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Determination of methylmercury and inorganic mercury in shark fillets†

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Three samples of deep-frozen shark fillets were analysed according to the following procedure: dissolution in tetramethylammonium hydroxide, derivatization/ethylation with sodium tetraethylborate, extraction into iso-octane and measurement with gas chromatography hyphenated to inductively coupled plasma mass spectrometry (GC–ICPMS) for the identification and quantification of methylmercury ($MeHg^+$) and inorganic mercury (Hg^{2+}). For the correction of procedural errors, two internal standards were used. The sample pretreatment was corrected by spiking with dibutyldipentyltin (DBT-pe), and the GC–ICPMS measurements were controlled by the signal stability of xenon which was added to the GC carrier gas.

Furthermore, for comparisons, the total amount of mercury was determined by an independent technique, i.e. atomic fluorescence spectroscopy. Standard reference materials, which are only available in the form of lyophilisates and not as fresh fish materials, were also analysed to ensure the procedural quality control. The concentration range of total mercury measured in the shark fillets was between 0.9 and 3.6 μg mercury per gram thawed-out shark fillet. Two samples contained higher concentrations than the European legislation. Speciation analysis leads to ≥94% mercury bound as $MeHg^+$. Since $MeHg^+$ is known as one of the most toxic compounds, this conclusion is of importance with respect to bioaccumulation of mercury species via fish into the human food chain.

KEYWORDS: methylmercury; inorganic mercury; speciation; deep-frozen fish; shark; IMEP-20; gas chromatography; inductively coupled plasma mass spectrometry; atomic fluorescence spectroscopy

INTRODUCTION

Speciation analysis is of growing interest because different species have quite different degrees of toxicity.[1] As for many elements, for mercury the organic species are also more poisonous than the corresponding free inorganic mercury(II) species.[2] This study is focused on the analysis of methylmercury ($MeHg^+$) and inorganic mercury (Hg^{2+}). Because $MeHg^+$ is known as a highly toxic compound, it must be handled with greatest precautions.[3,4]

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Natural methylation in the environment is now well established for a number of elements. Mercury was one of the first cases studied owing to its methylation potential and the high toxicity associated with the final product of $MeHg^+$.[5] Besides the mainly low natural concentration, mercury is introduced into the environment anthropogenically. If methylated species occur, then their concentrations may increase through bioaccumulation processes into the food chain. Analyses of different parts of the environment show different ratios between the abundance of $MeHg^+$ and Hg^{2+}, e.g. seawater contains around 5% of total mercury in the form of $MeHg^+$, whereas in phytoplankton it is around 15% and in zooplankton it is around 20%.[6,7] This contribution increases in herbivorous fish to approximately 70%,[8] and in fish of prey a maximum of nearly 100% as $MeHg^+$ is possible.[9] Nevertheless, the concentration of $MeHg^+$ varies between the different parts of the body.[7,10] Investigations about the

binding forms of $MeHg^+$ in fish, especially in swordfish skeletal muscle, were recently published. Results obtained by X-ray absorption near-edge spectra show binding of mercury directly with a carbon atom and a sulfur atom, which is most likely $MeHg^+$-cysteine (or a structurally related species), that may have to different toxicological implications.[11]

European legislation controls the maximum permitted concentration of mercury in different kinds of food: e.g. fish of prey and eel, 1.0 μg g^{-1} wet weight; mackerel, herring, sprat and other fish, 0.5 μg g^{-1} wet weight. There is no differentiation between $MeHg^+$ and inorganic mercury.[12] The World Health Organization (WHO) advises limits for maximum safe consumption. The limit for mercury is 300 μg per week, from which 200 μg $MeHg^+$ are the most allowed.[13]

