059643.

Hatfield, D.L., Berry, M.J., and Gladyshev, V.N. 2011. Glutathione Peroxidase 1 and Diabetes. in Selenium: its molecular biology and role in human health. Springer, ISBN: 978-1-4614-1024-9.

Hatfield, D.L., Tsuji, P.A., Carlson, B.A., and Gladyshev, V.N. 2014. Selenium and selenocysteine: roles in cancer, health, and development. Trends Biochem. Sci. **39**(3): 112–120. doi: 10.1016/j.tibs.2013.12.007.

He, P.P., Lv, X.Z., and Wang, G.Y. 2004. Effects of Se and Zn supplementation on the antagonism against Pb and Cd in vegetables. Environ. Int. **30**(2): 167–172. doi: 10.1016/S0160-4120(03)00167-3.

Hirschfeld, T. 1980. The hy-phen-ated methods. Anal. Chem. **52**(2): 226–232.

Van Hoewyk, D. 2013. A tale of two toxicities: Malformed selenoproteins and oxidative stress both contribute to selenium stress in plants. Ann. Bot. **112**(6): 965–972. doi: 10.1093/aob/mct163.

Van Hoewyk, D., Takahashi, H., Inoue, E., Hess, A., Tamaoki, M., and Pilon-Smits, E.A.H. 2008. Transcriptome analyses give insights into selenium-stress responses and selenium tolerance mechanisms in Arabidopsis. Physiol. Plant. **132**(2): 236–253. doi: 10.1111/j.1399-3054.2007.01002.x.

Hoffmann, E. de, and Stroobant, V. 2007. Mass Spectrometry: Principles and Applications. John Wiley & Sons.

Holmes, J.C., and Morrell, F.A. 1957. Oscillographic mass spectrometric monitoring of gas chromatography. Appl. Spectrosc. **11**(2): 86–87.

Houk, R.S. 1980. Inductively Coupled Argon Plasma as an Ion Source for Mass Spectrometric Determination of Trace Elements. Anal. Chem. **52**(14): 2283–2289.

Huerta, V.D., Szpunar, J., Lobinski, R., Sánchez, M.L.F., and Sanz-Medel, A. 2003. Sample preparation for identification of selenocompounds in urine by electrospray-MS/MS. J. Anal. At. Spectrom. **18**(12): 1471–1476. doi: 10.1039/B306020A.

D'Ilio, S., Violante, N., Majorani, C., and Petrucci, F. 2011. Dynamic reaction cell ICP-MS for determination of total As, Cr, Se and V in complex matrices: Still a challenge? A review. Anal. Chim. Acta **698**(1–2): 6–13. doi: 10.1016/j.aca.2011.04.052.

Imai, H., and Nakagawa, Y. 2003. Biological significance of phospholipid hydroperoxide glutathione peroxidase (PHGPx, GPx4) in mammalian cells. Free Radic. Biol. Med. **34**(2): 145–169.

Internet. 1. Introduction to ICP-MS (cited: 05.08.2014) - Available from: http://crustal.usgs.gov/laboratories/icpms/intro.html. USGS Crustal Geophysics and Geochemistry Science Center.

Internet. 2. An Introduction to Mass Spectrometry. Quadrupole Mass Analyzers. (cited at 10.08.2014) - Available from: http://science.widener.edu/svb/massspec/intro_to_ms/.

Internet. 3. MetWorks™ Automated Metabolite Identification Software - Thermo Scientific (cited: 10.10.2014) - Available from: http://www.thermoscientific.com/content/tfs/en/product/metworks-automated-metabolite-identification-software.html.

Internet. 4. Selenium and Health (2001) - Available from: http://www.nutrition.org.uk/attachments/145_Selenium%20and%20health.pdf. British Nutrition Foundation.

Iskandar, I.K., and Kirkham, M.B. 2001. Trace Elements in Soil: Bioavailability, Flux, and Transfer. CRC Press, London, UK.

Jayasinghe, S.B., and Caruso, J.A. 2011. Investigation of Se-containing proteins in Bertholletia excelsa H.B.K. (Brazil nuts) by ICPMS, MALDI-MS and LC-ESI-MS methods. Int. J. Mass Spectrom. **307**(1-3): 16–27. doi: 10.1016/j.ijms.2010.12.005.

Kaim, W., and Schwederski, B. 1994. Bioinorganic Chemistry: Inorganic Elements in the Chemistry of Life: An Introduction and Guide. Wiley.

Kannamkumarath, S.S., Wrobel, K., Wrobel, K., Vonderheide, A., and Caruso, J.A. 2002. HPLC-ICP-MS determination of selenium distribution and speciation in different types of nut. Anal. Bioanal. Chem. **373**(6): 454–460. doi: 10.1007/s00216-002-1354-3.

Kápolna, E., and Fodor, P. 2007. Bioavailability of selenium from selenium-enriched green onions (Allium fistulosum) and chives (Allium schoenoprasum) after "in vitro" gastrointestinal digestion. Int. J. Food Sci. Nutr. **58**(4): 282–296. doi: 10.1080/09637480601154335.

Kaur, N., Sharma, S., Kaur, S., and Nayyar, H. 2014. Selenium in agriculture: A nutrient or contaminant for crops? Arch. Agron. Soil Sci. **60**(12): 1593–1624. doi: 10.1080/03650340.2014.918258.

Kelleher, N.L., Lin, H.Y., Valaskovic, G.A., Aaserud, D.J., Fridriksson, E.K., and McLafferty, F.W. 1999. Top down versus bottom up protein characterization by tandem high-resolution mass spectrometry. J. Am. Chem. Soc. **121**(4): 806–812. doi: 10.1021/ja973655h.

Kellie, J.F., Tran, J.C., Lee, J.E., Ahlf, D.R., Thomas, H.M., Ntai, I., Catherman, A.D., Durbin, K.R., Zamdborg, L., Vellaichamy, A., Thomas, P.M., and Kelleher, N.L. 2010. The emerging process of Top Down mass spectrometry for protein analysis: biomarkers, protein-therapeutics, and achieving high throughput. Mol. Biosyst. **6**(9): 1532–1539. doi: 10.1039/c000896f.

Kim, H.-Y., Fomenko, D.E., Yoon, Y.-E., and Gladyshev, V.N. 2006. Catalytic advantages provided by selenocysteine in methionine-S-sulfoxide reductases. Biochemistry (Mosc.) **45**(46): 13697–13704.

Kirihara, M., Asai, Y., Ogawa, S., Noguchi, T., Hatano, A., and Hirai, Y. 2007. A Mild and Environmentally Benign Oxidation of Thiols to Disulfides. Synthesis **2007**(21): 3286–3289. doi: 10.1055/s-2007-990800.

Klein, M., Ouerdane, L., Bueno, M., and Pannier, F. 2011. Identification in human urine and blood of a novel selenium metabolite, Se-methylselenoneine, a potential biomarker of metabolization in mammals of the naturally occurring selenoneine, by HPLC coupled to electrospray hybrid linear ion trap-orbital ion trap MS. Metallomics **3**(5): 513–520. doi: 10.1039/c0mt00060d.

Korotkov, K.V., Kumaraswamy, E., Zhou, Y., Hatfield, D.L., and Gladyshev, V.N. 2001. Association between the 15-kDa selenoprotein and UDP-glucose: glycoprotein glucosyltransferase in the endoplasmic reticulum of mammalian cells. J. Biol. Chem. **276**(18): 15330–15336.

Kotrebai, M., Birringer, M., Tyson, J.F., Block, E., and Uden, P.C. 1999. Identification of the principal selenium compounds in selenium-enriched natural sample extracts by ion-pair liquid chromatography with inductively coupled plasma- and electrospray ionization-mass spectrometric detection. Anal. Commun. **36**(6): 249–252.

Kotrebai, M., Birringer, M., Tyson, J.F., Block, E., and Uden, P.C. 2000. Selenium speciation in enriched and natural samples by HPLC-ICP-MS and HPLC-ESI-MS with perfluorinated carboxylic acid ion-pairing agents. Analyst **125**(1): 71–78. doi: 10.1039/a906320j.

Kubachka, K.M., Meija, J., Leduc, D.L., Terry, N., and Caruso, J.A. 2007. Selenium volatiles as proxy to the metabolic pathways of selenium in genetically modified Brassica juncea. Environ. Sci. Technol. **41**(6): 1863–1869. doi: 10.1021/es0613714.

Kumaraswamy, E., Malykh, A., Korotkov, K.V., Kozyavkin, S., Hu, Y., Kwon, S.Y., Moustafa, M.E., Carlson, B.A., Berry, M.J., Lee, B.J., Hatfield, D.L., Dimond, A.M., and Gladyshev, V.N. 2000. Structure-expression relationships of the 15-kDa selenoprotein gene Possible role of the protein in cancer etiology. J. Biol. Chem. **275**(45): 35540–35547.

Labunskyy, V.M., Hatfield, D.L., and Gladyshev, V.N. 2014. Selenoproteins: Molecular pathways and physiological roles. Physiol. Rev. **94**(3): 739–777. doi: 10.1152/physrev.00039.2013.

Larsen, E.H., Lobinski, R., Burger-Meÿer, K., Hansen, M., Ruzik, R., Mazurowska, L., Rasmussen, P.H., Sloth, J.J., Scholten, O., and Kik, C. 2006. Uptake and speciation of selenium in garlic cultivated in soil amended with symbiotic fungi (mycorrhiza) and selenate. Anal. Bioanal. Chem. **385**(6): 1098–1108. doi: 10.1007/s00216-006-0535-x.

LeDuc, D.L., AbdelSamie, M., Móntes-Bayon, M., Wu, C.P., Reisinger, S.J., and Terry, N. 2006. Overexpressing both ATP sulfurylase and selenocysteine methyltransferase enhances selenium phytoremediation traits in Indian mustard. Environ. Pollut. **144**(1): 70–76. doi: 10.1016/j.envpol.2006.01.008.

