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CHALLENGES IN MERCURY SPECIATION

THESIS OF THE PHD DISSERTATION OF

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

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The Local Doctoral Council for Life Science of the Corvinus University of Budapest has been assigned in the resolution 02/10/2007 the following Thesis Committee for the public defense.

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1. INTRODUCTION

The biogeochemical cycling of mercury, the investigation of biotic and abiotic processes facilitating mercury cycling in the environment, the toxicity of mercury species are the most important subjects of the medical and biomedical researches.

There is a growing interest in mercury speciation in samples of human food products due to various levels of toxicity of the different chemical forms of mercury. Organic mercury compounds, of which methylmercury is the most common, are of special concern because of their enhanced toxicity. The route of human exposure to methylmercury is mainly through the diet especially via fish and shellfish that bioaccumulate this compound.

There are several terms to describe the mercury intake, of which the most commonly used for chemical contaminants are the Provisional Tolerable Weekly Intake (PTWI) in the UK/EU and the Reference Dose (RfD) in the USA. There are differences in the precise definitions of these terms, absolute safety cannot be guaranteed, these safety guidelines represent an intake where there is essentially no risk, as far as can be judged from the available scientific evidence.

Although there are numerous studies published in this subject, there is not uniform scientific opinion about the methylmercury exposures and the danger of consuming harmful levels of methylmercury from fish. Due to the past occurred poisonings European Commission regulated the limit for total mercury content in all fish and in other foodstuff.

The real challenge of the mercury speciation is the very low mercury content of the samples. Therefore very sensitive and selective analytical techniques are needed to be developed that meet these requirements. At the same time the development of analytical systems is less predominate in mercury speciation. Still the cold vapour atomic absorption spectrometry and atomic fluorescence spectrometry are the most commonly used methods due to their relatively low cost and high sensitivity and selectivity.

Numerous analytical methods have been described in the literature to measure mercury species in biological samples. Although there are non-chromatography speciatiation techniques most of the methods consist on home-made hyphenations between an appropriate separation technique (gas or liquid chromatography, electrophoresis) and a selective and sensitive detector. Gas chromatography is the most widely employed technique to separate different mercury species present in the sample due to its easy use and uncomplicated hyphenation with appropriate detectors. Since three or maximum four mercury species are present in real life samples, the separation of these compounds can be carried out simply.

As organomercury compounds are usually present as ionic species in the matrix sample or extract, these species are needed to be converted into more volatile ones. More commonly used methods to derivatise organomercury compounds are alkylation by sodium tetraalkylborate or phenylation by sodium tetraphenylborate, due to their use in aqueous phase and their realization is uncomplicated.

The key step of the pre-treatment of the sample is to liberate and isolate the required compounds from the matrix using a correct and efficient method. The extraction of mercury species from a complex matrix is limited by incomplete extraction, species transformation, sample contamination or analyte losses that can be produced during sample treatment.

For these reasons the efficiency and adaptability of the diverse improved procedures are highly disputed points of this subject.

Hyphenated techniques used for the determination of chemical forms of elements are based on successive steps which may vary from one procedure to another and these methods have their own particular source of error. Nowadays the speciation analyses are performed routinely in many laboratories to control the quality of the environment, food and health. It is now widely recognised that the lack of quality control of analyses performed in routine industrial and research laboratories creates strong economic losses due to the possible misuse of inaccurate data (e.g. in decision on the management of the quality of environment, food and health) and consequent burdens (e.g. action courts, doubts of the public on government decisions).

The analysts should use an analytical procedure on a routine basis only after it has been validated for the range of concentrations and matrices by using relevant reference materials and certified reference materials. In the absence of these materials, there are some other strategies that can be adopted by the analyst to achieve, improve and/or maintain quality of a method. Some of these are the different quality systems (accreditation), application of control charts, participation in interlaboratory studies (external quality control measures), or uncertainty evaluation of the improved methods.