Generally, the analytical procedures for speciation analysis involving gas chromatography (GC) are based on three steps: dissolution (or at least extraction of) the species, derivatization and measurements for quantification. For the dissolution of fish tissues, different procedures are reported. Acid digestion, alkaline digestion or solvent extraction is usually used for the separation of mercury species from the biological matrix.[14–16] Furthermore, many different enzymes, like trypsin, protease type XIV, lipase and/or cellulase, are used for enzymatic hydrolysis.[15] For several years derivatization by alkylation (especially as ethylation, but also as propylation) has been applied to transfer mercury species into volatile mercury species.[17,18] Hyphenated techniques are commonly used for speciation measurements. Formerly, on-line speciation of Hg and $MeHg^+$ by chromatography–atomic fluorescence spectrometry (AFS) hydride generation was used.[19] Nowadays, the measurement of mercury species by the two hyphenated techniques of high-performance liquid chromatography coupled to inductively coupled plasma mass spectrometry (HPLC–ICPMS)[20–23] and GC–ICPMS[24] dominate. If a higher sensitivity is requested then GC–ICPMS is the method of choice.

Studies about possible species transformation, e.g. during the analytical procedure, have been done with isotope-specific determination methods. The results showed that a direct ethylation of $MeHg^+$ in an atmospheric precipitation sample by sodium tetraethylborate ($NaBEt_4$) produced no significant amount of artifactual $MeHg^+$.[25] Others investigated the species transformation processes using synthetic solutions to simulate environmental matrices. From the experiments it could be shown that the species conversion, e.g. of $MeHg^+$ into Hg(0), depends on halide concentration levels.[17] Furthermore, the procedural order is of great importance, e.g. ethylation should be done after addition of the organic phase to avoid species transformation (K. G. Heumann, personal communication, 2003).

This work describes a procedure for the analysis of $MeHg^+$ and Hg^{2+} in fresh fish material. In this case, shark fillets had to be controlled for the concentrations of mercury species before possible import to The Netherlands. The experiments were carried out with three samples of deep-frozen shark fillets. Standard reference materials, which are only available in form of lyophilisates and not as fresh fish materials, were analysed to assure analytical quality control. Methodical aspects were improved, e.g. by using two internal standards for the correction of possible procedural errors. Total mercury was determined using AFS as an independent technique. These results, as well as the balance of speciation, were used for confirmation and interpretation of the outcomes.

EXPERIMENTAL

Reagents, standards and reference materials

Deionized water was purified by a Millipore system (Milli-Q, 18 MΩcm). All chemicals were of analytical grade or of higher purity. Hydrochloric acid (HCl), acetic acid (HAc), sodium acetate (NaAc), nitric acid (HNO_3), Titrisol® solution of bromide–bromate, hydroxylammonium chloride ($HONH_3Cl$) and tin(II) chloride dihydrate ($SnCl_2{\cdot}2H_2O$) were purchased from Merck, Darmstadt, Germany. Tetramethylammonium hydroxide (TMAH) was supplied by Fluka, Buchs, Switzerland. $NaBEt_4$ was purchased from ABCR, Karlsruhe, Germany. Iso-octane and a stock solution of 1000 μg ml^{-1} mercury as $Hg(NO_3)_2$ (atomic absorption standard) were obtained from Baker, Deventer, The Netherlands. $MeHg^+$ standards were prepared from solid methylmercury chloride (MeHgCl) from Riedel de Haën, Seelze, Germany. (*Safety note*: organic mercury compounds are extremely toxic. Direct contact with skin can lead to death. During handling, precautions are absolutely necessary, e.g. inhalation must be avoided and protective clothes must be worn.[3,17])

The internal standard for the sample pretreatment was dibutyl-dipentyltin (DBT-pe), which was synthesized at the IVM Laboratory of the Free University of Amsterdam, The Netherlands.[26] The reference material of lyophilized tuna fish is CRM463 supplied by IRMM, Geel, Belgium. For the International Measurement Evaluation Program (IMEP) the certified test sample of lyophilized tuna fish (IMEP-20) was also obtained from IRMM, Geel, Belgium.

Instrumentation

The instrumentation used for speciation consisted of an on-line coupled system of GC via a heated interface to an HP 4500 quadrupole-based ICP mass spectrometer. All parts for GC–ICPMS were purchased from Agilent Technologies, formerly Hewlett Packard, Amstelveen, The Netherlands.

