Ph D Thesis

Practical methods for the quality assurance of speciation analysis

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INTRODUCTION

In order to circumscribe the domain of element speciation, it is mandatory to quote the IUPAC (International Union for Pure and Applied Chemistry)recommendations for the definition of terms related to the chemical speciation of elements. Chemical species are specific forms of an element defined as to isotopic composition, electronic or oxidation state, and/or complex or molecular structure, while speciation analysis defines the analytical activities of identifying and/or measuring the quantities of one or more individual chemical species in a sample.

In recent years, trace element speciation has become an important concern in a wide variety of fields: health, environment, food etc. It is no longer a purely academic subject, but various industrial sectors, the government and legislative bodies are all involved. The reason for this is that by today it has become scientifically proved that mobility, bioavailability, retention, storage and toxicity of trace elements in living systems, food and environment depends on the chemical form in which it enters the system, the transformations it goes through – e.g.: metabolic processes - and the final form in which it is present. The form clearly governs its biochemical and geochemical behaviour.

The intensive developments in the field of speciation analysis are attributed to the information the determination of the chemical forms of elements is able to provide, as these determine most of their properties. At the nuclear level, isotope distribution can provide information about the geochemical sources of certain elements, while their redox state of elements has a strong influence on properties like solubility and reactivity. At the organometallic compound level, the nature and number of covalently bound ligands strongly influences the elements properties and at an even higher level of complexity, binding larger units such as proteins has a profound influence on factors like mobility, stability, bioavailability and toxicity. Speciation, therefore, defines the distribution of elements in different forms and provides information on the potential toxicity or essentiality of the investigated sample.

Speciation analysis is often mentioned as the greatest challenge of analytical chemistry in the 21. century. The underlying reasons are many. As the total trace element concentrations are already very low in the majority of natural matrices e.g.: in the ng L^{-1} range for vanadium in human serum, in case of speciation analysis when the identification and quantification of the individual species had to be carried out, concentrations of even a hundred or thousand times less have to be determined e.g. in biomolecules or microorganisms. This puts them out of scope of the existing analytical techniques. Therefore the ultimate goal would be to setup analytical systems with detection limits three to six orders of magnitude lower than whatever has been achieved up till now. The other problem is that the in-situ biochemical processes of species are not yet well understood. It is not clear what intermediate

states the species go through before reaching their final form, how they are incorporated in macromolecules and how and to what extent they are excreted. Have the answers been all given, it was obvious to what questions speciation analysis should answer and which are those specific fields of science that would benefit most of detemining the various chemical forms of elements. The high number of unanswered questions and the intensive development of speciation analysis together result that there are certain speciation problems for which still only one speciation technique is available. Therefore the validation of methods, the control of the trueness and the precision of speciation results is already difficult and is complicated even more by the fact that the certified synthetic form of many species is still not available and calibration can only be carried out with in-house synthesized compounds. In this case the purity of the synthesized form is questionable.

The recent years have seen remarkable advances in analytical instrumentation. The development of speciation analysis is interrelated with a number of other dynamically developing branches of natural sciences which basically determine the evolution route of speciation analysis.

The implementation of the quality assurance measures of the already existing speciation techniques which no longer serve purely academic interests is an imminent task. The quality of a vast number of decisions is determined by the reliability of analytical data and today by speciation data as well.

In contrast to earlier methods only capable of determining total elemental concentrations, analytical methods are nowadays capable of distinguishing and detecting various elemental species. In most cases the analytical methods apply hyphenated techniques – from extraction through separation to element selective detection – in which a suitable chromatographic or electrophoretic technique is coupled with an ideally element- or molecule-specific detector. In each step of speciation the specific sources of errors have to be eliminated and, in addition, in each single step the stability of the species has to be assured, undesired chemical reactions have to be avoided and the signal modifying matrix effects have to be eliminated.

