Ph.D. dissertation

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

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INTRODUCTION

Selenium is an essential microelement for humans. The bioavailability of selenium depends strongly on the compound speciation. Organic forms are usually more bioavailable and less toxic. The minimum necessary level of intake and the already poisonous doses are close together; therefore selenium supplementation needs to be monitored closely.

Selenised yeast is the main source of organic origin that the European Food Safety Authority approved for selenium supplementation; therefore thousands of tons of it are used every year. About 80% of its selenium content is present in the form of selenomethionine. As the bioavailability of this amino acid depends strongly on its chirality, an accurate method for the determination of the enantiomer ratios is indispensable to prevent the use of racemic selenomethionine to forge selenised yeast.

Selenised yeast also contains a large number of small-molecular-weight seleno-compounds. Out of more than 70 discovered species less than ten are available as standards. Being able to synthesize a number of these components would enable selenised yeast batch identification.

While selenised yeast is a good source for selenium supplementation it is not suitable for direct consumption. The ideal functional food would be a species of classic mushrooms that like yeast, converts inorganic selenium into organic forms.

AIMS

My work can be grouped around three main topics. The aim of the first subject was to examine the effect of sample preparation on the D,L-enantiomer ratio of extracted selenomethionine through the following steps:

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

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

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

The third purpose was to examine and identify the selenium metabolites of *Hericium erinaceus*, - also called lion's mane mushroom - through the following steps:

10. to moderately enrich *Hericium erinaceus* with inorganic selenium,
11. to develop and optimise a method for extraction and sample cleanup,
12. to enrich the sample extract in selenium compounds,
13. to examine the extracts with HPLC-ESI-QTOF-MS for the identification of selenium compounds,
14. to compare the selenium metabolites with those of selenised yeasts and the S-metabolic pathways of the mushroom to assign S-Se counterparts.

MATERIALS AND METHODS

ICP- MS Agilent 7500cs (Agilent, Santa Clara, CA, USA) was used to monitor the isotopes of Se. The instrument was coupled to an Agilent 1200 HPLC system equipped with an optional extended loop (+400 μL). The HPLC-ICP-MS analysis was executed with 5% O_2 as optional gas when using organic solvent based eluents and H_2 as collision gas.

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

Intermediate products of syntheses of 2,3-dihydroxy-propionyl-glutathione were also monitored with an HPLC-ESI-MS coupling where a QTRAP 3200 triple quadrupole – linear ion trap mass spectrometer (ESI-QQQ-MS; Applied Biosystems/Sciex; Foster City, CA, USA) was used.

For the identification of selenium species an Agilent 6530 Accurate-Mass ESI-QTOF-MS was used with an Agilent 6220 derived dual ion spray source. The instrument was coupled to an Agilent 1200 HPLC system.

During my experiments about the effect of sample preparation methods on the D,L-enantiomer ratio of extracted selenomethionine I treated my samples (CRM SELM-1 yeast and monkeypot-nut (*Lecythis minor*, Jacq.)) with the two most commonly used methods: enzymatic digestion and methansulphonic acid (MSA) hydrolysis. In the first case samples were dissolved in neutral pH buffer and incubated for 24 hours, in the later concentrated acid was used in reflux for 8 hours. Before enantiomer separation an anion-exchange chromatographic cleanup step was necessary. Enantiomer separation was achieved using derivatisation with *O*-phthalaldehyde and *N*-isobutyril-cysteine and chromatographic separation.

During the validation of the 2,3-dihydroxy-propionyl group in selenium speciation by chemical synthesis and liquid chromatography - mass spectrometry analyses glyceric acid was freed from its calcium salt, activated through coupling with pentachlorophenol and subjected to chromatographic clean-up. Selenocystine was dissolved in DMF (*N,N'*-dimethylformamide) with the help of HCl. Water was removed through overnight freezing. Selenocystine and glyceric acid were coupled in DMF in the presence of 4-methylmorpholine. The synthesis products were cleaned-up with fraction collection. The conjugate was coupled

with glutathione in an oxidative reaction induced by H_2O_2, catalysed by NaI. Selenocystine was also coupled with glutathione with the help of dithiothreitol (DTT), H_2O_2, and NaI.

