SZENT ISTVÁN UNIVERSITY

DEVELOPMENT OF SAMPLE PREPARATION METHODS AND PRODUCTION OF REFERENCE MATERIALS FOR SPECIATION ANALYSES

Theses of the doctoral dissertation of

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1. INTRODUCTION

1.1 History of speciation analysis and its development

Mercury was the first element to prove (Minamata Bay, Japan, 1956) the fact that an element and its forms of different oxidation state or ligands may show even extremely different behaviour in living organisms. Chromium was the second one to be addressed in front page articles at the same time (1959) about the extremely different physiological roles of its (vital) trivalent and (toxic) hexavalent forms. Arsenic and its non-toxic form of arseno-betain came next in 1977 by Edmonds and Francesconi to solve the contradistinction between the well-known toxicity of arsenic in general and the relatively high arsenic content of usual seafood products. 6 years later arrived the following element, tin, considered neutral to organisms in inorganic state but genotoxic for not only target organisms in organic form derived from tributyl-tin compounds used in anti-fouling paintings on ships. Finally, selenium and iron should be noted as examples for a new approach on characterising elements based not on some features like "toxic / non-toxic: inorganic As *vs*. organic As" or "toxic / vital: CrVI *vs*. CrIII" but on features like "less or more bioavailable", "having optional useful effects on human health / no optional advantages".

The former examples have already indicated that the total concentration of an element can not provide sufficient information any more, about any kind of samples – about anything containing different chemical-physical-physiological forms of an element. A new branch of analysis has arisen on the edge of several scientific specialities covering (bio)analytical-food-environmental-clinical-physiological chemistry: the speciation analysis, having no specialised techniques for quite a long time. IUPAC finally published the exact definitions in 2000 supplemented with a series of examples. According to the recommendations, a chemical species is "a specific form of an element defined as to isotopic composition, electronic or oxidation state, and/or complex or molecular structure", while speciation analysis is "the analytical activities of identifying and/or measuring the quantities of one or more individual chemical species in a sample." Ni, Pb, Pt and Pd have meanwhile joined the series of speciation elements to make up and refresh the former list of Hg, Cr, As, Sn, Se and Fe.

Speciation analyses, however developed for a long a time individually and separately focusing on one element at a time, have already contributed to our knowledge with results of paramount importance in the field of pharmacy, food science, occupational topics and environmental issues, along with biochemical processes responsible for either healthy or toxic metabolic activity in human physiology.

1.2 Objectives

As- and Se-speciation analyses are considered quite a new field of science, thus possessing several teething problems two of which have already been addressed by former PhD dissertations of the Department of Applied Chemistry ("Quality Control in Speciation Analyses" by Ildikó Ipolyi, PhD, 2003 and "Sample Introduction Techniques in Speciation Analyses" by Zsolt Stefánka, PhD, 2003). This dissertation covers a special part of speciation, the development of sample preparation methods for As- and Se-speciation for the analysis of real samples including a

special one considered applicable for the production of a reference material for Se-speciation purposes. The scientific activity of the PhD candidate Mihály Dernovics can be divided into three main objectives:

- the development of sample preparation methods for As- and Se-speciation analyses by enlarging the scope of enzymatic applications, along with the assessment of general applicability of enzymes in this field;
- the assessment of the possibility of setting up a universal protocol for the sample preparation of samples of organic origin for As- and Se-speciation purposes;
- the development of reference materials by natural or artificial enrichment for speciation purposes with the help of usual labware.

2. MATERIALS AND METHODS

2.1 Samples of interest

All the samples analysed in the frame of this dissertation are considered "usual" targets of speciation studies:

- As-enriched green algae (*Chlorella vulgaris*)
- As-enriched flatfish (*Pleuronoctes platessa*)
- Se-enriched yeast (*Saccharomyces cerevisiae*)
- Se-enriched mushroom (*Agaricus bisporus*)
- Se-containing Brazil nuts (*Bertholletia excelsa*)

The samples listed above were obtained from different sources containing different levels of the given analyte in order to be able to draw general conclusions.

2.2 Reagents and enzymes

Several kinds of enzymes were applied in the study including the use of proteases (pepsin, trypsin, pronase, protease XIV), crude extracts for cell wall digestion (Driselase, lysing enzyme), and lipase. For certain analyses protease inhibition cocktails were also addressed. Usual buffer solutions (phosphate, citrate, TRIS) served as reaction media in most cases.

2.3 General approach of sample preparation

It was practical to set up a general enzymatic sample preparation technique that could be easily adapted for any kind of samples not analysed before. Finally, a 3-step approach was accepted:

(i) extraction with buffered solutions or deionized water to recover soluble As- and Se-species;

(ii) enzymatic cell wall digestion to get access to species blocked by or bound to cell wall components; this step can be carried out with optional protease inhibition;

(iii) enzymatic lipid and protein hydrolysis to brake down sample matrix and to hydrolyse proteins carrying species of interest.

This approach served as a basic method providing the possibility to be adapted to the given sample to analyse. The stepwise technique helped to optimise the extraction procedure and to insert/delete the useful/useless sample preparation levels by setting elemental (As and Se) mass balance at each step. The whole procedure was supplemented with spiking for quality control purposes.