Leustek, T. 2002. Sulfate Metabolism. Arab. Book Am. Soc. Plant Biol. **1**. doi: 10.1199/tab.0017.

Li, H.-F., McGrath, S.P., and Zhao, F.-J. 2008. Selenium uptake, translocation and speciation in wheat supplied with selenate or selenite. New Phytol. **178**(1): 92–102. doi: 10.1111/j.1469-8137.2007.02343.x.

Lintschinger, J., Fuchs, N., Moser, J., Kuehnelt, D., and Goessler, W. 2000. Selenium-enriched sprouts. A raw material for fortified cereal-based diets. J. Agric. Food Chem. **48**(11): 5362–5368. doi: 10.1021/jf000509d.

Lippman, S.M., Klein, E.A., Goodman, P.J., Lucia, M.S., Thompson, I.M., Ford, L.G., Parnes, H.L., Minasian, L.M., Gaziano, J.M., Hartline, J.A., Parsons, J.K., Bearden III, J.D., Crawford, E.D., Goodman, G.E., Claudio, J., Winquist, E., Cook, E.D., Karp, D.D., Walther, P., Lieber, M.M., Kristal, A.R., Darke, A.K., Arnold, K.B., Ganz, P.A., Santella, R.M., Albanes, D., Taylor, P.R., Probstfield, J.L., Jagpal, T.J., Crowley, J.J., Meyskens Jr., F.L., Baker, L.H., and Coltman Jr., C.A. 2009. Effect of selenium and vitamin E on risk of prostate cancer and other cancers: The selenium and vitamin E cancer prevention trial (SELECT). JAMA - J. Am. Med. Assoc. **301**(1): 39–51. doi: 10.1001/jama.2008.864.

Liu, Y., Lopez-Avila, V., Zhu, J.J., Wlederin, D.R., and Beckert, W.F. 1995. Capillary electrophoresis coupled on-line with inductively coupled plasma mass spectrometry for elemental speciation. Anal. Chem. **67**(13): 2020–2025.

Lobanov, A.V., Fomenko, D.E., Zhang, Y., Sengupta, A., Hatfield, D.L., and Gladyshev, V.N. 2007. Evolutionary dynamics of eukaryotic selenoproteomes: Large selenoproteomes may associate with aquatic life and small with terrestrial life. Genome Biol. **8**(9). doi: 10.1186/gb-2007-8-9-r198.

Lobinski, R. 1998. Speciation - targets, analytical solutions and markets. Spectrochim. Acta Part B At. Spectrosc. **53**(2): 177–185. doi: 10.1016/S0584-8547(97)00146-8.

Lobinski, R., Edmonds, J.S., Suzuki, K.T., and Uden, P.C. 2000. Species-selective determination of selenium compounds in biological materials (Technical report). Pure Appl. Chem. **72**(3): 447–461. doi: 10.1351/pac200072030447.

Lobinski, R., Moulin, C., and Ortega, R. 2006. Imaging and speciation of trace elements in biological environment. Biochimie **88**(11): 1591–1604. doi: 10.1016/j.biochi.2006.10.003.

Lobiński, R., and Szpunar, J. 1999. Biochemical speciation analysis by hyphenated techniques. Anal. Chim. Acta **400**(1-3): 321–332. doi: 10.1016/S0003-2670(99)00628-5.

Van Loon, J.C., Radziuk, B., and Kahn, N. 1977. Metal speciation using atomic absorption spectroscopy. At. Absorpt Newslett **16**(4): 79–83.

Makarov, A., Denisov, E., Kholomeev, A., Balschun, W., Lange, O., Strupat, K., and Horning, S. 2006. Performance Evaluation of a Hybrid Linear Ion Trap/Orbitrap Mass Spectrometer. Anal. Chem. **78**(7): 2113–2120. doi: 10.1021/ac0518811.

Mallick, P., and Kuster, B. 2010. Proteomics: A pragmatic perspective. Nat. Biotechnol. **28**(7): 695–709. doi: 10.1038/nbt.1658.

Mann, M., Kulak, N.A., Nagaraj, N., and Cox, J. 2013. The Coming Age of Complete, Accurate, and Ubiquitous Proteomes. Mol. Cell **49**(4): 583–590. doi: 10.1016/j.molcel.2013.01.029.

McClung, J.P., Roneker, C.A., Mu, W., Lisk, D.J., Langlais, P., Liu, F., and Lei, X.G. 2004. Development of insulin resistance and obesity in mice overexpressing cellular glutathione peroxidase. Proc. Natl. Acad. Sci. U. S. A. **101**(24): 8852–8857.

McConnell, K.P. 1963. Metabolism of selenium in the mammalian organism. J. Agric. Food Chem. **11**(5): 385–388. doi: 10.1021/jf60129a011.

McLafferty, F.W., Breuker, K., Jin, M., Han, X., Infusini, G., Jiang, H., Kong, X., and Begley, T.P. 2007. Top-down MS, a powerful complement to the high capabilities of proteolysis proteomics. FEBS J. **274**(24): 6256–6268. doi: 10.1111/j.1742-4658.2007.06147.x.

McSheehy, S., Kelly, J., Tessier, L., and Mester, Z. 2005. Identification of selenomethionine in selenized yeast using two-dimensional liquid chromatography-mass spectrometry based proteomic analysis. Analyst **130**(1): 35–37. doi: 10.1039/b414246b.

McSheehy, S., Pannier, F., Szpunar, J., Potin-Gautier, M., and Lobinski, R. 2002a. Speciation of seleno compounds in yeast aqueous extracts by three-dimensional liquid chromatography with inductively coupled plasma mass spectrometric and electrospray mass spectrometric detection. Analyst **127**(2): 223–229. doi: 10.1039/b108680b.

McSheehy, S., Pohl, P., Szpunar, J., Potin-Gautier, M., and ŁObiński, R. 2001. Analysis for selenium speciation in selenized yeast extracts by two-dimensional liquid chromatography with ICP-MS and electrospray MS-MS detection. J. Anal. At. Spectrom. **16**(1): 68–73. doi: 10.1039/b007427f.

McSheehy, S., Szpunar, J., Haldysb, V., and Tortajada, J. 2002b. Identification of selenocompounds in yeast by electrospray quadrupole-time of flight mass spectrometry. J. Anal. At. Spectrom. **17**(5): 507–514. doi: 10.1039/b201015c.

McSheehy, S., Yang, W., Pannier, F., Szpunar, J., Lobiński, R., Auger, J., and Potin-Gautier, M. 2000. Speciation analysis of selenium in garlic by two-dimensional high-performance liquid chromatography with parallel inductively coupled plasma mass spectrometric and electrospray tandem mass spectrometric detection. Anal. Chim. Acta **421**(2): 147–153. doi: 10.1016/S0003-2670(00)01039-4.

Meija, J., Montes-Bayón, M., Le Duc, D.L., Terry, N., and Caruso, J.A. 2002. Simultaneous monitoring of volatile selenium and sulfur species from se accumulating plants (wild type and genetically modified) by GC/MS and GC/ICPMS using solid-phase microextraction for sample introduction. Anal. Chem. **74**(22): 5837–5844. doi: 10.1021/ac020285t.

Mester, Z., Sturgeon, R., and Pawliszyn, J. 2001. Solid phase microextraction as a tool for trace element speciation. Spectrochim. Acta - Part B At. Spectrosc. **56**(3): 233–260. doi: 10.1016/S0584-8547(00)00304-9.

Mester, Z., Willie, S., Yang, L., Sturgeon, R., Caruso, J.A., Fernández, M.L., Fodor, P., Goldschmidt, R.J., Goenaga-Infante, H., Lobinski, R., Maxwell, P., McSheehy, S., Polatajko, A., Sadi, B.B.M., Sanz-Medel, A., Scriver, C., Szpunar, J., Wahlen, R., and Wolf, W. 2006. Certification of a new selenized yeast reference material (SELM-1) for methionine, selenomethinone and total selenium content and its use in an intercomparison exercise for quantifying these analytes. Anal. Bioanal. Chem. **385**(1): 168–180. doi: 10.1007/s00216-006-0338-0.

Michalski, A., Cox, J., and Mann, M. 2011. More than 100,000 detectable peptide species elute in single shotgun proteomics runs but the majority is inaccessible to data-dependent LC-MS/MS. J. Proteome Res. **10**(4): 1785–1793. doi: 10.1021/pr101060v.

Miernyk, J.A., and Hajduch, M. 2011. Seed proteomics. J. Proteomics **74**(4): 389–400. doi: 10.1016/j.jprot.2010.12.004.

Miraliakbari, H., and Shahidi, F. 2008. Lipid Class Compositions, Tocopherols and Sterols of Tree Nut Oils Extracted with Different Solvents. J. Food Lipids **15**(1): 81–96. doi: 10.1111/j.1745-4522.2007.00104.x.

Möckel, H.J., Höfler, F., and Melzer, H. 1985. The retention of selenium chains in reversed-phase liquid chromatography. Chromatographia **20**(8): 471–476. doi: 10.1007/BF02344788.

Montaser, A. 1998. Inductively Coupled Plasma Mass Spectrometry. John Wiley & Sons, Hoboken (NJ), US.

Montes-Bayón, M., Molet, M.J.D., González, E.B., and Sanz-Medel, A. 2006. Evaluation of different sample extraction strategies for selenium determination in selenium-enriched plants (Allium sativum and Brassica juncea) and Se speciation by HPLC-ICP-MS. Talanta **68**(4): 1287–1293. doi: 10.1016/j.talanta.2005.07.040.

Montes-Bayón, M., Yanes, E.G., De León, C.P., Jayasimhulu, K., Stalcup, A., Shann, J., and Caruso, J.A. 2002. Initial studies of selenium speciation in Brassica juncea by LC with

ICPMS and ES-MS detection: An approach for phytoremediation studies. Anal. Chem. **74**(1): 107–113. doi: 10.1021/ac0106804.

