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

ANIKÓ NÉMETH

Supervisor
Mihály Dernovics, PhD

Department of Applied Chemistry
Faculty of Food Science
Corvinus University of Budapest

Budapest, 2015
### PhD School/Program

<table>
<thead>
<tr>
<th>Name</th>
<th>PhD School of Life Sciences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field</td>
<td>Food Science</td>
</tr>
</tbody>
</table>

**Head**

Prof. József Felföldi, PhD  
Department of Physics and Control  
Faculty of Food Science  
Corvinus University of Budapest

**Supervisor**

Mihály Dernovics, PhD  
Department of Applied Chemistry  
Faculty of Food Science  
Corvinus University of Budapest

The applicant met the requirements of the PhD regulations of the Corvinus University of Budapest and the thesis is accepted for the defence process.
INTRODUCTION

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 (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 foodstuffs or food supplements. However, 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.

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. 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. Brazil nut (Bertholletia excelsa) and monkeypot nut (Lecythis minor) were selected for the analysis since they are important sources of dietary selenium as natural food and feed supplements, respectively.
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. Recent studies are based on the application of high resolution mass spectrometric methods that enable the large-scale identification of selenocompounds.

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 identify new selenometabolites based on their accurate mass, chromatographic behavior and MS/MS fragmentation pattern
- 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
- 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.
MATERIALS AND METHODS

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).

Water extraction was applied 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.

**Identification of selenocompounds**

A 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. 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. 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.
Proteomic analysis

The aim of our proteomic study was the fingerprinting of a complex mixture of proteins by the application of the shotgun proteomics approach. A complex protein fraction was obtained through a straightforward sample preparation procedure and its tryptic digest was analyzed by high resolution mass spectrometry preceded by a HPLC separation. The identification of proteins is based on the identification of the peptides derived from them. This peptide-based shotgun method is the far most widely used and the most generic proteomic approach (Cravatt et al. 2007, Cox and Mann 2011).

The database-dependent identification of peptides is based on the in silico digestion of known proteins. Ideally, a unique protein database should be created from reviewed UniProtKB entries of the studied organism. It is a rather challenging task in case of non-model, less investigated organisms, especially with forest trees (Neale and Kremer 2011). As 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).

Furthermore, selenopeptides were screened by an exact mass database of known and putative Se-containing peptides. The tandem MS spectra were also manually searched for Se-containing peptides based on their characteristic isotopic distribution. The sulfur analogues of selenopeptides were also screened and their fragmentation facilitated the identification of selenopeptides.

RESULTS AND DISCUSSION

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 1).

![Figure 1](image-url). 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 step.

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 SeCys₂ metabolism through the formation of selenodiglutathionines). 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 Lecythisaceae 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.

**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\textsuperscript{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 \textit{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 \textit{L. minor} plant from the toxic effects of Se.
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-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*. 
References


List of relevant publications

1. *Scientific articles*

Articles in international journals with impact factor


- Cumulated impact factor: 13,792
- 8 independent citations

1. *Conference abstracts*

International conferences


Hungarian conferences


2. Scientific awards

   Best poster award of the European Young Chemist Network (2011)
   Best poster award at the 32nd Informal Meeting on Mass Spectrometry (2014)