One of the factors hampering the continuous development of Se-speciation is the lack of reference materials, which makes the validation of speciation analyses sometimes extremely difficult. There have been publications on experiments carried out to prepare adequate raw materials to serve as a base for lower or higher level reference materials, and preliminary studies connected to this dissertation have also covered the analysis of Se-enriched yeast, lactic acid bacteria and mushroom. These studies ended up with problems that could not be solved under the actual circumstances, e.g. batch-to-batch inhomogeneity arising from laboratory scale experiments, insufficient strain selection and low column recovery issues. Afterwards, samples containing selenium of natural origin were screened and Brazil nuts were chosen to carry out the study intended for reference material production.

3. RESULTS AND DISCUSSION

3.1 Results of the experiments carried out with enzymatic sample preparation

Analysis of As-enriched algae and fish samples through enzymatic sample preparation

Extraction with methanol is practically the most often applied sample preparation method in As-speciation, although there have been publications on the use of enzymes as well. A study presented in this dissertation was focused on the assessment of the ability of enzymatic methods including cell wall and protein digestion for the analysis of algae and flatfish samples. The enzymatic methods were applied in batch and in sequential set-ups and parallel extraction with methanol was also carried out. The results were evaluated with the help of one or multivariate statistical methods that revealed strong matrix dependence: while cell wall digestion helped to increase the extraction efficiency in the case of algae samples, none of the enzymes in question proved to be of crucial importance.

Analysis of Se-enriched yeast samples through enzymatic sample preparation

Se-enriched yeast fermented and treated in general downstream processes in industrial scale – besides its protein content accounting for a considerable amount of selenium accumulated – usually contain lipid and cell wall components that might be broken down to an unknown extent. These components may block the process of sample preparation by hindering the access of extraction agents to Se-species. In case these components are partly or completely broken down, e.g. in an enzymatic way, there is a theoretical possibility to increase the extraction efficiency of selenium, which is often considered unsatisfactory in relevant papers. On the other hand, a careful and precise optimisation process to select the protease applied in the mainstream of the sample preparation process may offer additional benefits.

The results of this study revealed that the application of cell wall digestion and lipid hydrolysing enzymes did not increase significantly the extraction efficiency of selenium and the sequential enzymatic technique did not offer unambiguous advantages over batch techniques. The comparison of different proteases ended up in the introduction of pronase E (Merck) in Se-speciation studies that proved to be better in several practical fields, e.g. cost-effective sample preparation, easier handling, and was selected for all the further experiments, replacing protease XIV (Sigma), referred mostly in relevant technical papers.

Analysis of inorganic Se-enriched mushroom samples through enzymatic sample preparation

Soils in Hungary are poor in selenium, that is why traditional Hungarian cuisine is usually not able to provide the optimal daily selenium intake. This problem can be solved with the introduction of Se-enriched food or near food products like selenised mushroom grown on compost supplemented with some kind of selenium source. A chapter of this dissertation deals with the Se-speciation analysis of inorganic Se-enriched *Agaricus bisporus* to achieve the main goal: by developing a sample preparation method for this sample, there should be an opportunity to estimate the selenium bioavailability of a cultivated mushroom in an indirect way to test whether this type of cultivation results in a different Se-availability pattern compared to natural grown mushroom (considered almost useless in terms of selenium supplementation by several authors).

Unlike yeast samples, sequential enzymatic sample preparation turned up to be the most efficient for the extraction of Se-enriched mushroom samples, reaching up to 75% Se-recovery. As the enzymes applied in this study (pepsin, trypsin) are also present in human digestion, this high value may indicate the possibly good Se-bioavailability and the possible role of this food sample in dietary selenium supplementation.

The quality control of the sample preparation and speciation analysis of this sample was done through a spiking technique that proved to be useful in the differentiation of the extraction behaviour of Se-species originally present in the sample and those added during the spiking procedure. On the other hand, spiking helped to indirectly identify one of the Se-species (Se(IV)) recovered from the mushroom sample.

Analysis of organic Se-enriched mushroom samples through enzymatic sample preparation

Another chapter of this dissertation presents the examination of Se-enriched mushroom samples grown on compost supplemented with Se-yeast, an organic Se-source. This sample contained selenium in the same order of magnitude than the other Se-mushroom; however, the different selenium supplementation caused a significantly different selenium distribution pattern and extraction behaviour according to the results of this study. The highest Se-extraction efficiency (around 90%) could be achieved by a 3-step sequential enzymatic method including cell wall digestion, but the highest column recovery was observed in the case of a one-step method applying a non-specific protease treatment only. The reason behind this phenomenon was the possibly different protein size distribution that can have an effect on the elimination of Se-

carrying macromolecules during centrifugation steps involved in multi-step methods, thus resulting in smaller amount of available and recoverable selenium in the sample.

The effect of cell wall digestion on the extraction of total selenium was confirmed with specific inhibition of proteolytic activities: the application of protease inhibitors helped to justify the fact that the higher extraction efficiency achieved by the insertion of cell wall digestion is independent from optional protein degradation. Of note is the fact that the use of different cell wall degrading enzyme mixtures ended up in the recovery of different Se-species.