Moreno, F.J., Jenkins, J.A., Mellon, F.A., Rigby, N.M., Robertson, J.A., Wellner, N., and Mills, E.N.C. 2004. Mass spectrometry and structural characterization of 2S albumin isoforms from Brazil nuts (Bertholletia excelsa). Biochim. Biophys. Acta - Proteins Proteomics **1698**(2): 175–186. doi: 10.1016/j.bbapap.2003.11.007.

Mounicou, S., Dernovics, M., Bierla, K., and Szpunar, J. 2009a. A sequential extraction procedure for an insight into selenium speciation in garlic. Talanta **77**(5): 1877–1882. doi: 10.1016/j.talanta.2008.10.028.

Mounicou, S., Szpunar, J., and Lobinski, R. 2009b. Metallomics: The concept and methodology. Chem. Soc. Rev. **38**(4): 1119–1138. doi: 10.1039/b713633c.

Mukherjee, S.P., Lane, R.H., and Lynn, W.S. 1978. Endogenous hydrogen peroxide and peroxidative metabolism in adipocytes in response to insulin and sulfhydryl reagents. Biochem. Pharmacol. **27**(22): 2589–2594.

Münchberg, U., Anwar, A., Mecklenburg, S., and Jacob, C. 2007. Polysulfides as biologically active ingredients of garlic. Org. Biomol. Chem. **5**(10): 1505–1518. doi: 10.1039/b703832a.

Nakamura, R., and Teshima, R. 2013. Proteomics-based allergen analysis in plants. J. Proteomics **93**: 40–49. doi: 10.1016/j.jprot.2013.03.018.

Neale, D.B., and Kremer, A. 2011. Forest tree genomics: growing resources and applications. Nat. Rev. Genet. **12**(2): 111–122. doi: 10.1038/nrg2931.

Németh, A., García Reyes, J.F., Kosáry, J., and Dernovics, M. 2013. The relationship of selenium tolerance and speciation in Lecythidaceae species. Metallomics **5**(12): 1663–1673. doi: 10.1039/c3mt00140g.

Neuhierl, B., and Böck, A. 1996. On the mechanism of selenium tolerance in selenium-accumulating plants. Purification and characterization of a specific selenocysteine methyltransferase from cultured cells of Astragalus bisculatus. Eur. J. Biochem. **239**(1): 235–238.

Nilsson, T., Mann, M., Aebersold, R., Iii, J.R.Y., Bairoch, A., and Bergeron, J.J.M. 2010. Mass spectrometry in high-throughput proteomics: ready for the big time. Nat. Methods **7**(9): 681–685. doi: 10.1038/nmeth0910-681.

Nordberg, M., Duffus, J., and Templeton, D.M. 2004. Glossary of terms used in toxicokinetics (IUPAC Recommendations 2003). Pure Appl. Chem. **76**(5). doi: 10.1351/pac200476051033.

Ogra, Y., and Anan, Y. 2009. Selenometabolomics: Identification of selenometabolites and specification of their biological significance by complementary use of elemental and molecular mass spectrometry. J. Anal. At. Spectrom. **24**(11): 1477–1488. doi: 10.1039/b910235c.

Ogra, Y., and Anan, Y. 2012. Selenometabolomics explored by speciation. Biol. Pharm. Bull. **35**(11): 1863–1869. doi: 10.1248/bpb.b212016.

Ogra, Y., Ishiwata, K., Iwashita, Y., and Suzuki, K.T. 2005. Simultaneous speciation of selenium and sulfur species in selenized odorless garlic (Allium sativum L. Shiro) and shallot (Allium ascalonicum) by HPLC-inductively coupled plasma-(octopole reaction system)-mass spectrometry and electrospray ionization-tandem mass spectrometry. J. Chromatogr. A **1093**(1-2): 118–125. doi: 10.1016/j.chroma.2005.07.081.

Ogra, Y., Kitaguchi, T., Ishiwata, K., Suzuki, N., Iwashita, Y., and Suzuki, K.T. 2007. Identification of selenohomolanthionine in selenium-enriched Japanese pungent radish. J. Anal. At. Spectrom. **22**(11): 1390–1396. doi: 10.1039/b707348h.

Ouerdane, L., Aureli, F., Flis, P., Bierla, K., Preud'Homme, H., Cubadda, F., and Szpunar, J. 2013. Comprehensive speciation of low-molecular weight selenium metabolites in

mustard seeds using HPLC-electrospray linear trap/orbitrap tandem mass spectrometry. Metallomics **5**(9): 1294–1304. doi: 10.1039/c3mt00113j.

Pappin, D.J.C., Hojrup, P., and Bleasby, A.J. 1993. Rapid identification of proteins by peptide-mass fingerprinting. Curr. Biol. **3**(6): 327–332.

Papp, L.V., Holmgren, A., and Khanna, K.K. 2010. Selenium and selenoproteins in health and disease. Antioxid. Redox Signal. **12**(7): 793–795. doi: 10.1089/ars.2009.2973.

Papp, L.V., Lu, J., Holmgren, A., and Khanna, K.K. 2007. From selenium to selenoproteins: Synthesis, identity, and their role in human health. Antioxid. Redox Signal. **9**(7): 775–806. doi: 10.1089/ars.2007.1528.

Parker, D.R., Page, A.L., and Bell, P.F. 1992. Contrasting Selenate-Sulfate Interactions in Selenium-Accumulating and Nonaccumulating Plant Species. Soil Sci. Soc. Am. J. **56**(6): 1818. doi: 10.2136/sssaj1992.03615995005600060028x.

Pedrero, Z., Elvira, D., Cámara, C., and Madrid, Y. 2007a. Selenium transformation studies during Broccoli (Brassica oleracea) growing process by liquid chromatography-inductively coupled plasma mass spectrometry (LC-ICP-MS). Anal. Chim. Acta **596**(2): 251–256. doi: 10.1016/j.aca.2007.05.067.

Pedrero, Z., Encinar, J.R., Madrid, Y., and Cámara, C. 2007b. Identification of selenium species in selenium-enriched Lens esculenta plants by using two-dimensional liquid chromatography-inductively coupled plasma mass spectrometry and [77Se]selenomethionine selenium oxide spikes. J. Chromatogr. A **1139**(2): 247–253. doi: 10.1016/j.chroma.2006.11.031.

Pedrero, Z., and Madrid, Y. 2009. Novel approaches for selenium speciation in foodstuffs and biological specimens: A review. Anal. Chim. Acta **634**(2): 135–152. doi: 10.1016/j.aca.2008.12.026.

Phillips, K.M., Ruggio, D.M., and Ashraf-Khorassani, M. 2005. Phytosterol composition of nuts and seeds commonly consumed in the United States. J. Agric. Food Chem. **53**(24): 9436–9445. doi: 10.1021/jf051505h.

Pickering, I.J., Wright, C., Bubner, B., Ellis, D., Persans, M.W., Yu, E.Y., George, G.N., Prince, R.C., and Salt, D.E. 2003. Chemical form and distribution of selenium and sulfur in the selenium hyperaccumulator Astragalus bisulcatus. Plant Physiol. **131**(3): 1460–1467. doi: 10.1104/pp.014787.

Pickering, T.L., Saunders, K.J., and Tobolsky, A.V. 1967. Disproportionation of organic polysulfides. J. Am. Chem. Soc. **89**(10): 2364–2367.

Pietschmann, N., Rijntjes, E., Hoeg, A., Stoedter, M., Schweizer, U., Seemann, P., and Schomburg, L. 2014. Selenoprotein P is the essential selenium transporter for bones. Metallomics **6**(5): 1043–1049. doi: 10.1039/C4MT00003J.

Platz, E.A., and Lippman, S.M. 2009. Selenium, genetic variation, and prostate cancer risk: Epidemiology reflects back on selenium and vitamin E cancer prevention trial. J. Clin. Oncol. **27**(22): 3569–3572. doi: 10.1200/JCO.2009.22.2117.

Pramanik, B.N., Mirza, U.A., Ing, Y.H., Liu, Y.-H., Bartner, P.L., Weber, P.C., and Bose, A.K. 2002. Microwave-enhanced enzyme reaction for protein mapping by mass spectrometry: A new approach to protein digestion in minutes. Protein Sci. **11**(11): 2676–2687. doi: 10.1110/ps.0213702.

Preud'Homme, H., Far, J., Gil-Casal, S., and Lobinski, R. 2012. Large-scale identification of selenium metabolites by online size-exclusion-reversed phase liquid chromatography with combined inductively coupled plasma (ICP-MS) and electrospray ionization linear trap-Orbitrap mass spectrometry (ESI-MSn). Metallomics **4**(5): 422–432. doi: 10.1039/c2mt00172a.

Prins, C.N., Hantzis, L.J., Quinn, C.F., and Pilon-Smits, E.A.H. 2011. Effects of selenium accumulation on reproductive functions in Brassica juncea and Stanleya pinnata. J. Exp. Bot. **62**(15): 5633–5640. doi: 10.1093/jxb/err247.

Quinn, C.F., Freeman, J.L., Reynolds, R.J.B., Cappa, J.J., Fakra, S.C., Marcus, M.A., Lindblom, S.D., Quinn, E.K., Bennett, L.E., and Pilon-Smits, E.A.H. 2010. Selenium hyperaccumulation offers protection from cell disruptor herbivores. BMC Ecol. **10**. doi: 10.1186/1472-6785-10-19.

Quinn, C.F., Prins, C.N., Freeman, J.L., Gross, A.M., Hantzis, L.J., Reynolds, R.J.B., in Yang, S., Covey, P.A., Bañuelos, G.S., Pickering, I.J., Fakra, S.C., Marcus, M.A., Arathi, H.S., and Pilon-Smits, E.A.H. 2011. Selenium accumulation in flowers and its effects on pollination. New Phytol. **192**(3): 727–737. doi: 10.1111/j.1469-8137.2011.03832.x.