2. OBJECTIVES

The facilities of mercury speciation through development, study and quality assurance of two irrespective analytical methods were investigated in this PhD work. The objectives of the study can be defined as follows:

- Development of a fast and cost-efficient analytical method for the determination of methylmercury in different foodstuff.
  - — Investigation of less time consuming and more efficient sample preparation treatments.
  - — Optimization of phenylation reaction and investigation of volatility of phenylated compounds.
  - — Investigation of the kinetics of SPME.
  - — Optimization of dilution, investigation of the effect of methanol and potassium in case of "low-content" samples.
- Evaluation of uncertainty statement of the improved method.
- Participation in international interlaboratory studies in order to control the quality of this method.
- Application of the method for the determination of mercury and methylmercury in typical fish-containing food samples commercially available in Hungary.
  - — Analysis of the three most highly consumed and pooled marine fish species.
  - — Analysis of individual wrapped marine fish products currently available in a local supermarket.
  - — Analysis of two freshwater fish species.
- Application and quality control of an irrespective analytical method by making mass balance.
- Investigation of the stability and species-specific degradation behaviour of methylmercury and ethylmercury under microwave irradiation in alkaline media and under ultrasonic irradiation in acid solution.

3. MATERIALS AND METHODS

3.1 Sample preparation

In the case of alkaline digestion-SPME-GC-AFS method 250 mg of lyophilised sample using 5 ml of 18 % NaOH in methanol was digested. After the digested solution was diluted varied depending on the MeHg content of the sample, 1 ml of the diluted solution was pipetted to 10 ml of 1 M, pH=5 acetate buffer and 1 ml of 1 % $NaBPh_4$ was added. Finally during vigorous stirring headspace SPME extraction was carried out. After extraction the fiber was introduced into the heated inlet port of the GC for thermal desorption.

In the case of acid digestion-solvent extraction-GC-MS method 200 mg of previously defatted sample using 5 ml of 6 M hydrochloric acid was digested. The liberated compounds were extracted first with toluene and then with cysteine. The derivatization reaction was carried out after acidification and adding 1 ml of $CuSO_4$ solution and 0.2 ml of 1% $NaBPh_4$ solution, finally 1 ml of hexane. After agitation the phases were separated and the organic phase analysed.

3.2. Reagents and standards

The following reagents and standards were used during the work **:** deionized water, KOH, NaOH, methanol, $NaBPh_4$, sodium-acetate, hexane, toluene, acetone, cysteine, $CuSO_4$, NaCl, hydrochloric acid, sulphuric acid, nitric acid, MeHgCl, EtHgCl, $HgCl_2$ and BCR-CRM-464, TORT-2, BCR-710 and DORM-2 certified reference materials.

3.3. Instrumentation

The following instruments were applied : freeze-drying system, drying box, household coffee-grinder, ultrasonic bath, microwave digestion system, magnetic stirring plate, centrifuge, manual SPME device equipped with a fused silica fiber coated with a 100 μm film of PDMS, GC-AFS and GC-MS hyphenated systems, DB-1, HP-1 and HP-5 capillary columns, finally AMA 254 direct mercury analyzer.

3.4 Samples

Typical marine fish-containing food samples commercially available in Hungary and freshwater fish samples were analysed in the frame of my studies.

4. RESULTS

4.1 Development of alkaline sample digestion-SPME-GC-pyrolysis-AFS method

Alkaline sample preparation-phenylation derivatization-SPME-GC-pyrolysis-AFS system was developed for determination of mercury species in food samples during my studies.

Development of the alkaline digestion treatment and the aqueous phase phenylation derivatization were optimised during the method. The kinetics of SPME, the effect of methanol and potassium and the thermal desorption characteristic of the components from the SPME fiber in the heated inlet port of the GC were investigated. Additionally the volatility of phenylated compounds were investigated and the separation with GC was optimised.

Three different CRMs were used for validation purposes.

4.2 The uncertainty evaluation of method

The uncertainty statement of the improved method was evaluated and presented according to the principles of ISO/GUM in the course of my PhD studies. The aim of this work was to define the critical steps of this method in terms of reproducibility.

As a result of these experiments it can be concluded that the alkaline digestion and the calibration are the most critical steps regarding reproducibility of measurements. The phenylation detrivatization, the SPME and the volume- and mass measurement during sample preparation are less dominant process in this respect.

4.3 Participation in interlaboratory studies for quality control of method

Using the improved method I participated in two international interlaboratory studies coordinated by the Joint Research Centre-Institute for Reference Materials and Measurements (IRMM) of the European Commission. The results confirmed that using my improved method, reliable measurements of high metrological quality can be performed.

4.4 Measurement of seafood and freshwater fish samples

Freshwater fish samples and marine fish-containing food products commercially available in Hungary were analysed. The first part of the samples involved the three most commonly consumed marine fish species, herring imported from Poland, sardines imported from Thailand and hake imported from Argentina. The second set of samples — containing individual wrapped marine fish products — was arbitrarily selected from local supermarkets. Finally two freshwater fish species, carp and catfish were analysed.