3.2 Results of the study aimed at the production of reference materials for speciation analysis

During the preliminary studies of this part of PhD experimental work the Se-speciation analysis of samples mentioned above (see section 2.3; lactic acid bacteria, Se-enriched yeast, etc.) was implemented in order to assess their ability to serve as raw materials for the production of Se-reference materials. The in-house production (fermentation) of this kind of biomass went together with the chance of controlling the final total selenium content to a certain extent; however, the resulting products did not fit the basic requirements set before.

Fortunately, there was a possibility to start to analyse Brazil nuts samples, a natural food product containing selenium up to even 0.5 mg g^{-1}. This shelled fruit possesses relatively high lipid content (71 m/m %) that had to be removed by Soxhlet extraction to obtain a stable product. This raw material was treated afterwards according to the general protocol designed for the production of reference material including the development of sample preparation, the preliminary and final Se-speciation measurements (by a hyphenated HPLC-UV-HG-AFS set up), milling, homogenisation, sorting, designing the storage and stability experiments, sterilisation, and finally the implementation of the analyses for homogeneity and stability testing. The outcome of all these experiments was successful, as the candidate reference material prepared from Brazil nuts proved to be adequate for the quality control purposes of Se-speciation. The real value of this result is highlighted by the fact that this has been the first laboratory reference material for Se-speciation that has been published in a relevant peer-reviewed quality scientific journal.

3.3 New scientific achievements

- The extraction efficiency of total As and As-species can be significantly increased with the application of cell wall digestion enzymes (Driselase) in the case of As-enriched algae samples. In the case of natural As-containing fish samples, the use of proteolytic enzymes (trypsin) did not increase significantly the As-extraction efficiency compared to the methods that were not based on the application of enzymes.
- When carrying out the sample preparation of Se-enriched yeast samples (fermented and downstream treated in industrial scale to meet the requirements of usual LRM production) for Se-speciation analysis with non-specific proteolytic enzymes, the application of enzymes intended for cell wall digestion and lipid hydrolysis did not improve significantly Se-extraction efficiency. Through the comparison of proteolytic crude enzyme mixtures (of *Streptomyces griseus* origin) used in one-step Se-speciation sample preparation of Se-enriched yeast samples, the experiments and statistical evaluation have verified that the one produced by Merck (pronase E) could achieve higher Se-extraction efficiency than the one usually referred in relevant papers (protease XIV, Sigma).
- A sample preparation method based on sequential enzymatic treatments was developed for Se-speciation that could achieve 75% extraction efficiency from mushroom samples grown on compost supplemented with inorganic selenium. The procedure of spiking followed by sequential enzymatic sample preparation was successfully applied to differentiate between the extraction behaviour Se-species originally present in the sample and those added during spiking, denoting to the degradation of the protein matrix of the sample. Spiking was also applied to indirectly identify one of the Se-species (Se(IV)) recovered during Se-speciation analysis.
- In the case of organic Se-enriched mushroom samples, multi-step enzymatic sample preparation methods including the application of cell wall degradation (even together with parallel protease inhibition) provided significantly higher Se-extraction efficiency compared to the methods based on the use of proteolytic enzymes or buffered solutions only. A series of experiment focusing on the application of protease inhibition agents proved the significance and presence of proteolysis during cell wall digestion, i.e. the specific inhibition of protein degradation was used to differentiate the role of cell wall digestion in Se-extraction. The application of "lysing enzyme" resulted in the recovery of a Se-species (identified as $SeCys_2$ by HPLC analysis) that could not be extracted by any other sample preparation methods addressed in the study.
- Brazil nuts containing high amount of selenium (80 μg g^{-1}) and lipid (71 m/m %) can be defatted with the help of organic solvent mediated solid-liquid extraction without decreasing significantly its selenium content. This defatted product proved to be an adequate raw material for the production of the first reference material in scientific literature intended for Se-speciation purposes as (i) its SeMet content – the Se-species that is one of the most studied Se-molecules in our days – is high enough to be determined quantitatively and qualitatively as well, i.e. its use would fulfil real needs in speciation analyses; (ii) the

defatted raw material can be easily prepared and homogenised; (iii) its SeMet-content proved to be stable during γ-irradiation and under different temperature conditions, and finally, (iv) the most often applied one-step proteolytic treatment of Se-speciation recovers almost 100% of selenium from the reference material, providing the possibility of sample preparation with low standard deviation parameters.

4. CONCLUSIONS AND SUGGESTIONS

The general sample preparation approach (based on the use of different enzymes) presented in this dissertation can be easily adapted to serve as a basic method for the Se- and As-speciation analyses of samples of biological origin never or rarely analysed before.

The candidate laboratory reference material that was prepared from Brazil nuts can be presumably used for quality control purposes at any kind of Se-speciation analyses where the analyte possesses similar composition, e.g. in the case of the most often studied sample of Se-speciation, Se-enriched yeast that usually contains similar amount of protein, total Se and SeMet.

5. PUBLICATIONS RELATED TO THE DISSERTATION

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