For the examination of the metabolism of selenium in *Hericium erinaceus* (lion's mane mushroom), the selenised fungi was created by injecting the mushroom bags with SeIV solution. Fot the different stages of the research different sample preparations were used. For determination of total Se content the sample was microwave digested with HNO_3 and H_2O_2. Enzymatic digestion with pronase E was completed for the determination of selenomethionine content. For the identification of small molecular weight Se-containing compounds an aqueous ultrasonic extraction was executed followed by a size exclusion chromatography cleanup. The collected fractions were subjected to further cleanup by ion-pairing - reversed phase liquid chromatography, then injected to HPLC-ESI-QTOF-MS for identification and fragmentation.

RESULTS

In our study about the about the effect of sample preparation methods on the D,L-enantiomer ratio of extracted selenomethionine we have attempted to execute sample concentration, which proved to be unsuccessful because of the irreversible coagulation caused by the methansulphonic acid. For the validation of the developed method enantiomer separation was also executed on commercially available D,L-selenomethionine too. A good chromatographic resolution of 2.4 was achieved, that would have enabled either standard addition or external calibrations. As the D-selenomethionine content of the real samples proved to be too low for standard addition, external calibration had to be finally executed for the two enantiomers. The slopes of the calibrations were significantly different, 6561 and 4127 counts/ng Se for L- and D- selenomethionine, respectively.

Two different samples, the CRM SELM-1 and the protein fraction of monkeypot nut were treated with two different commonly used sample preparation methods, and the D,L-enantiomer ratios acquired were subjected to statistical analysis. It was found that there was no significant difference between the enantiomer ratios measured from different samples using the same method, which means that neither sample preparation methods were matrix dependent. The analysis also revealed significant differences in the D,L-enantiomer ratio of selenomethionine depending on the sample preparation method. Namely, the ratio of D-selenomethionine was determined to be 2.2-2.7% and 0.5-0.6% after MSA based and enzymatic hydrolyses, respectively. Regarding the fact that the use of MSA digestion is

widespread in selenium speciation and applied in certification campaigns with or without subsequent derivatisation techniques, special care should be taken of quantitative selenomethionine determination. Namely, calibration processes carried out with a selenomethionine standard the enantiomer ratio of which is not inherently identical to what is obtained after the sample preparation steps may lead to biased results. The difference is not negligible and might have remained hidden in previous studies because of the relatively high uncertainty. According to our results, these differences should be taken into account in the evaluation of results because their extent is comparable to measurement uncertainties.

During the validation of the 2,3-dihydroxy-propionyl group in selenium speciation by chemical synthesis and liquid chromatography - mass spectrometry analyses the greatest challenges of the synthesis were the commercial unavailability of any active ester form of glycerine and the tendency of polyols for condensation/polymerization. The use of pentafluorophenol (PFP) might be preferred over pentachlorophenol, as PFP esters react faster and the removal of pentachlorophenol may be difficult. However, in our research it was found that the reaction of glyceric acid with PFP did not yield any detectable amount of ester; therefore, the step was repeated with the use of pentachlorophenol. During the experiment it was also found that the reaction was relatively slow; the second 24 hours of incubation yielded an order of magnitude more synthesis products.

Active ester coupling to selenocystine yields a mixture of non-derivatized, single and double derivatized species, thus requiring a clean-up step. Both single- and double-esterised compounds were collected and subjected to fragmentation. The fragmentation data are in agreement with literature data. Both compounds can be used for further synthesis, and can be found in all selenised yeast batches.

The difficulty of the chemical synthesis of selenocysteine-glutathione conjugate is the effective oxidative conjugation in the presence of the huge excess of dithiothreitol required for the solubilisation of selenocystine. In literature, articles can be found where the author claims that reduced glutathione can be oxidized solely in the presence of H_2O_2. Based on this information, the first experiment for the conjugation included mixing the selenocystine solution with reduced glutathione and adding H_2O_2 drop by drop to the mixture in hope of being able to form Se-S bridges. Results were evaluated with ESI-QQQ-MS. The acquired data showed no change in the form of glutathione. Even when 25-fold more H_2O_2 has been added the method did not yield any of the conjugate nor a detectable amount of oxidized glutathione. Reduced glutathione would start decomposing long before it would start

producing the oxidized form. It was finally a literature based study that offered the solution: the use of NaI as catalyst was necessary. The purified selenocysteine-glutathione was characterized with HPLC-ESI-QTOF-MS. It is important to mention that the synthesis of selenocysteine-glutathione was addressed both as an optimization step and as an independent method for the synthesis of a commercially unavailable compound.

