International Conference on Metrology of Environmental, Food and Nutritional Measurements

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2nd IMEKO TC19 Conference on Environmental Measurements
1st IMEKO TC23 Conference on Food and Nutritional Measurements

10 – 12 September 2008, Budapest, Hungary,

DEVELOPMENT OF A REFERENCE PROCEDURE FOR THE DETERMINATION OF METHYLMERCURY IN FISH PRODUCTS

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Abstract: The purpose of this work is to present the development of an analytical procedure for the speciation analysis of methylmercury in fish products. The paper is focused on the extraction of the sample, considered as one of the most difficult step in the speciation process, for which quantitative recovery and preservation of the integrity of the species should be considered.

Keywords: Speciation analysis, Methylmercury, Isotope dilution.

1. INTRODUCTION

Speciation analysis, *i.e.* the identification, quantification, and characterisation of the chemical forms of a given element, is one of the key challenges of modern analytical chemistry. Mechanisms of accumulation, storage, or expulsion within an organism are, for instance, strongly dependent on the chemical species.
Undoubtedly, one of the most important applications of elemental speciation is to be found in the area of toxicology. In fact, information on the total content of an element is very often not sufficient to provide information related to toxicity.
Among the heavy metals, mercury is one of the most studied environmental pollutants. This is largely the result of its high toxicity and mobility in the environment. Due to the ability to travel over long distances in the atmosphere as gaseous elemental specie, mercury is regarded as a 'global pollutant' [1]. The high toxicity of mercury is given, *inter alia*, by its methylated form, methylmercury (hereafter $MeHg^+$), which is widely recognized as a neurotoxin affecting humans [1].
The main pathway of contamination of the humans with mercury is the food chain. The great ability to be bioaccumulated in the aquatic food chain leads to considerably elevated levels of $MeHg^+$ in aquatic organisms in higher levels of the food chain, despite nearly immeasurable quantities of Hg^{2+} in the water.
Regulatory authorities are required to measure Hg in a variety of biological, industrial and food samples for reasons of public health. For example, it is an obligation for EU member states to control concentrations of contaminants in foodstuffs [2]. In addition to total Hg measurement, the specific measurement of $MeHg^+$ is of interest to regulatory organisations, as this specie has a mammalian LD50 (Lethal Dose 50) 1000 times lower than elemental Hg.
In addition, many foodstuffs, particularly fish contain the majority of the Hg as $MeHg^+$. The 2003 recommendation from JECFA (Joint Expert Committee on Food Additives) [3] is that the provisionally tolerable human consumption of $MeHg^+$ is limited to 1.6 μg per kg body mass, per week. As the current wet weight concentration of $MeHg^+$ in certain species of fish is permitted up to 1.0 μg g^{-1}, and the greater proportion can be present as $MeHg^+$, a fish consumer could easily exceed such a recommendation [4].

PURPOSE

Despite the large amount of scientific literature on the topic, very few papers deal with the metrological approach of speciation analysis. The purpose of our work is the validation of a reference procedure for the determination of

$MeHg^+$ in fish matrices.
Sample preparation is considered as a difficult step of the speciation process. It should essentially provide the species in a medium compatible with the separation approach, but in this process the integrity of the species should be preserved. With other words, the first action to be taken with regards to sample preparation when analyzing solid samples is the release of the analytes from the matrix.

METHODS

The analysis of mercury species is achieved by hyphenated techniques, namely high performance liquid chromatography (HPLC) coupled with inductively coupled plasma-mass spectrometry (ICP-MS). The analytical procedures developed by NMIs can rely on the isotope dilution mass spectrometry (IDMS), a powerful strategy capable of highly accurate results traceable to the "Système International d'Unités" (SI) and recognised by the "Comité Consultatif pour la Quantité de Matière" (CCQM) as a primary method of measurement.
A method for the extraction of mercury species was developed at LNE on a tuna fish. The protocol is based on a thiol-containing reagent, known to bind tightly mercury [5]. Firstly, the optimization of the reagent content with an incubation in water bath for 2 hours at 80 °C [6] was achieved. Secondly, the acceleration of the extraction time is investigated by the use of ultrasounds and microwaves. Different exposure durations and several microwave powers were tested.

Sample

The tests have been performed on a certified reference material of tuna (BCR-463) certified for the total amount of mercury and the amount of $MeHg^+$ (Table 1).

Table 1: certified values of BCR-463 given in equivalent of mercury.

	Certified value / µg(Hg)/g	Uncertainty / µg(Hg)/g
Total Hg	2.85	0.16
$MeHg^+$	2.83	0.15

Extraction

The first extraction protocol [6] consisted in a 2-hour incubation of the sample (200mg) in a water bath with 40 ml of 0.1% (w/v) L-Cystein and 0.1 % (w/v) de L-Cys, HCl, H_2O.
The extract phase was analysed by HPLC-ICP-MS (Figure 1).
Investigations were leaded to reduce the time of the sample treatment. Tests were therefore performed by using either an ultrasonic bath with different exposition time (from 15 minutes to 1 hour) or a microwave field (at different powers and exposition times) in closed vessel (Ethos 900, Milestone). In order to verify the extraction efficiency, the amount of $MeHg^+$ was used instead of the total amount of Hg due to the risk of species evolution during the analysis protocol. An extraction percentage of $MeHg^+$ was obtained calculating the ratio between the determined and the certified values. For each test, three replicates were performed.