The instrumental details for GC and the interface are given in Table 1. The temperature program used for GC is summarized in Table 2. The operating conditions and the method set-up for ICPMS are given in Table 3. For chromatographic data analysis, 'ICP-MS Chromatographic Software C.01.00' from Agilent Technologies, Amstelveen, The Netherlands, was used.

For the determination of total mercury, the sample material was digested using a CEM MDS 2000 microwave system that was supplied by Beun de Ronde BV, Abcoude, The

Table 1. Instrumental details of GC and GC–ICPMS interface

GC instrumentation	HP6890
V (injection)	1 µl; splitless
T (injection)	250 °C
Carrier gas	Helium with 0.1% xenon (which is used as instrumental internal standard) supplied by Hoek Loos BV, Amsterdam, The Netherlands
Flow rate	6.5 ml min^{-1} with constant flow
Column	HP-1 (polydimethylsiloxane)
GC–ICPMS interface	Transfer line
Temperature control of interface via GC	
T (port 1)	280 °C
T (port 2)	280 °C

Table 2. GC temperature program

T rate (°C min^{-1})	*T* (°C)	*t* (min)
	50	1
10	60	
80	140	
40	170	
120	270	1.5
	280	

Table 3. Operating conditions and method set-up for ICPMS

RF power	1220 W
Cool gas flow	15 l min^{-1} Ar
Auxiliary gas flow	1 l min^{-1} Ar
Sample gas flow	0.9 l min^{-1} Ar
Additional gas flow	25 ml min^{-1} air
Operation mode	With shield torch
Measured isotope	Integration time
$^{200}Hg^{+}$	70 ms
$^{202}Hg^{+}$	70 ms
As internal standards	
$^{120}Sn^{+}$	70 ms
$^{126}Xe^{+}$	50 ms
Total run time of method	8 min

Netherlands. Subsequent measurements were carried out using a Merlin Plus System atomic fluorescence spectrometer from PS Analytical supplied by Landré Intechmij BV, Vianen, The Netherlands. The fluorescence intensity of mercury(0) was measured at 253.7 nm.

Sample preparation for speciation and measurements with GC–ICPMS

The three different samples of deep-frozen cut shark fillets (Shark 1, Shark 2, Shark 3) were analysed in triplicate. To avoid inhomogeneity effects in the sample material, the shark fillets were totally thawed out and homogenized by stirring before taking subsamples of fresh fish material. 0.5 g was weighed into a 50 ml tube and 5 ml TMAH was added. The sample material was totally dissolved after shaking the samples for 12 h (overnight).

Afterwards, the sample was diluted with water to a total volume of 50 ml. An aliquot of 25 ml was transferred into a 50 ml tube and 8 ml buffer solution of 2 M HAc–2 M NaAc was added. DBT-pe, used as internal standard for possible evaporation of the extract during the sample pretreatment, was dissolved in the organic solvent iso-octane with a concentration of 2%. Of this, 3 ml was added to the sample mixture. For derivatization, 3 ml of a freshly prepared solution of 1% $NaBEt_4$ in water was added and the mixture was shaken for 30 min. To achieve complete derivatization, 1 ml of 1% $NaBEt_4$ in water was added and also shaken for 30 min. After centrifugation both phases were separated and an aliquot of the upper layer (organic phase) was transferred into a GC vial. The measurements were carried out with the GC–ICPMS system according to the procedure described. Raw data were corrected with both standards used: DBT-pe for the sample pretreatment and xenon (from the GC carrier gas) for the measurements (see also Fig. 1).

A standard stock solution was prepared by dissolving MeHgCl in 0.04% HCl with a concentration of 100 µg l^{-1} mercury as $MeHg^{+}$. For the calibration of $MeHg^{+}$, different dilutions were prepared from the stock solution. For the calibration of Hg^{2+} the stock solution of 1000 µg ml^{-1} mercury (atomic absorption standard) was diluted. In addition, a blank was prepared. These solutions were pretreated (ethylated and extracted) according to the same procedure described of samples.