Combining the optimized processes of selenocysteine-glutathione conjugation and the clean-up of 2,3-DHP-containing selenocystine species were the prerequisites to arrive at a detectable amount of 2,3-DHP-selenocysteine-glutathione. However, this compound is slightly retained on special reversed phase HPLC columns intended for use with eluents with low organic solvent content; therefore, a more robust clean-up technique with anion exchange chromatography - HPLC was chosen. The fragments of 2,3-DHP- selenocysteine-glutathione match those of reported in literature.

While some (*e.g.*, the γ-Glu specific) of the MS/MS fragments of selenocysteine-lutathione and 2,3-DHP-selenocysteine-glutathione are shared, the majority of the fragments are different. The most significant difference is the high abundance of the SeCys residue that appears both in native (*m/z* 167.95) and 2,3-DHP-derivatised (*m/z* 255.97) forms during the fragmentation of 2,3-DHP-selenocysteine-glutathione but appears only as a minor fragment during the fragmentation of selenocysteine-glutathione. This great difference in fragmentation pattern is unusual, as the two compounds share their basic structure.

During the experiments about the metabolism of selenium in *Hericium erinaceus* (lion's mane mushroom) total selenium content was found to be 42.3 μg g^{-1} (d.w.)

The two most abundant peaks of the ion-pairing-reversed phase-ICP-MS chromatogram acquired could be identified and quantified with standard addition as selenomethionine (20.7 μg g^{-1} d.w.) and *Se*-methylselenocysteine (0.3 μg g^{-1} d.w.). Their cumulated amount almost reaches 50% of total selenium content, which is unprecedented in any classic mushrooms grown on inorganic selenium source. This suggests that the metabolism of *H. erinaceus* is atypical of what is known of the pathways of *Agaricomycetes,* and more similar to that of yeast, *S. cerevisiae*. To support this theory, further investigations were carried out in search for typical yeast selenium metabolites, especially for seleno-adenosyl compounds.

The most abundant and the late eluting fractions of the water extractable part of selenium were collected from S and further purified with ion-pairing - reversed phase HPLC separation with ICP- MS. In the late eluting low molecular weight fraction two peaks were

collected and subjected to HPLC-ESI-QTOF-MS analyses. Three selenium-containing components were found with the mass-to-charge ratios (*m/z*) of 362.03623, 360.05707 and 346.04163, respectively. The most abundant one of these, *m/z* 346.04163 eluted at 7.5 minutes. The targeted MS/MS fragmentation of this compound indicated it to be *Se*-methyl-5-selenoadenosine.

The identification of the compound eluting at t_R=1.3 min with *m/z* 360.05707 is not as simple as that of *Se*-methyl-5-selenoadenosine. According to the elemental composition, there is only one possible composition: an additional CH_2 group compared to *Se*-methyl-5-selenoadenosine, that is, $C_{12}H_{18}N_5O_3Se^+$ /theoretical m/z 360.0569; δ + 0.47 ppm/. The MS/MS data indicated the losses of adenosyl, adenine and ribose groups. This information may relate basically to two possible structures, *Se*-ethyl-5-selenoadenosine and *Se*-dimethyl-5-selenonium-adenosine, a cationic compound that has not been described before. As stable cations are known among selenium species (such as trimethylselenonium ion, *Se*-adenosyl-selenomethionine and *Se*-methylselenomethionine), none of the possible structures can be excluded based on accurate mass and MS/MS results. Therefore, additional information must be taken into account: (1) the retention time of the *m/z* 360.05707 compound was close to the void, while an ethyl group on the Se moiety would not result in a retention time so much shorter; (2) while *Se*-methyl-5-selenoadenosine was found in oxidized form in the sample, no compound was found at the theoretical mass of *m/z* 376.0519 (3) while *Se*-methyl-5-selenoadenosine was not found in doubly charged form in the actual ESI full scan spectra at any level of abundance, the compound at *m/z* 360.05707 was continuously present in single and doubly charged states, which suggests an ease of second ionisation probably due to the stable cationic structure; (4) only *Se*-methyl-5-selenoadenosine could be detected as the $[M-H]^-$ pseudomolecular ion and as a formic acid adduct. All these observations indirectly prove the compound with m/z 360.05707 is *Se*-dimethyl-5-selenonium-adenosine.