In order to preserve the original speciation of the sample while permitting the analysis, several compromises have to made. Due to the compromises and the inherently unstable character of elemental and organic species, speciation analysis has to be performed with special care, otherwise the accuracy of produced data will be questionable. The greatest challenge in speciation is not to assure precision, but to assure the trueness of results, which is the closeness of agreement between the ‘true value’ and the measured value. Trueness relies on the true value of the substance to be measured, which is defined as ‘a value, which would be obtained by measurement, if the quantity could be completely defined and if all measurement imperfections and stability problems could be eliminated. It appears that the best

possible way to preserve the original speciation information of the investigated samples is the elimination of the sources of errors already in the first, planning stage of the experiment.

All aspects and considerations of quality control that apply to trace element/organic analysis, also apply to speciation analysis, but in addition, particular precautions have to be taken in this field due to the properties – above all the stability – of elemental and organometallic species.

AIMS

The aim of my work was the development of speciation systems and methods; and the implementation of quality assurance measures in order to produce valid data with the application of the novel systems and methods in the speciation analysis of different matrices.

In order to establish a suitable quality control system, the possible errors related to each particular step of speciation analysis have to be evaluated:

- Planning – element-specificity;
- Sampling, conservation and storage – stability, determination of conservation & storage conditions;
- Subsampling – homogeneity, subsample size;
- Extraction – choice of extractant, sample:extractant ratio; matrix effect; dilution extract for analysis; suitability to separation;
  Sequential extraction – modification of procedure, application of method-specific CRM;
- Separation – choice of sample introduction technique; quality of chromatograms; gradient elution; standard addition; application of two different chromatographic techniques;
- Detection – element-specificity; elimination of interferences; AFS detector in multielement systems;
- Calibration – external calibration; external calibration with underivatised calibrants and internal standard; internal calibration
- Use of control cards.

After solving the element- and sample-specific difficulties occurring in the steps of the newly developed speciation methods, quality assurance measures were implemented to maintain the integrity of the species and to monitor method performance.

EXPERIMENTAL

During my PhD work I contributed to the development of several speciation systems and methods and applied the optimized methods to the speciation analysis of various sample matrices. According to the instrumentation used, my work was divided into three distinct areas:

1. development, optimization and application of systems applying high performance liquid chromatography coupled with atomic fluorescence detection for the arsenic and selenium speciation of different matrices;
2. development, optimization and application of two time-saving variations of the BCR three-step sequential extraction procedure on environmental samples;
3. development of speciation methods for the determination of methylmercury and organotin concentrations in environmental samples with GC-MS

Samples of arsenic speciation:

- natural waters,
- oyster,
- soil,
- mussels,
- chicken meat.

Samples of selenium speciation:

- yeast,
- mushroom (Agaricus bisporus),
- urine,
- brasil nut.

Samples used for the development of the time-saving versions of the BCR three-step sequential extraction procedure:

- RM S7 – estuarine sediment reference material,
- CRM 601 – lake sediment certified reference material.

Samples of methylmercury and organotin analysis:

- Mussels – Mytillus edulis, Mytillus galloprovincialis,
- CRM 477 – butyltin compounds in mussel tissue,
- BCR 710 – oyster tissue candidate reference material.

RESULTS

The results of my experimental work can also be divided into three groups based on the instrumentation used:

1. Various systems for the determination of arsenic and selenium species with the hyphenation of high performance liquid chromatography and atomic fluorescence detection, were developed depending on the concentration range of the species to be determined. The suitability of the different systems for real sample analysis was proved with speciation of different matrices.
2. Two alternative versions of the BCR three-step sequential extraction method were developed and optimized with the exploitation of ultrasound and microwave energy for the fractionation of heavy metals in sediments of different origin. The analytical performance of the alternative versions were evaluated by using the method-specific BCR 601 certified reference material. According to the statistical evaluation of the results the proposed accelerated sequential extraction are valid alternatives to the conventional method in rutine analysis.
3. A method was developed for the extraction and GC-MS determination of the methylmercury content of biological samples and the long-term quality assurance of the method was implemented. The performance monitoring of a method validated for the determination of the organotin content of biological samples was carried out with the use of certified reference materials and control cards.