Sample preparation for determination of total mercury and measurements with AFS

The three different samples of deep-frozen cut shark fillets (Shark 1, Shark 2, Shark 3) were analysed in triplicate. To avoid inhomogeneity effects in the sample material the shark fillets were totally thawed out and homogenized by stirring before taking subsamples of fresh fish material. 0.5 g of fish material was digested after adding 7 ml 10% HNO_3 under microwave conditions, which are given in Table 4. Afterwards, the samples were diluted with water to a total volume of 50 ml. An aliquot of 1 ml was mixed with 2.5 ml of bromide–bromate solution (0.05 M; equivalent to 0.05 M bromine) in 4.6 ml 37% HCl. This converted all mercury species (especially organo mercury species) into Hg^{2+}. A possible surplus of bromide was eliminated by adding 12% $HONH_3Cl$ in H_2O. Finally, the mixture was diluted with water to a total volume of 100 ml. In the flow-injection system for AFS, Hg^{2+} was reduced to mercury(0) by adding Sn^{2+} as a solution of 2% $SnCl_2 \cdot 2H_2O$ in 1.8 M HCl, transferred from the solution into an argon gas phase and measured. For calibration, standards were prepared of the stock solution of 1000 µg ml^{-1} mercury (atomic absorption standard), pretreated and