Rayman, M.P. 2000. The importance of selenium to human health. The Lancet **356**(9225): 233–241. doi: 10.1016/S0140-6736(00)02490-9.

Rayman, M.P. 2008. Food-chain selenium and human health: Emphasis on intake. Br. J. Nutr. **100**(2): 254–268. doi: 10.1017/S0007114508939830.

Rayman, M.P. 2012. Selenium and human health. The Lancet **379**(9822): 1256–1268. doi: 10.1016/S0140-6736(11)61452-9.

Rayman, M.P., Infante, H.G., and Sargent, M. 2008. Food-chain selenium and human health: Spotlight on speciation. Br. J. Nutr. **100**(2): 238–253. doi: 10.1017/S0007114508922522.

Read, R., Bellew, T., Yang, J.G., Hill, K.E., Palmer, I.S., and Burk, R.F. 1990. Selenium and amino acid composition of selenoprotein P, the major selenoprotein in rat serum. J. Biol. Chem. **265**(29): 17899–17905.

Reinders, J. 2013. Pharmaceutical proteomics: A journey from discovery and characterisation of targets to development of high-throughput assays. Eur. Pharm. Rev. **18**(6): 33–37.

Reyes, L.H., Mar, J.L.G., Rahman, G.M.M., Seybert, B., Fahrenholz, T., and Kingston, H.M.S. 2009. Simultaneous determination of arsenic and selenium species in fish tissues using microwave-assisted enzymatic extraction and ion chromatography-inductively coupled plasma mass spectrometry. Talanta **78**(3): 983–990. doi: 10.1016/j.talanta.2009.01.003.

Rigby, M.C., Lemly, A.D., and Gerads, R. 2014. Fish toxicity testing with selenomethionine spiked feed - What's the real question being asked? Environ. Sci. Process. Impacts **16**(3): 511–517. doi: 10.1039/c3em00612c.

Rotruck, J.T., Pope, A.L., Ganther, H.E., Swanson, A.B., Hafeman, D.G., and Hoekstra, W.G. 1973. Selenium: biochemical role as a component of glutathione peroxidase. Science **179**(4073): 588–590.

Rubino, F.M., Verduci, C., Giampiccolo, R., Pulvirenti, S., Brambilla, G., and Colombi, A. 2004. Characterization of the disulfides of bio-thiols by electrospray ionization and triple-quadrupole tandem mass spectrometry. J. Mass Spectrom. **39**(12): 1408–1416. doi: 10.1002/jms.745.

Rundqvist, L., Tengel, T., Zdunek, J., Björn, E., Schleucher, J., Alcocer, M.J.C., and Larsson, G. 2012. Solution Structure, Copper Binding and Backbone Dynamics of Recombinant Ber e 1-The Major Allergen from Brazil Nut. PLoS ONE **7**(10). doi: 10.1371/journal.pone.0046435.

Sabbagh, M., and Van Hoewyk, D. 2012. Malformed selenoproteins are removed by the ubiquitin-proteasome pathway in stanleya pinnata. Plant Cell Physiol. **53**(3): 555–564. doi: 10.1093/pcp/pcs015.

Schroeder, H.A., Frost, D.V., and Balassa, J.J. 1970. Essential trace metals in man: Selenium. J. Chronic Dis. **23**(4): 227–243. doi: 10.1016/0021-9681(70)90003-2.

Schwarz, K., Bieri, J.G., Briggs, G.M., and Scott, M.L. 1957. Prevention of exudative diathesis in chicks by factor 3 and selenium. Proc. Soc. Exp. Biol. Med. Soc. Exp. Biol. Med. N. Y. N **95**(4): 621–625. doi: 10.3181/00379727-95-23308.

Secor, C.L., and Lisk, D.J. 1989. Variation in the Selenium Content of Individual Brazil Nuts. J. Food Saf. **9**(4): 279–281. doi: 10.1111/j.1745-4565.1989.tb00527.x.

Senn, M., Venkataraghavan, R., and McLafferty, F.W. 1966. Mass Spectrometric Studies of Peptides. III.1 Automated Determination of Amino Acid Sequences. J. Am. Chem. Soc. **88**(23): 5593–5597. doi: 10.1021/ja00975a044.

Shah, M., Meija, J., and Caruso, J.A. 2007. Relative mass defect filtering of high-resolution mass spectra for exploring minor selenium volatiles in selenium-enriched green onions. Anal. Chem. **79**(3): 846–853. doi: 10.1021/ac060703k.

Shamberger, R.J., and Frost, D.V. 1969. Possible protective effect of selenium against human cancer. Can. Med. Assoc. J. **100**(14): 682.

Shibagaki, N., Rose, A., McDermott, J.P., Fujiwara, T., Hayashi, H., Yoneyama, T., and Davies, J.P. 2002. Selenate-resistant mutants of Arabidopsis thaliana identify Sultr1;2, a sulfate transporter required for efficient transport of sulfate into roots. Plant J. **29**(4): 475–486. doi: 10.1046/j.0960-7412.2001.01232.x.

Sors, T.G., Ellis, D.R., Gun, N.N., Lahner, B., Lee, S., Leustek, T., Pickering, I.J., and Salt, D.E. 2005a. Analysis of sulfur and selenium assimilation in Astragalus plants with varying capacities to accumulate selenium. Plant J. **42**(6): 785–797. doi: 10.1111/j.1365-313X.2005.02413.x.

Sors, T.G., Ellis, D.R., and Salt, D.E. 2005b. Selenium uptake, translocation, assimilation and metabolic fate in plants. Photosynth. Res. **86**(3): 373–389. doi: 10.1007/s11120-005-5222-9.

De Souza, M.P., Pilon-Smits, E.A.H., Mel Lytle, C., Hwang, S., Tai, J., Honma, T.S.U., Yeh, L., and Terry, N. 1998. Rate-limiting steps in selenium assimilation and volatilization by Indian mustard. Plant Physiol. **117**(4): 1487–1494.

Stadlober, M., Sager, M., and Irgolic, K.J. 2001. Effects of selenate supplemented fertilisation on the selenium level of cereals — identification and quantification of selenium compounds by HPLC–ICP–MS. Food Chem. **73**(3): 357–366. doi: 10.1016/S0308-8146(01)00115-7.

Stadtman, T.C. 1977. Biological Function of Selenium. Nutr. Rev. **35**(7): 161–166. doi: 10.1111/j.1753-4887.1977.tb06579.x.

Sun, S.S., Altenbach, S.B., and Leung, F.W. 1987. Properties, biosynthesis and processing of a sulfur-rich protein in Brazil nut (Bertholletia excelsa H.B.K.). Eur. J. Biochem. **162**(3): 477–483.

Sun, W., Gao, S., Wang, L., Chen, Y., Wu, S., Wang, X., Zheng, D., and Gao, Y. 2006. Microwave-assisted Protein Preparation and Enzymatic Digestion in Proteomics. Mol. Cell. Proteomics **5**(4): 769–776. doi: 10.1074/mcp.T500022-MCP200.

Swearingen Jr., J.W., Frankel, D.P., Fuentes, D.E., Saavedra, C.P., Vásquez, C.C., and Chasteen, T.G. 2006. Identification of biogenic dimethyl selenodisulfide in the headspace gases above genetically modified Escherichia coli. Anal. Biochem. **348**(1): 115–122. doi: 10.1016/j.ab.2005.10.007.

Syka, J.E.P., Coon, J.J., Schroeder, M.J., Shabanowitz, J., and Hunt, D.F. 2004. Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. Proc. Natl. Acad. Sci. U. S. A. **101**(26): 9528–9533. doi: 10.1073/pnas.0402700101.

Szabó, G., Bálint, S., Nyeste, E., Medgyesi, I., Tamáskovics, A., and Podmaniczky, G. 1991. Trace element deficiency in healthy subjects based on multi-element analysis of serum and plasma. Orv. Hetil. **132**(8): 395–400.

Szpunar, J. 2005. Advances in analytical methodology for bioinorganic speciation analysis: Metallomics, metalloproteomics and heteroatom-tagged proteomics and metabolomics. Analyst **130**(4): 442–465. doi: 10.1039/b418265k.

Szpunar, J., and Łobiński, R. 2002. Multidimensional approaches in biochemical speciation analysis. Anal. Bioanal. Chem. **373**(6): 404–411. doi: 10.1007/s00216-002-1282-2.

Szpunar, J., and Lobinski, R. 2003. Hyphenated Techniques in Speciation Analysis. Royal Society of Chemistry.

Szpunar, J., Lobinski, R., and Prange, A. 2003. Hyphenated techniques for elemental speciation in biological systems. Appl. Spectrosc. **57**(3): 102A–112A. doi: 10.1039/9781847551092.

Takatera, K., and Osaki, N. 1994. HPLC/ICP mass spectrometric study of the selenium incorporation into cyanobacterial metallothionein induced under heavy-metal stress. Anal. Sci. **10**(4): 567–572. doi: 10.2116/analsci.10.567.

Tamaoki, M., Freeman, J.L., and Pilon-Smits, E.A.H. 2008. Cooperative ethylene and jasmonic acid signaling regulates selenite resistance in Arabidopsis. Plant Physiol. **146**(3): 1219–1230. doi: 10.1104/pp.107.110742.

Tanner, S.D., Baranov, V.I., and Bandura, D.R. 2002. Reaction cells and collision cells for ICP-MS: a tutorial review. Spectrochim. Acta Part B At. Spectrosc. **57**(9): 1361–1452. doi: 10.1016/S0584-8547(02)00069-1.

Taylor, H.E. 2001. Inductively Coupled Plasma-mass Spectrometry: Practices and Techniques. Academic Press, Elsevier.