The step-by-step quality control of my speciation tasks was carried out and the following conclusion could be drawn:

- Mobility, bioavailability, retention and storage are element, moreover species-specific characteristics, therefore already the first step of speciation analysis – planning - requires element-specificity.
- The conservation of species in their original state requires the determination of specific storage condition in each sample individually. If the samples are kept under the optimal storage conditions or their speciation analysis is carried out as soon as possible, the integrity of species can be maintained.
- Since modern methods of speciation analysis are highly sensitive, homogeneity has significant influence on the precision of results. If subsamples taken from one sampling point are homogenized together, a loss of environmental information might have to be encountered.
- The evaluation of parameters affecting the accuracy and the precision of results was carried out during the development of the alternative, time-saving versions of the BCR three-step sequential extraction procedure utilizing ultrasonic and microwave energy and.
- In speciation analysis the optimal parameters and the limits of the chosen separation technique are highly influenced by the applied extraction technique. In case the modification of the extraction

parameters decreased the quality of quantitative measurements, changes in the separation conditions should be considered as alternative solution. The sample matrix and the concentration of species to be quantified determines the suitability of extraction techniques significantly.

- The most frequently occurring chromatographic problems in speciation are peak identification and peak purity for which spiking or the use of two independent chromatographic techniques offer quality control alternatives in case no certified reference material and detector capable of providing molecular information is available. In complicated systems such as HPLC-HHPN/USN-AFS and HPLC-TO-UV-HG-AFS, the problems making chromatogram evaluation difficult are often not of chromatographic origin, but the preceeding steps affect the quality of peaks.
- The on-line derivatization of species after liquid chromatographic separation is a difficult task, requires optimization with significant compromises.

Organoselenium compounds are able to form hydrides directly, without pretreatment. The advantages of the HPLC-HG-AFS system are based on this phenomenon. The inclusion of a pretreatment – prereduction – step in the system by ensuring the complete destruction of selenium species to Se(IV), therefore greatly enhances the sensitivity of the system and increases the number of identifiable species, however, with pretreatment the system becomes more complicated and the number of sources of errors also increases.

- Despite atomic fluorescence detection is an element-specific technique, in case of using high efficiency nebulizer as sample introduction we have to count with the occurrence of non-specific background signals on the chromatograms. For the identification of these signals the application of hydride generation is suggested if detectors capable of molecular identification are not available.
- Several calibration approaches have been suggested and applied for the quantification of speciation results. The suitability of each is determined by sample matrix, the number of samples to be analyzed and the applied analytical method.
- The long-term performance of a speciation method can be monitored by the regular use of RM or CRM samples and application of control charts.

It can be concluded that even though the critical points in each step of speciation analysis have to be controlled individually, the steps of speciation cannot be treated be treated independently. In order to avoid the altering of the species information originally present in the sample, the harmonization of steps has to be solved.

NEW SCIENTIFIC OBSERVATIONS

1. The speciation analysis of environmental samples requires element specific experiment planning. My observations underline that the experiments planned for the monitoring of coastal sea water for organotin contamination are not suitable for the similar monitoring of arsenic species concentrations because of the differences in the biochemical reactions and bioavailability of the two elements and their species.
2. The results of my experiments prove that the samples taken for speciation analysis have to be analyzed possibly immediately or the specific storage conditions have to be provided in order to maintain the original species integrity in the sample. This requires the careful determination of the species- and matrix-specific conservation and storage conditions of the samples. However, conservation rarely assures 100% stability.

   - While the stability of the investigated inorganic arsenic species cannot be assured in natural water samples, the organoselenium species found in brazil nut are stable enough to render the production of reference material possible.
3. Sample homogeneity and the size of subsamples significantly affect the precision of speciation results. The effect of sample homogeneity on the reproducibility of results was determined by the mercury speciation of mussel and the selenium speciation of brazil nut samples. While the homogenization of subsamples in one batch significantly improves sample homogeneity, it might as well lead to losses of environmental information also in speciation analysis.
4. It was concluded that the determination of extraction efficiency is inevitable in speciation, however, even the species addition/recovery values have to treated with special care.

   - Two, significantly shorter versions of the conventional BCR three-step sequential extraction procedure was developed with the utilization of ultrasonic and microwave energy. The parameters affecting the accuracy and precision of the novel methods was determined by using BCR 601 certified reference material.