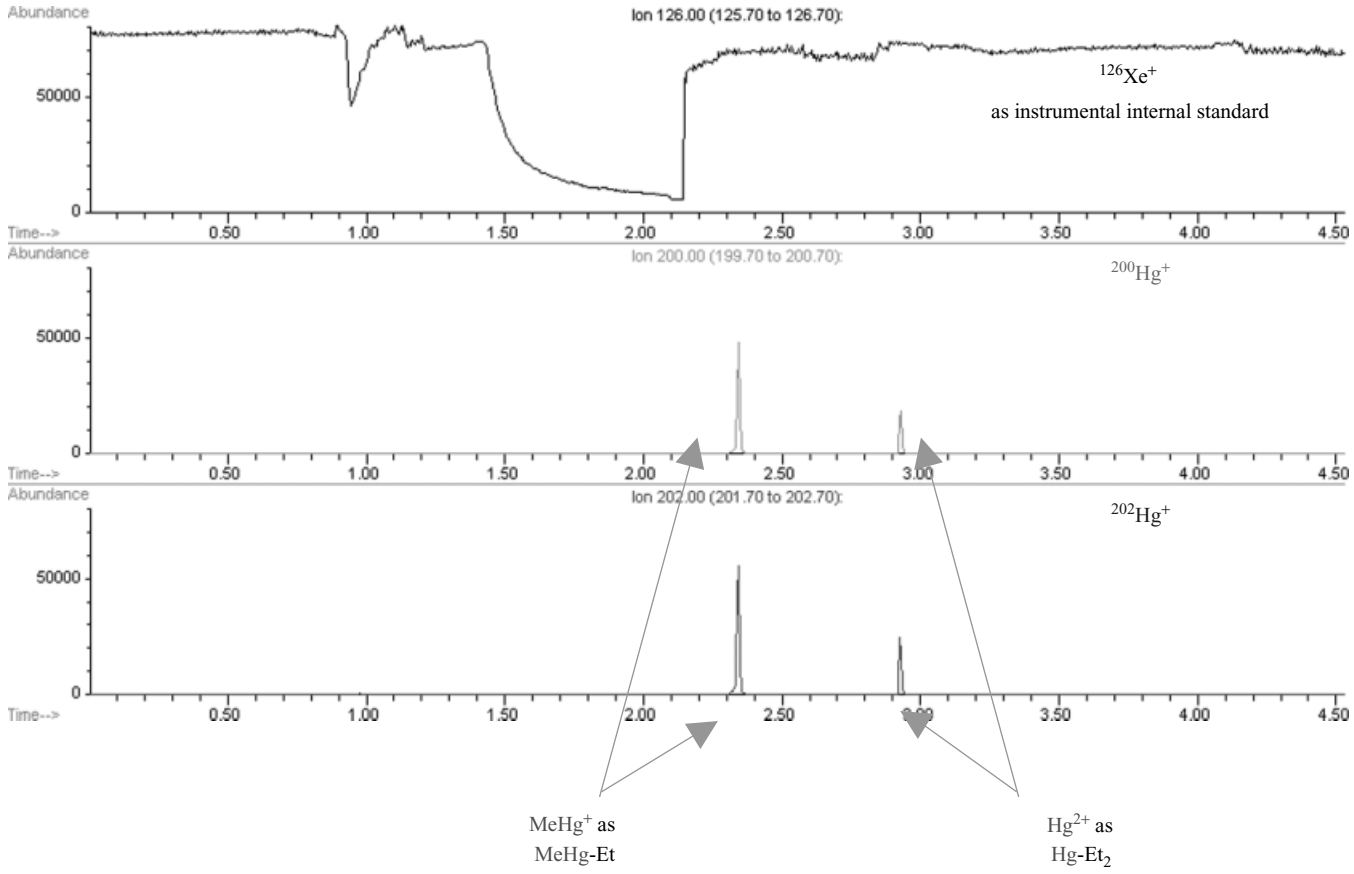

Figure 1. GC–ICPMS chromatogram of $MeHg^+$ and Hg^{2+}, both as ethylated species for the retention time range 0 to 4.5 min.

Table 4. Program for digestion with microwave system

Stage	1	2	3	4
Power (W)	330 (50%)	510 (80%)	390 (60%)	0 (0%)
Pressure (bar)	5.6	9	9	0
Run time (min)	10	8	52	30

measured according to the same procedure described for samples.

Accuracy and quality control aspects

In both analytical methods, GC–ICPMS and AFS, analysis of standard reference materials according to the same analytical procedures was performed for true evaluation of the procedure and quality control. Nevertheless, available reference materials are not fresh or deep-frozen fish materials, but lyophilized material like CRM 463 tuna fish. These lyophilized materials were only used for a general control of procedures. For the speciation by GC–ICPMS, 0.1 g of CRM 463 was taken and treated according to the procedure above described for GC–ICPMS. For AFS, 0.5 g of CRM 463 was analysed.

Recently, the certified test sample IMEP-20 tuna fish was analysed and the first published certified values were only used for general methodological aspects.[27]

Comparison of results for fresh/deep-frozen fish material was done by comparing the results of the different techniques.

RESULTS AND DISCUSSION

Results of speciation by GC–ICPMS

Figure 1 shows the chromatogram in the retention time range from 0 to 4.5 min for both ethylated mercury species, which were measured simultaneously for two isotopes ($^{200}Hg^+$ and $^{202}Hg^+$). The correlation between $^{200}Hg^+$ and $^{202}Hg^+$ is in good agreement and, therefore, no interference on either of the two masses was determined. Furthermore, the signal of the instrumental internal standard (xenon) is given in Fig. 1. The internal standard of sample pretreatment (DBT-pe) has a retention time of 5.4 min. The GC-program developed, with its total run time of 8 min, as well as all the other measurement conditions, is not only applicable for the determination of mercury species, it also allows us to perform the separation of commonly abundant organotin species like mono-, di-, tri-butyltin, as well as mono-, di-, tri-phenyltin.

Table 5. Results and balances of GC–ICPMS data with respect to the determination of $MeHg^+$ and Hg^{2+} in shark fillets

Sample	$c(MeHg^+) = c(MeHg^+)$		$c(Hg^{2+})$	$c(\Sigma Hg)$	Part ($MeHg^+$) in ΣHg [%]
	$\mu g\ g^{-1}$ $MeHg^+$	$\mu g\ g^{-1}$ Hg	($\mu g\ g^{-1}$ Hg)	($\mu g\ g^{-1}$ Hg)	
Shark 1	1.54 ± 0.08[a]	1.43 ± 0.08[a]	0.08 ± 0.02	1.52	94.0
Shark 2	1.01 ± 0.18[a]	0.94 ± 0.17[a]	0.01 ± 0.002	0.95	98.9
Shark 3	3.68 ± 0.30[a]	3.42 ± 0.28[a]	0.11 ± 0.02	3.53	96.9

[a] Standard deviation from replicate determinations.