Interestingly, the most abundant seleno-adenosyl species of *S. cerevisiae*, *Se*-adenosyl-selenohomocysteine, has not been detected in the sample. Whether the lack of *Se*-adenosyl-selenohomocysteine is caused by significantly different metabolic pathways or merely the lower level of selenium enrichment (*i.e.*, 2000 ppm *vs*. 43 ppm) still needs to be confirmed. On the other hand, the high level of contribution of selenomethionine and *Se*-methyl-selenocysteine to the total selenium content and the presence of seleno-adenosyl compounds definitely make this mushroom unique among the *Agaricomycetes* group. This unusual metabolism might offer the possibility for the development of a functional food product.

NOVEL SCIENTIFIC RESULTS

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

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

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

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

LIST OF RELEVANT PUBLICATIONS

Peer reviewed journal articles:

1. **Orsolya Egressy-Molnár**, Anna Magyar, Attila Gyepes and Mihály Dernovics, 2014, Validation of the 2,3-dihydroxy-propionyl group in selenium speciation by chemical synthesis and LC-MS analyses, RSC Advances Issue 52,4, 27532-27540 I.F: 3.708

2. **Egressy-Molnár, O**., Vass, A., Németh, A., García-Reyes, J.F., Dernovics, M, 2011, Effect of sample preparation methods on the D,L-enantiomer ratio of extracted selenomethionine, Analytical and Bioanalytical Chemistry, 401 (1) , pp. 373-380 I. F: 3.778

National and international conference abstracts

1. Dernovics Mihály - Németh Anikó - **Egressy-Molnár Orsolya** (2011): "Kapcsolt tömegspektrometriai rendszerek szerepe az újonnan felfedezett szelénmódosulatok azonosításában." Mikroelem Miniszimpózium, Budapest. 18/10/2011

2. Németh Anikó - **Egressy-Molnár Orsolya** - Winfried Behr - Juan F. García-Reyes - Dernovics Mihály (2011): "Növényi kén- és szelénanyagcsere folyamatok analog intermedierjeinek azonosítása ortogonális és kapcsolt tömegspektrometriai módszerekkel." MKE 1. Nemzeti Konferencia, Sopron, 22-25/05/2011

3. Dernovics Mihály - **Egressy-Molnár Orsolya** - Juan Francisco García-Reyes - Németh Anikó - Shuxun Shao (2011): "Food-Related Phytoremediation Initiative in the Seleniferous Area of Jianshi County, Enshi T.M.A.P., China: Challenges for Selenium Speciation and LC/MS Based Food Analysis". Chinese-European Cooperation for a Long-Term Sustainability – International Conference at the Corvinus University of Budapest. 10-11/11/2011

4. **Egressy-Molnár Orsolya** - Vass Andrea - Dernovics Mihály (2012): Unique metabolism of selenium in Hericium erinaceus (lions's mane mushroom). TEFC Conference, Visegrád, 15-17/11/2012

5. **Orsolya Egressy-Molnár**, József Lénárt, Júlia Győrfi, Mihály Dernovics, (2013) Hericium erinaceus: a mushroom with yeast-like Se-metabolism, European Winter Conference on Plasma Spectrochemistry, Krakow, Poland, 10-15/02/2013

6. **Orsolya Egressy-Molnár**, Attila Gyepes, Anna Magyar Mihály Dernovics (2014) Validation of the 2,3-dihydroxi-propionyl group in selenium speciation by chemical synthesis and LC-MS analyses. 8th International Franco-Spanish Workshop, Pau, France, 08-10/07/2014