Separation

Separation was performed on a reverse phase C_{18} column (Synergi Hydro-RP, Phenomenex) after formation of a complex between the mercury in its different species and a sulphur compound. The mobile phase was composed by 0.05% (w/v) L-Cysteine and 0.05% (w/v) L-Cys, HCl, H_2O (BioUltra, Sigma-Aldrich) with a pH value fixed at 2.3 by drop wise addition of HCl.
50 µl were injected and the flow was set at 1 ml min^{-1}.
Detection was done on a quadrupole ICP-MS, PQ-ExCell (Thermo Fisher) directly coupled with the HPLC (Spectra system, Thermo Fisher).

RESULTS

As shown from the chromatogram presented in figure 1, only two species of Hg are present: Hg^{2+} and $MeHg^+$. Their determination was performed firstly by extern calibration (Table 2). The value of the total mercury was obtained by adding all the Hg species.

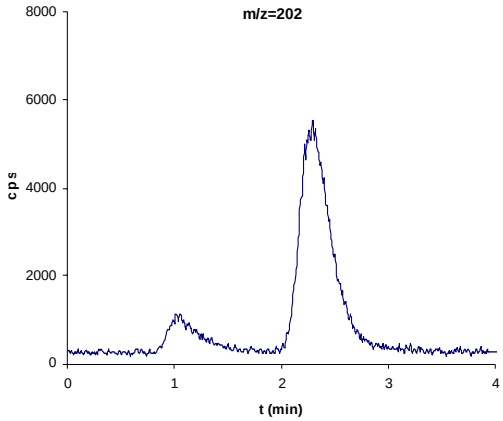

Fig. 1: Chromatograph of the extract phase from BCR-463

Table 2: Concentrations found in BCR-463

	Content / µg(Hg)/g	Standard deviation / µg(Hg)/g
$MeHg^+$	2.79	0.08
Hg^{2+}	0.065	0.002
Total Hg	2.86	X

Contents of $MeHg^+$ and inorganic Hg are compatible with certified values, allowing us to validate this extraction protocol for mercury in a matrix of fish, in particular the leach out of the sample.
Then, the improvement of the time for the sample treatment was investigated. The first series of tests allowed evaluating the efficiency of the microwave field and ultrasonic bath with respect to the results obtained with the first extraction protocol water bath. Results are reported in Figure 2.
The figure shows that for an action from 15 min to 1 hour, the ultrasonic bath does not allow any improvement of the extraction. The extraction recovery on $MeHg^+$ slightly improves with the time, but it never exceeds the 45%. The action of the microwave field, on the contrary, looks interesting because after only 6 min at 70 W a recovery of about 70% is obtained.
The error bars on the graph represent the standard deviation over the three replicates.

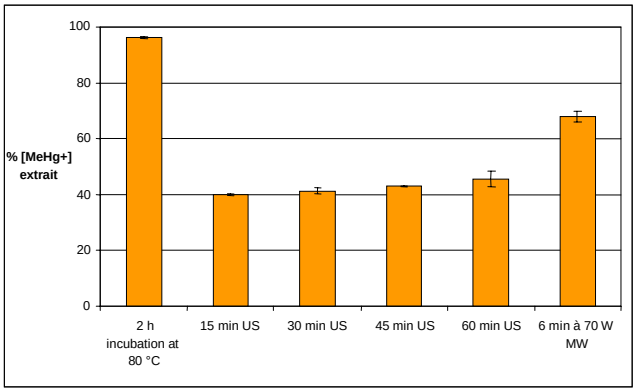

Fig. 2: Extraction efficiency of $MeHg^+$ with respect to different protocols (US= ultrasonic bath, MW = microwave assistance)

A second series of tests was therefore focused on the control of extraction with respect of the microwave field (time and power). A time of 11 min (instead of 6 min) was used for different powers. Results are shown in Figure 3.

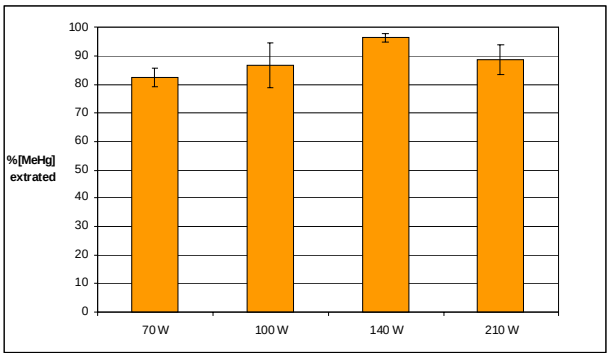

Fig. 3: Efficiency of $MeHg^+$ extraction with respect to the different microwave powers applied.

The increase of the exposition time allowed an increase of the extraction efficiency (comparing Fig. 2 and Fig. 3 for the protocol at 70 W). Moreover, the increase of the power improves at first the extraction of $MeHg^+$. For power higher than 140 W on the contrary, the extraction decreases, likely due to a process of demethylation of $MeHg^+$ during the analysis.
Finally, the protocol chosen for the treatment of samples was the exposition at 140 W for 11 min.

CONCLUSIONS

The work presents the analytical procedure developed at LNE for the analysis of methylmercury from a certified reference material of tuna fish. The sample preparation was improved by the use of microwaves to reduce the time of extraction. The analysis of extracts was realized by a HPLC-ICP-MS system. The optimization of the analysis procedure was achieved with the use of a CRM, certified in methylmercury.
The next step of development will be the application of specie-specific isotope dilution under the aspects of a primary method of measurement. Therefore, it will first allow the identification of potential demethylation of $MeHg^+$ during the analysis. Then, it will enable the traceability of the measure.

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