Templeton, D.M., Ariese, F., Cornelis, R., Danielsson, L.-G., Muntau, H., Van Leeuwen, H.P., and Łobiński, R. 2000. Guidelines for terms related to chemical speciation and fractionation of elements. Definitions, structural aspects, and methodological approaches (IUPAC recommendations 2000). Pure Appl. Chem. **72**(8): 1453–1470.

Terry, N., Zayed, A.M., De Souza, M.P., and Tarun, A.S. 2000. Selenium in higher plants. Annu. Rev. Plant Biol. **51**: 401–432.

Trelease, S.F., and Trelease, H.M. 1938. Selenium as a Stimulating and Possibly Essential Element for Indicator Plants. Am. J. Bot. **25**(5): 372–380. doi: 10.2307/2436763.

Vaudel, M., Barsnes, H., Berven, F.S., Sickmann, A., and Martens, L. 2011. SearchGUI: An open-source graphical user interface for simultaneous OMSSA and X!Tandem searches. Proteomics **11**(5): 996–999. doi: 10.1002/pmic.201000595.

Vaudel, M., Sickmann, A., and Martens, L. 2014. Introduction to opportunities and pitfalls in functional mass spectrometry based proteomics. Biochim. Biophys. Acta BBA - Proteins Proteomics **1844**(1, Part A): 12–20. doi: 10.1016/j.bbapap.2013.06.019.

Virupaksha, T.K., and Shrift, A. 1965. Biochemical differences between selenium accumulator and non-accumulator astragalus species. Biochim. Biophys. Acta BBA - Gen. Subj. **107**(1): 69–80. doi: 10.1016/0304-4165(65)90389-2.

Vonderheide, A.P., Wrobel, K., Kannamkumarath, S.S., B'Hymer, C., Montes-Bayón, M., De León, C.P., and Caruso, J.A. 2002. Characterization of selenium species in Brazil nuts by HPLC-ICP-MS and ES-MS. J. Agric. Food Chem. **50**(20): 5722–5728. doi: 10.1021/jf0256541.

Wang, J., Liu, C.-Q., Lu, H., Wei, C., and Zhou, T. 2007. Accurate measurement of trace selenium in fish by isotope dilution multiple collector inductively coupled plasma-mass spectrometry with hexapole collision cell. Fenxi Huaxue Chin. J. Anal. Chem. **35**(6): 814–818.

Watkinson, J.H. 1966. Fluorometric determination of selenium in biological material with 2,3-diaminonaphthalene. Anal. Chem. **38**(1): 92–97.

Westlake Jr., H.E., Laquer, H.L., and Smyth, C.P. 1950. The dipole moments and the interconvertibility of diethyl disulfide and trisulfide. J. Am. Chem. Soc. **72**(1): 436–438.

Whanger, P.D. 2002. Selenocompounds in plants and animals and their biological significance. J. Am. Coll. Nutr. **21**(3): 223–232.

White, P.J., Bowen, H.C., Marshall, B., and Broadley, M.R. 2007. Extraordinarily High Leaf Selenium to Sulfur Ratios Define "Se-accumulator" Plants. Ann. Bot. **100**(1): 111–118. doi: 10.1093/aob/mcm084.

Woller, Á., Garraud, H., Boisson, J., Dorthe, A.M., Fodor, P., and Donard, O.F.X. 1998. Simultaneous speciation of redox species of arsenic and selenium using an anion-exchange microbore column coupled with a micro-concentric nebulizer and an

inductively coupled plasma mass spectrometer as detector. J. Anal. At. Spectrom. **13**(2): 141–149. doi: 10.1039/A704772J.

Woodrow, C.C., Carmack, M., and Miller, J.G. 1951. The dipole moments and structures of a series of alkyl polysulfides. J. Chem. Phys. **19**(7): 951–954.

Wu, B., and Becker, J.S. 2012. Imaging techniques for elements and element species in plant science. Metallomics **4**(5): 403–416. doi: 10.1039/C2MT00002D.

Xue, T., and Hartikainen, H. 2000. Association of antioxidative enzymes with the synergistic effect of selenium and UV irradiation in enhancing plant growth. Agric. Food Sci. Finl. **9**(2): 177–186.

Xue, T., Hartikainen, H., and Piironen, V. 2001. Antioxidative and growth-promoting effect of selenium on senescing lettuce. Plant Soil **237**(1): 55–61. doi: 10.1023/A:1013369804867.

Xu, X., Carlson, B., Irons, R., Mix, H., Zhong, N., Gladyshev, V., and Hatfield, D. 2007. Selenophosphate synthetase 2 is essential for selenoprotein biosynthesis. Biochem J **404**: 115–120.

Yang, L., Maxwell, P., and Mester, Z. 2013. Microwave-assisted acid digestion protocol for the determination of methionine and selenomethionine in selenium-enriched yeast by species specific isotope dilution GC-MS. Anal. Methods **5**(2): 525–529. doi: 10.1039/c2ay25498k.

Yasumoto, K., Suzuki, T., and Yoshida, M. 1988. Identification of selenomethionine in soybean protein. J. Agric. Food Chem. **36**(3): 463–467. doi: 10.1021/jf00081a014.

Yathavakilla, S.V.K., Shah, M., Mounicou, S., and Caruso, J.A. 2005. Speciation of cationic selenium compounds in Brassica juncea leaves by strong cation-exchange chromatography with inductively coupled plasma mass spectrometry. J. Chromatogr. A **1100**(2): 153–159. doi: 10.1016/j.chroma.2005.09.025.

Yu, C., Cai, Q., Guo, Z.-X., Yang, Z., and Khoo, S.B. 2004. Simultaneous speciation of inorganic selenium and tellurium by inductively coupled plasma mass spectrometry following selective solid-phase extraction separation. J. Anal. At. Spectrom. **19**(3): 410–413. doi: 10.1039/B310318H.

Zheng, J., Goessler, W., and Kosmus, W. 1998. The chemical forms of selenium in selenium nutritional supplements: An investigation by using HPLC/ICP/MS and GF/AAS. Trace Elem. Electrocytes **15**(2): 70–75.

Zhu, Y.-G., Pilon-Smits, E.A.H., Zhao, F.-J., Williams, P.N., and Meharg, A.A. 2009. Selenium in higher plants: understanding mechanisms for biofortification and phytoremediation. Trends Plant Sci. **14**(8): 436–442. doi: 10.1016/j.tplants.2009.06.006.

Zoorob, G.K., McKiernan, J.W., and Caruso, J.A. 1998. ICP-MS for Elemental Speciation Studies. Mikrochim. Acta **128**(3--4): 145–168. doi: 10.1007/BF01243044.

Zubarev, R.A., Kelleher, N.L., and McLafferty, F.W. 1998. Electron capture dissociation of multiply charged protein cations. A nonergodic process. J. Am. Chem. Soc. **120**(13): 3265–3266. doi: 10.1021/ja973478k.

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10. APPENDICES

Table A1. Accurate mass database of known selenometabolites mainly from plant origin.

Formula	Molecular mass	Compound name
H_2O_3Se	127.9013	selenite
H_2O_4Se	145.9118	selenate
PH_3O_3Se	161.8985	selenophosphate
$C_3H_7NO_2Se$	168.9642	selenocysteine
$C_4H_9NO_2Se$	182.9799	selenohomocysteine
$C_4H_9NO_2Se$	182.9799	*Se*-methyl-selenocysteine
$C_5H_{11}NO_2Se$	196.9955	selenomethionine
$C_3H_7NO_4Se$	200.9540	selenocysteine selenate
$C_6H_{14}NO_2Se$	212.0190	*Se*-methyl-selenomethionine
$C_5H_{11}NO_3Se$	212.9904	selenomethionine-Se-oxide
$C_6H_{11}NO_3Se$	224.9904	Se-propylselenocysteinese-oxide
$C_7H_{14}N_2O_4Se$	270.0119	selenocystathionine
$C_8H_{16}N_2O_4Se$	284.0276	selenohomolanthionine
$C_9H_{16}N_2O_5Se$	312.0224	γ-glu-*Se*-methylselenocysteine
$C_9H_{16}N_2O_5Se$	312.0225	*N*-acetylselenocystathionine
$C_{10}H_{18}N_2O_5Se$	326.0381	γ-glu-SeMet
$C_6H_{12}N_2O_4Se_2$	335.9128	selenocystine
$C_{10}H_{17}N_3O_6Se$	355.0283	*Se*-glutathione
$C_8H_{16}N_2O_4Se_2$	363.9441	selenohomocystine
$C_{11}H_{19}N_3O_6Se$	369.0440	*Se*-methylseleno-glutathione
$C_{12}H_{21}N_3O_7Se$	399.0545	γ-glu-selenocystathionine
$C_{14}H_{20}N_6O_5Se$	432.0660	*Se*-adenosyl-selenohomocysteine
$C_{15}H_{23}N_6O_5Se$	447.0895	*Se*-adenosyl-selenomethionine
$C_{10}H_{14}N_5O_{10}PSe$	474.9644	adenosine-5'-phosphoselenate
$C_{14}H_{21}N_3O_9SSe$	487.0163	3-dihydroxypropionyl-SeCys
$C_{15}H_{23}N_3O_9SSe$	501.0320	3-dihydroxypropionyl-SeHCy
$C_{14}H_{21}N_3O_9Se_2$	534.9611	3-dihydroxypropionyl-SeCys
$C_{10}H_{15}N_5O_{13}P_2Se$	554.9307	3'-phosphoadenosine-5'-phosphoselenate
$C_{18}H_{29}N_5O_{11}SSe$	603.0750	γ-glu-selenocysteine

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Table A2: List of the mass-to-charge ratios of the selenocompounds from *L. minor* and *B. excelsa* that were present in too low concentration to be identified by fragmentation. Compounds in bold are the derivatives of SeHCy (they contain the in-source fragment *m/z* 181.9715).