   - It was presented with the arsenic speciation of chicken meat samples that in case the modification of extraction parameters decreases the quality of quantification, the modification of separation condition has to be taken in consideration as alternative solution. It was proved that the matrix of the analyzed sample and the concentration of the species to be determined influences the suitability of extraction techniques.

5. The efficiency of separation is significantly influenced by the parameters of sample preparation. The harmonization of sample preparation and the chromatographic conditions is a necessity in order to provide reliable speciation results.

   - My observations proved that in the speciation systems including high efficiency nebulization - HPLC-HHPN-AFS and HPLC-USN-AFS – the choice of extractant and sample introduction technique influences the quality of the obtained chromatogram significantly and might cause background signals which can be eliminated with hydride generation that assures complete gas-liquid separation.

   - It was demonstrated that standard addition and the application of two different chromatographic techniques gives a reliable quality assurance alternative in the identification of known species in case suitable certified reference materials and detectors providing molecular information are not available.

6. It can be stated that derivatization – applied either before of after separation – may enhance the selectivity and sensitivity of methods, but as one of the most critical in the sequence of steps in speciation analysis, it is loaded with a number of sources of errors – e.g: contamination, artifact formation etc.

   - It was proved that organoselenium species partially form hydride directly - without pretreatment. The on-line coupling of HPLC and AFS with HG without pretreatment is based on this phenomenon. Despite the decomposition and thus the hydride generability of organoselenium compounds in the HPLC-HG-AFS setup are partial, the sensitivity is greatly improved compared to the systems applying high efficiency nebulizer as sample introduction system.

   - However, the system becomes more complicated and the number of sources of errors increases in case a prereduction step is employed before hydride generation – HPLC-TO-UV-HG-AFS - sensitivity further improves and by the reduction of Se(VI) to Se(IV) the number of hydride generable species grows.

7. The need for molecular mass spectrometry in speciation was demonstrated.

   - The occurrence of non-element specific background signals was presented in case of employing nebulization in the HPLC-AFS systems as sample introduction, even though atomic fluorescence detection is an element selective technique.

   - It was demonstrated that with two element specific AFS detectors the development of a simultaneous multielement speciation system is possible.

8. It was also demonstrated that the calibration of speciation measurements has to be species-specific. It was proved that external calibration is rarely appropriate because of the effect of sample matrix; while calibration with standard addition or CRMs may significantly decrease the uncertainty of results.

   - The successful application of external calibration has been proved for simple matrices (natural water samples). By the quantification of the methylmercury content of the mussel samples it was demonstrated that the use of underivatized calibrants together with an internal standard is able to compensate for the losses which occur from sample preparation to detection. With the determination of the arsenobetaine content oyster samples it was demonstrated that despite the method of standard addition is rather time-consuming, it is the most reliable calibration method.

PUBLICATIONS

Hungarian

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   Előadás, MTA Spektrokémiai és Környezetkémiai Bizottságának ülése
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7. Sörös Cs., **Ipolyi I.,** Dernovics M., Fodor P.: *Arzén speciáció a gyakorlatban*
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International

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1. **I. Ipolyi**, P. Fodor*: Development of analytical systems for the simultaneous speciation of arsenic [As(III), methylarsonic acid, dimethylarsinic acid, As(V)] and selenium [Se(IV), Se(VI)]*
   Analytica Chimica Acta, 2000, **413**, 13-23.
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6. **I. Ipolyi**, P. Massanisso, S. Sposato, P. Fodor and R. Morabito: *Concentration levels of total and methyl mercury in mussel samples collected along the coasts of Sardinia Island (Italy)*
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   Journal of Analytical and Bioanalytical Chemistry, in press.

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2. **I. Ipolyi**, Zs. Stefánka, P. Fodor: *Possibilities of simultaneous Se and As speciation using HPLC-AFS system*
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5. Zs. Stefánka, **I.Ipolyi**, M.Dernovics, P.Fodor: *Comparison of sequential sample preparation methods of selenised mushroom (Agaricus bisporus)*
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