On the basis of the results it can be concluded that obtained methylmercury and mercury levels in each sample were to some degree less than the prescribed limit values found in the European Commission Regulation. Considering the average Hungarian consumption of these products, 3.1 kg of fish (in general) a year per person, the risk of methylmercury intoxication of an average consumer via fish consumption is negligible.

4.5 Acid digestion-solvent extraction-GC-MS method

During my studies the previously improved and validated method was investigated in order to explore the errors of the technique. It was found that using ethylmercury as internal standard the method was proved accurate but absence of internal standard it was not effective enough.

Consequently the efficiency of all steps of this method were defined by making mass balance. On the basis of the results of the experiments it was allocated that the accounted popular acid leaching is not effective enough to the extraction of methylmercury from samples, the recovery from CRM samples are between 50-60 %. At the same time in the course of other steps loss was not experienced.

After all the stability of the other organic species, ethylmercury was studied during this method in order to explain the differences between the results of measurements performed with and without internal standard. In the experiments ethylmercury appeared to be less tolerant towards acid leaching than methylmercury so it can be concluded that the use of ethylmercury as internal standard can result in false effects.

4.6 Investigation of the stability and species-specific degradation behaviour of methylmercury and ethylmercury

Preventing the original distribution of the target analytes in a sample during storage and preparation is still one of the most challenging problems in mercury speciation studies. Therefore the transformation, degradation and interconversion phenomena of inorganic and organomercury compounds have been widely investigated.

The aim of this study was to highlight and characterise the specific degradation features of methylmercury and ethylmercury during alkaline digestion under microwave irridation, in order to provide additional information that should be considered when ethylmercury is employed as internal standard in mercury speciation studies.

It was proved by my work that methylmercury and ethylmercury yield different degradation behaviours under microwave irradiation. The reason for this may be the different strengths of the Hg-C bonds in methylmercury and ethylmercury. In these experiments methylmercury appeared to be much more tolerant towards microwave energy than ethylmercury in aqueous solution. Consequently individual species-specific degradation studies should be carried out within the method validation protocol, particularly for any methods that use different alkylmercury compounds as internal standard.

5. NEW SCIENTIFIC ACHIEVEMENTS

1. A fast and cost-efficient analytical method was developed for the determination of methylmercury in food samples. The improved method is based on alkaline sample preparation followed by aqueous phase phenylation derivatization with $NaBPh_4$ and SPME, finally GC-AFS detection. Based on the results of international intercomparison studies the method is capable of performing reliable measurements of high metrological quality.

It can be established that using headspace SPME the method is selective for methylmercury because of the less volatility of diphenylmercury derived from inorganic mercury.

2. It was first established that KOH should be replaced by NaOH in the sample preparation procedure in order to avoid the potential precipitation reaction between potassium and sodium tetraphenylborate. This problem predominates especially in case of “low content” samples.

3. The uncertainty statement of above-mentioned method was evaluated according to the principles of ISO/GUM.

It can be concluded that the alkaline digestion and the calibration are the most critical steps regarding reproducibility of measurements. The phenylation detrivatization, the SPME and the volume- and mass measurement during sample preparation are less dominant processes in this respect.

4. To carry out an estimation of average Hungarian methylmercury exposures via marine fish, fish-containing food and freshwater fish consumption, several commercially available marine products — the three most consumed fish species in Hungary — and two freshwater fish species were analysed.

As a result it can be concluded that obtained methylmercury and mercury levels in each sample were to some degree less than the prescribed limit values found in the European Commission Regulation. Considering the average Hungarian consumption of these products, 3.1 kg of fish (in general) a year per person, the risk of methylmercury intoxication of an average consumer via fish consumption is negligible.

5. Based on the results of my research the acid digestion-solvent extraction-GC-MS method was proved not effective enough to determinate methylmercury content in food samples. Two different processes occurring at the same time, namely the not effective digestion of the sample and the degradation of internal standard during acid leaching, resulted accidentally in the previously accurate results.

6. The stability and the degradation behaviour of methylmercury and ethylmercury were investigated under microwave irradiation. It was proved that methylmercury and ethylmercury yield different degradation behaviours under microwave irridation. In these experiments methylmercury appeared to be much more tolerant towards microwave energy than ethylmercury in aqueous solution. Consequently, individual species-specific degradation studies should be carried out within the method validation protocol, particularly for any methods that use different alkylmercury compounds as internal standards.