Positive ion mode	
Experimental *m/z*	
319.0188	**490.1907**
370.1117	**501.1353**
385.0508	**512.2107**
392.9468	**514.9453**
400.1760	**548.2326**
408.2374	551.1601
410.1446	557.1459
421.0729	**566.0404**
422.0717	596.1972
428.2059	629.1666
481.2540	**648.2114**
486.1243	

Negative ion mode
Experimental *m/z*
290.9824
426.9641
428.1838
479.2410

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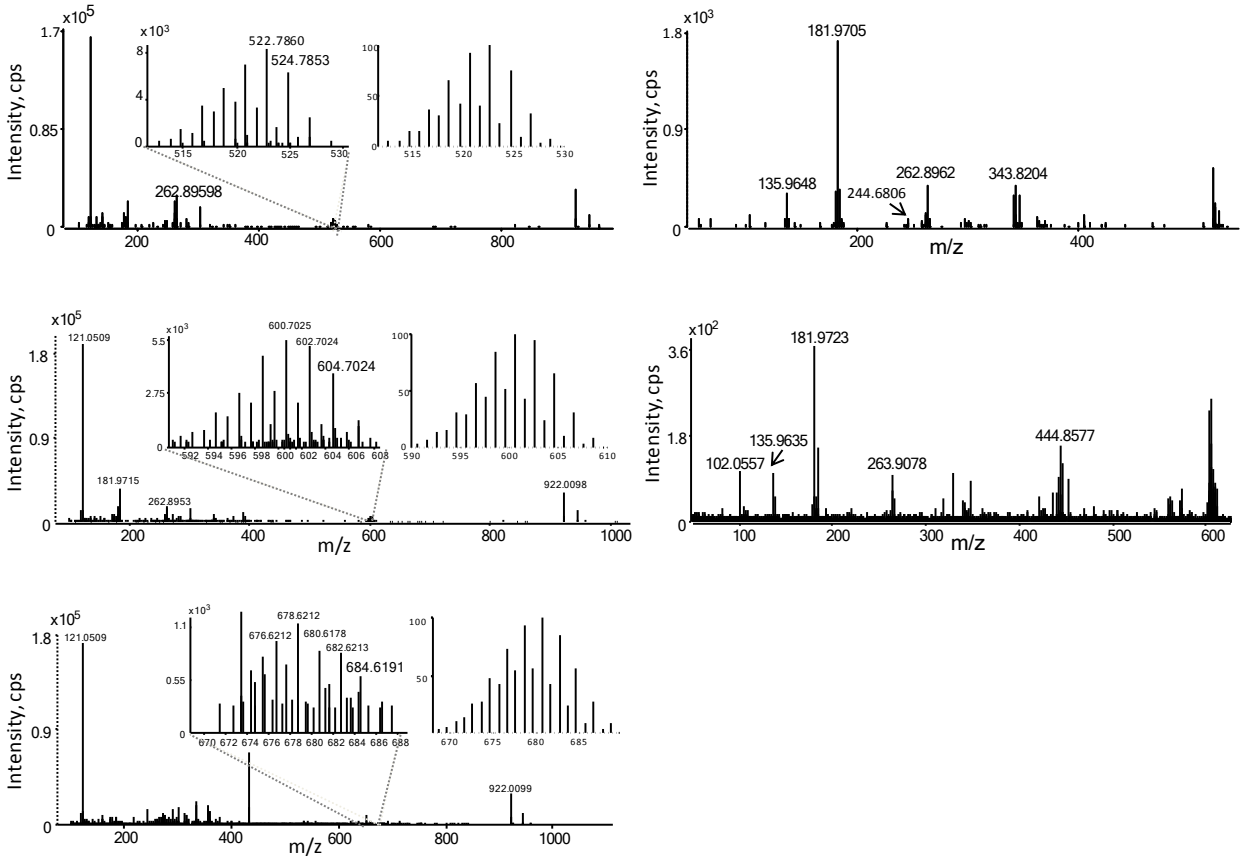

Figure A1. Fullscan and MS/MS spectra of the identified polyselenides. The intensity of compound *m/z* 685 was not sufficient for successful MS/MS experiments, therefore this compound was identified on the basis of its accurate mass, isotopic pattern and characteristic in-source fragments. The inserts show the experimental and theoretical isotopic patterns of the compounds.

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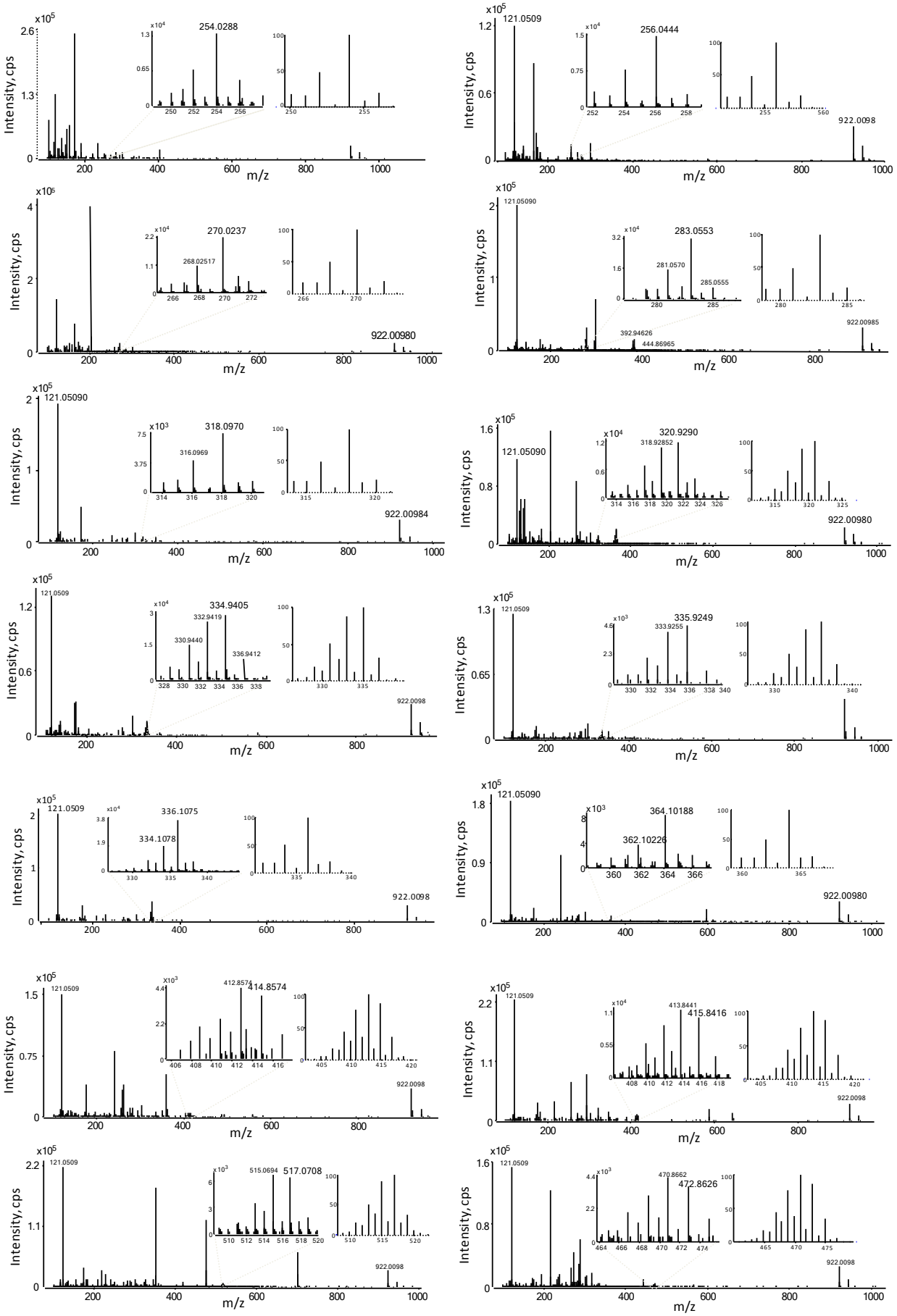

Figure A2. Fullscan spectra of other newly identified selenocompounds.

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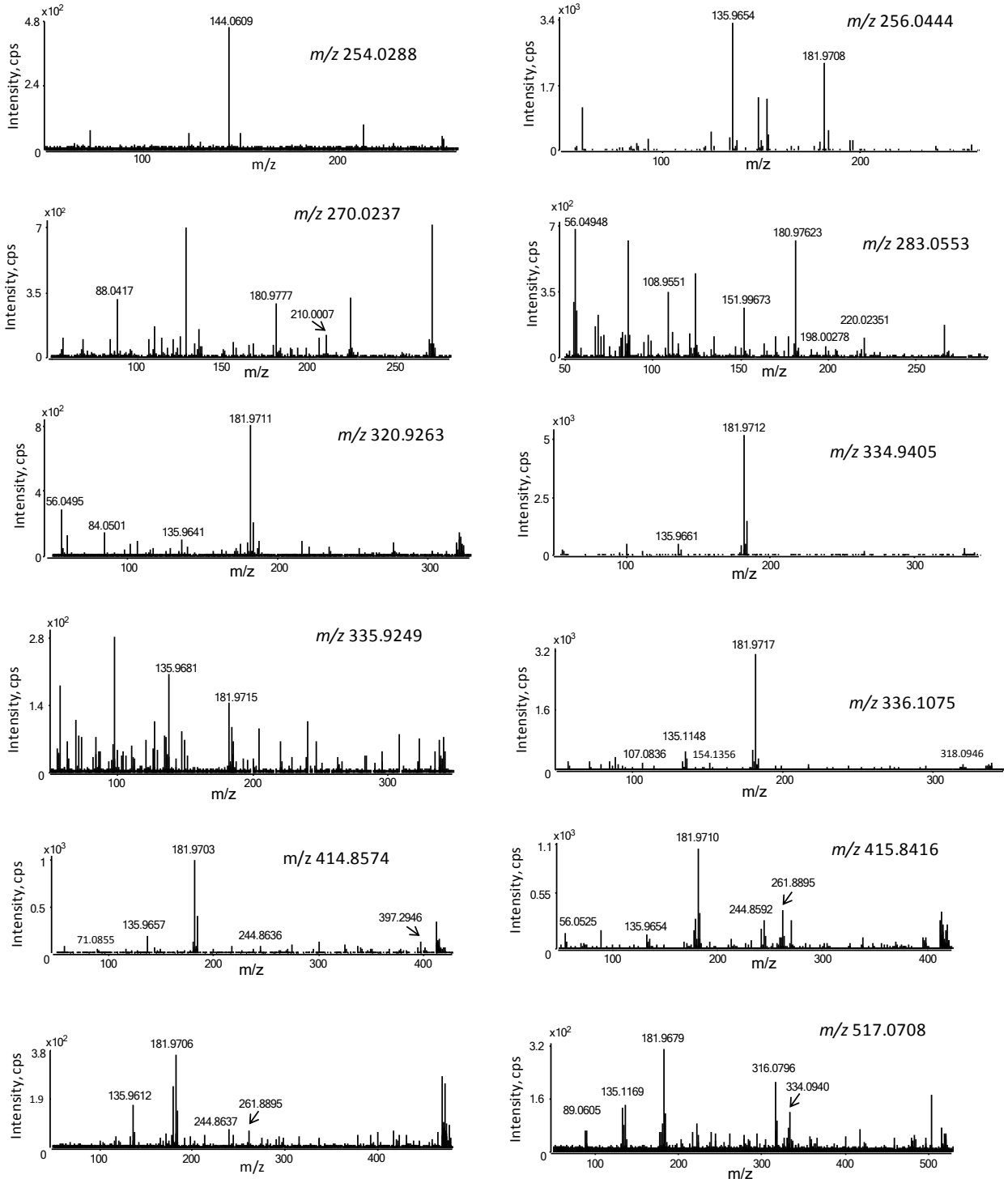

Figure A3. MS/MS spectra of other newly identified selenocompounds.

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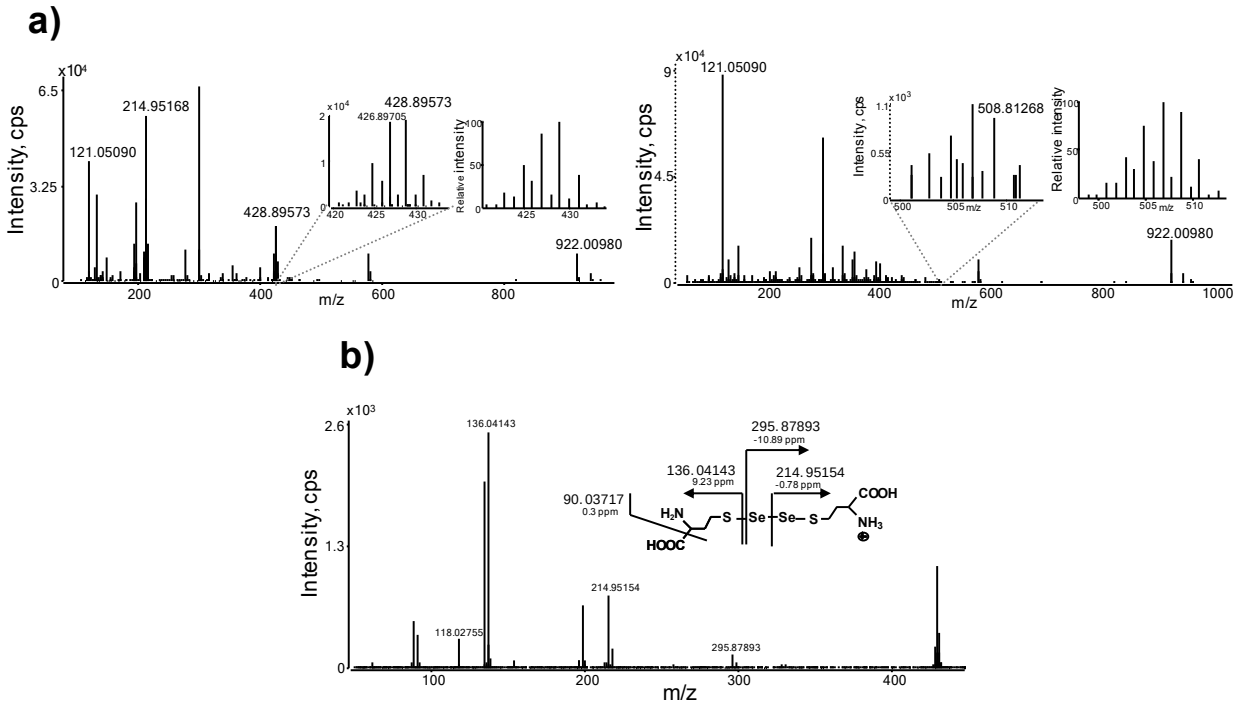

Figure A4. a) Fullscan spectra of the synthesized polychalcogenides. The inserts show the experimental and the theoretical isotopic distributions. b) The MS-MS spectra of the compound containing two Se atoms (m/z 428.8957). The insert shows the structure of the molecule and its fragmentation.

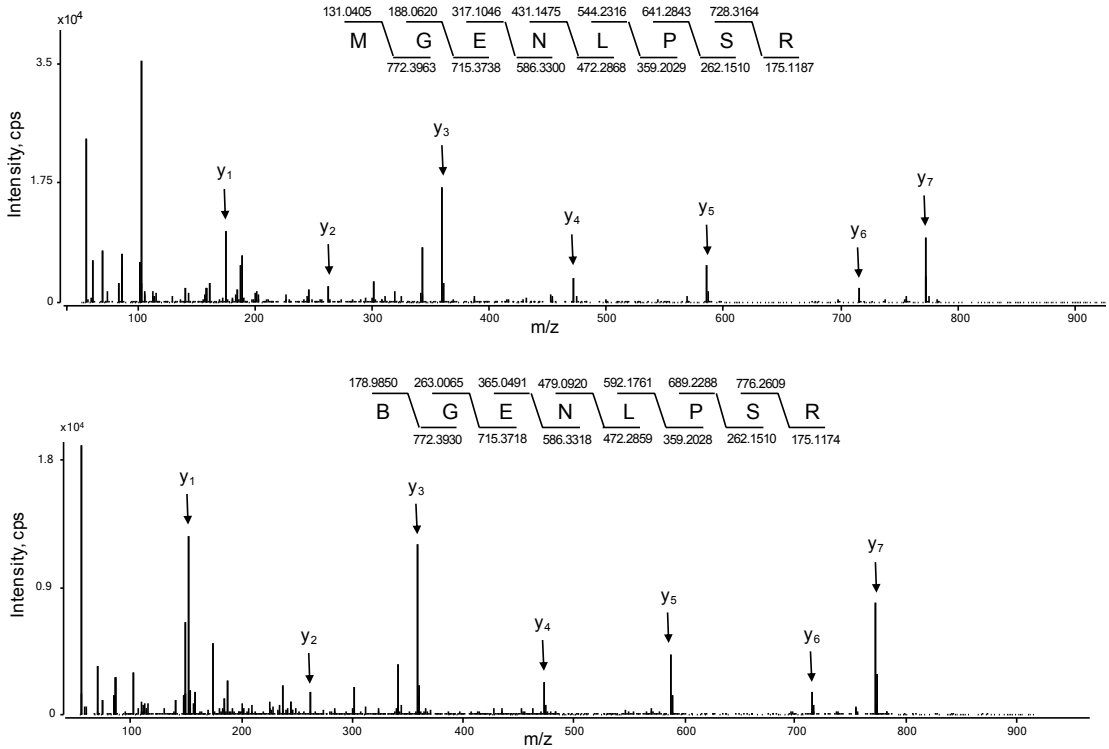

Figure A5. MS/MS spectra and details of *de novo* sequencing of selenopeptides.