6. PUBLICATIONS

***In journals with impact factor*.**

Zs. Jókai, J. Hegoczki, P. Fodor, Stability and Optimization of Extraction of Four Arsenic Species, *Microchemical Journal*, 59 (1998), 117-124, **(2006 ISI-impact factor: 1,558**) **Cited by 7.**

Zs. Jókai, L. Abrankó, P. Fodor, SPME-GC-Pyrolysis-AFS Determination of Methylmercury in Marine Fish Products by Alkaline Sample Preparation and Aqueous Phase Phenylation Derivatization, *J. Agric. Food Chem.* 53 (2005), 5499-5505, **(2006 ISI-impact factor: 2,322**) **Cited by 6.**

L. Abrankó, **Z. Jókai**, P. Fodor, Investigation of the species-specific degradation behaviour of methylmercury and ethylmercury under microwave irridation, *Anal. Bioanal. Chem.* 383 (2005), 448-453, **(2006 ISI-impact factor: 2,591**) **Cited by 2.**

R.C.Quétel, J.P.Snell, Y.Aregbe, L.Abrankó, **Zs. Jókai**, C.Brunori, R.Morabito, W.Hagan, S.Azemard, S. Wyse, V.Fajon, M.Horvat, M.Logar, O.F.X.Donard, E Krupp, J.Entwisle, R.Hearn, M.Schantz, K.Inagaki, A.Takatsu, P.Grinberg, S.Willie, B.Dimock, H.Hintelmann, J.Zhu, E.Blanzo Gonzalez, G.Centineo, J.Ignacio Garcia Alonso, A.Sanz-Medel and E.Björn, Methylmercury in tuna : demonstrating measurement capabilities and evaluating comparability of results worldwide from the CCQM-P39 comparison, *J. Anal. At. Spectrom.* 20 (2005), 1058-1066, **(2006 ISI-impact factor: 3,630) Cited by 3.**

In journals without impact factor.

Bertényi Zsuzsa, **Szatura Zsuzsa**, Tóth Árpád, Fodor Péter, Réztartalmú növényvédőszerek tisztaságvizsgálata, *Magyar Kémiai Folyóirat 101.évf. 10.sz. : 446-450, 1995*

Dr Kertész Tamás, Dr Balák Csaba, **Jókai Zsuzsa,** Kápolna Emese, Dr Lakner Géza, Dr Kempler Péter, Dr Gachályi Béla, A magnézium anyagcsere zavara 2.típusú diabetes mellitusban, *Magyar Belorvosi Archivum* 57 (2004), 4-9

In conference proceedings

Hungarian full-text

Jókai Zsuzsa, Martin Bittner, Fodor Péter, Mintaelőkészítési módszerek kidolgozása elemspeciációhoz, *39.* Magyar Spektrokémiai Vándorgyűlés, Mosonmagyaróvár, 1996. (előadás alapján)

Jókai Zsuzsa, Abrankó László, Fodor Péter, Magyarországon fogyasztott halak metilhigany tartalmának mérése SPME-GC-AFS rendszeren , *Analitikai Vegyészkonferencia, 47. Magyar Spektrokémiai Vándorgyűlés, Bioanalitika* (Balatonföldvár*),* 2004. (előadás alapján)

International abstract

Zsuzsa Jókai, László Abrankó, Ildikó Ipolyi, Péter Fodor, Determination of methylmercury in freshwater fish and marine food samples by alkaline sample preparation followed by SPME-GC-AFS detection, *3rd International Conference on Trace Element Speciation in Biomedical, Nutritional and Environmental Sciences,* (München), 2004 (poszter alapján)

Cs. Sörös, R. Schaffer, **Zs. Jókai** , L. Abrankó, M. Üveges, M. Dernovics, P. Fodor, The role of As, Hg, Se and Sn speciation in food analysis, *CE Food* (Budapest), 2004. (poszter alapján)

Zsuzsa Jókai, László Abrankó, Peter Fodor, Determination of methylmercury in tuna fish (CCQM-P.39) and the investigation of the limitation of ethylmercury as internal standard, *European Winter Conference on Plasma Spectrochemistry* (Budapest), 2005. (díjnyertes poszter alapján)

László Abrankó, **Zsuzsa Jókai**, Peter Fodor, Method development for mercury speciation studies in fish samples intended for human comsumption, *XII Hungarian-Italian Symposium on Spectrochemistry Environmental Pollution and Human Health, (*Pécs), 23-27 October 2005. (előadás alapján)