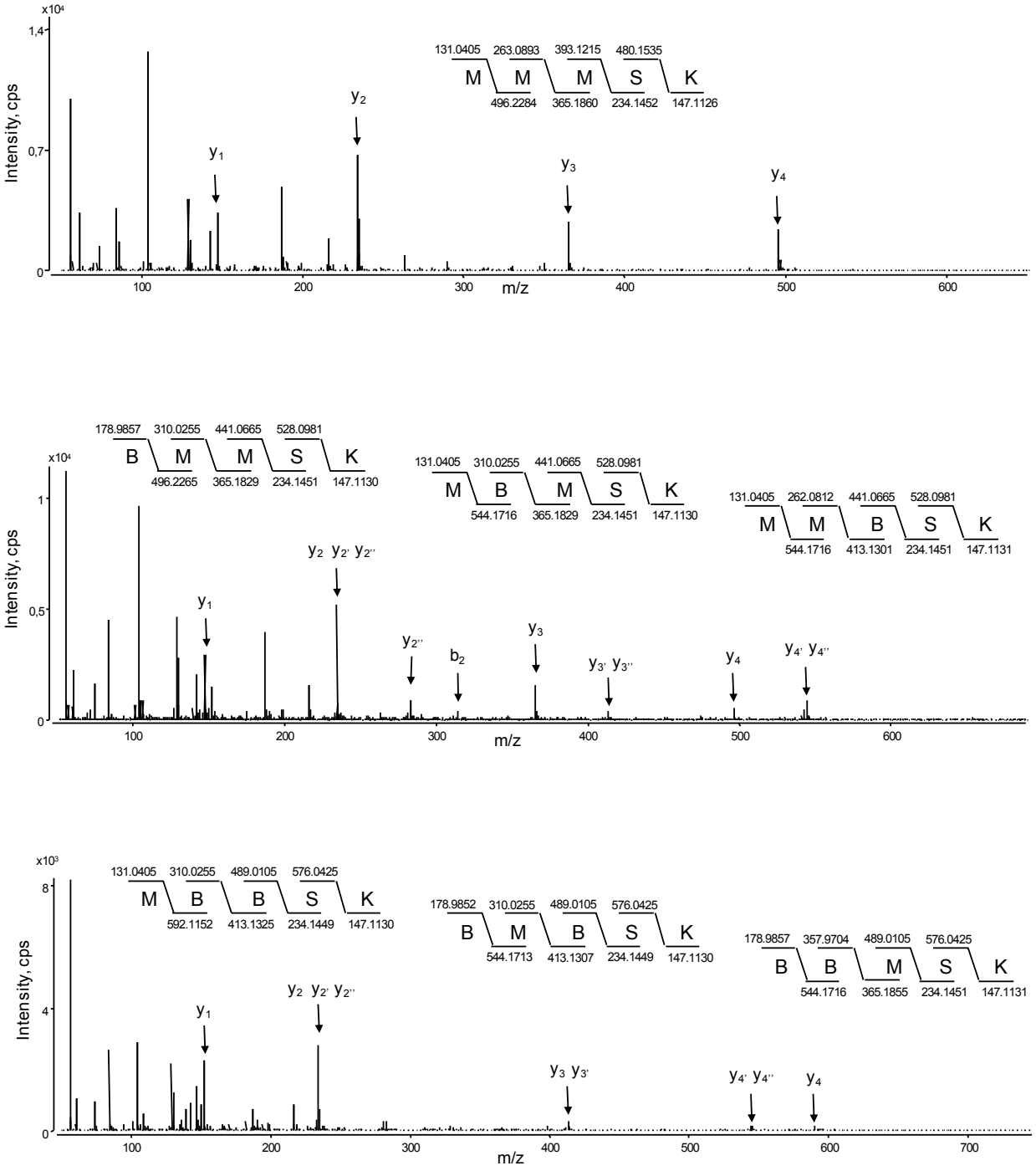

Figure A5. MS/MS spectra and details of *de novo* sequencing of selenopeptides (cont.).

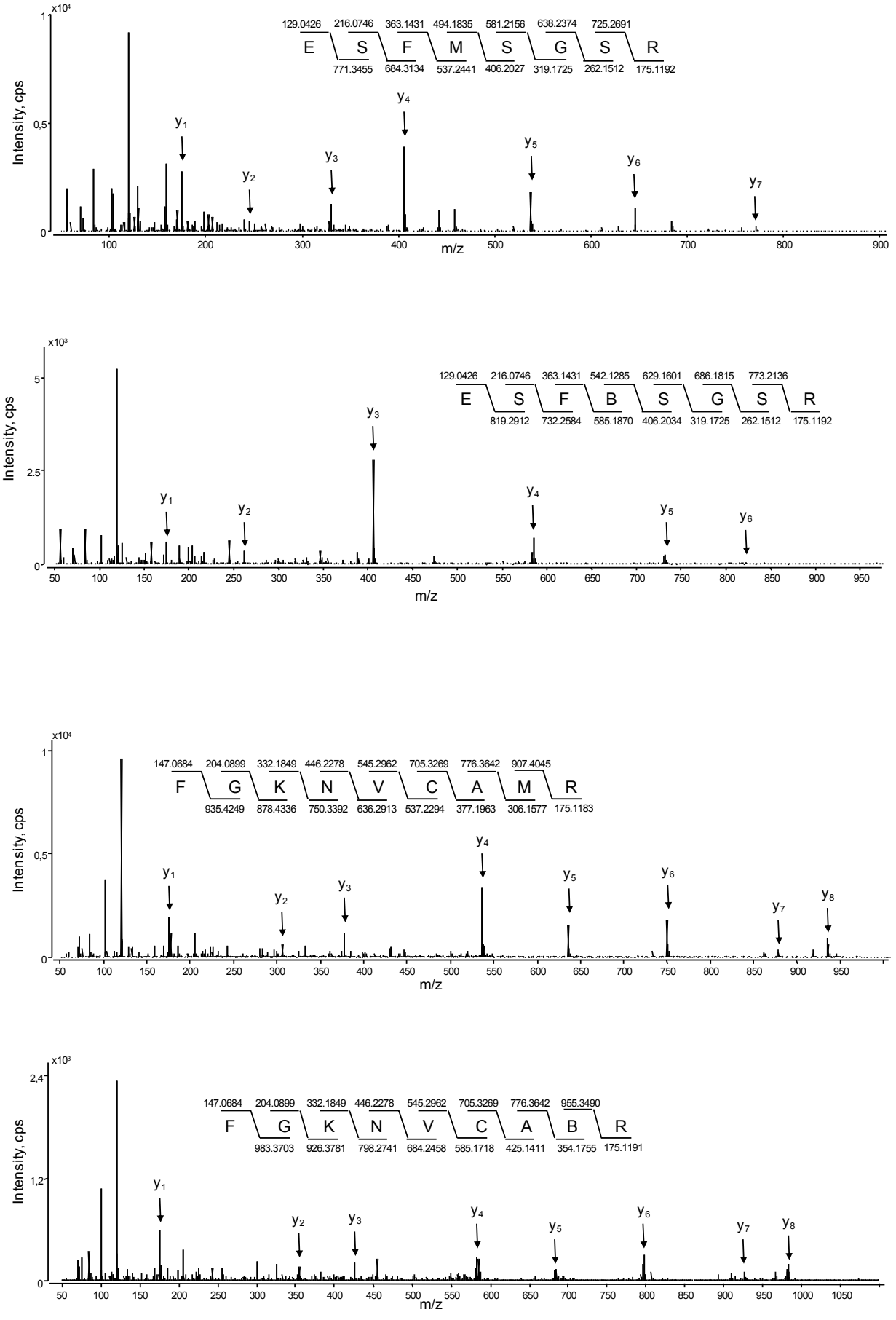

Figure A5. MS/MS spectra and details of *de novo* sequencing of selenopeptides (cont.).

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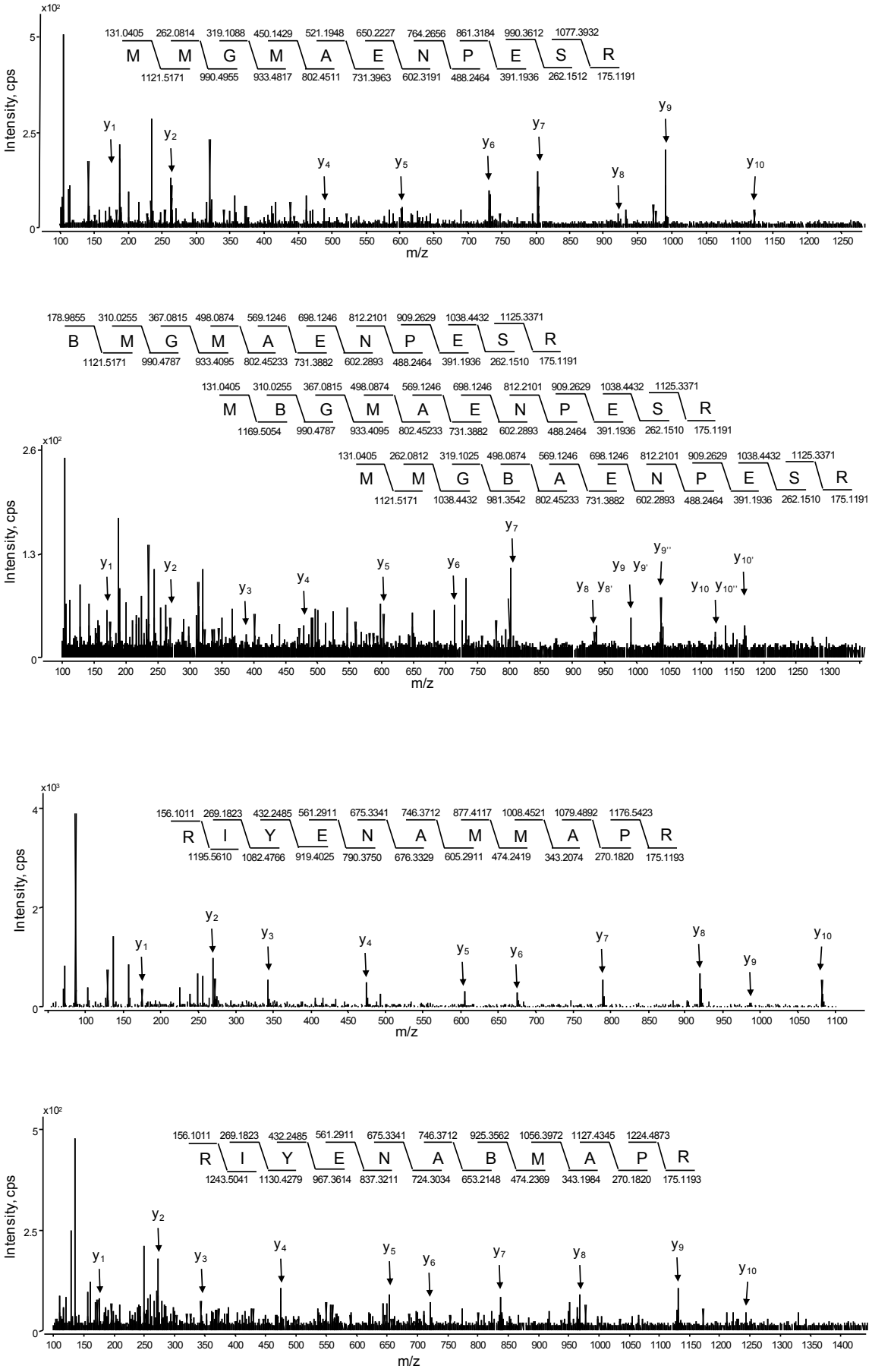

Figure A5. MS/MS spectra and details of *de novo* sequencing of selenopeptides (cont.).

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