Raw data were corrected for both internal standards and led to the results given in Table 5 for the (triplicate) shark fillets analysed. The concentrations of $MeHg^+$ were also converted to concentrations of mercury by using atomic mass ratios. This allows us to calculate the total sum of mercury, which is given by $MeHg^+$ and Hg^{2+}, in the fish samples. The sum of all mercury species was in the range from 0.95 to 3.52 μg mercury per gram thawed-out shark filet. Two samples were identified that contained higher concentrations mercury than allowed according to the European regulation.[12] Shark 1 contained 1.52 $\mu g\ g^{-1}$ mercury. This is significantly higher than the limit value of 1 $\mu g\ g^{-1}$ mercury. A concentration of more than three times the limit value was determined in Shark 3 (3.53 $\mu g\ g^{-1}$ mercury). Evaluating the ratio between both species, $MeHg^+$ and Hg^{2+}, showed a high abundance of ≥94% $MeHg^+$ in all shark samples. This is in agreement with the results of Potgeter[7] about the correlation between both species in fish of prey. Since $MeHg^+$ is known to be a very toxic compound, this conclusion is of importance with respect to bioaccumulation of mercury species via fish into the human food chain.

Evaluation and quality control was done by analysing a certified reference material (CRM 463) in each sequence and by participating in the evaluation of the certified test sample (IMEP-20). As already mentioned in the Introduction, both are lyophilized tuna fish, and this allows only a general procedure control. In each sequence CRM 463 was analysed and all recoveries of $MeHg^+$ were in the range 94 to 108% of the certified value (3.04 $\mu g\ g^{-1}$ $MeHg^+$). IMEP-20 was also analysed in triplicate according to the same procedure. The result obtained, i.e. 3.93 ± 0.17 $\mu g\ g^{-1}$ $MeHg^+$, was in the certified range of 4.24 ± 0.27 $\mu g\ g^{-1}$ $MeHg^+$.[27] Therefore, it could be concluded that the procedure applied for the determination of mercury species in these shark fillets is acceptable.

Results of determinations by AFS

The concentration of total mercury in the shark fillets (Shark 1, Shark 2 and Shark 3) was determined in triplicate and the results obtained are summarized in Table 6. It is significant that two samples had concentrations above the limit value of 1 $\mu g\ g^{-1}$ mercury, whereas Shark 2 was in the range of the limit value.

For the general procedure control with AFS, CRM 463 was analysed in each sequence. The recoveries of mercury were in the range 98 to 103% of the certified value (2.85 $\mu g\ g^{-1}$ total mercury).

Table 6. AFS results with respect to the determination of total mercury in shark fillets

Sample	c(Hg) ($\mu g\ g$)
Shark 1	1.85 ± 0.16[a]
Shark 2	1.05 ± 0.09[a]
Shark 3	3.33 ± 0.19[a]

[a] Standard deviation from replicate determinations.

Comparison of results determined by different methods

Within this study the results achieved by speciation were controlled by analysing the same samples by AFS. As a comparison, the total concentration of mercury determined by AFS versus the sum of the concentrations obtained by speciation ($MeHg^+$ and Hg^{2+}) was chosen. The results for Shark 2 and Shark 3 show a good agreement, especially with respect to a fresh sample material. The interpretation that Shark 1 and Shark 3 contained concentrations of mercury that were higher than the allowed level of 1 $\mu g\ g^{-1}$ mercury[12] is, therefore, confirmed. Therefore, fillets of Shark 1 and Shark 3 should not be distributed on the food market.

CONCLUSIONS

For the determination of $MeHg^+$ and Hg^{2+}, a multi-step pretreatment by dissolution, ethylation and extraction, as well as measurement with GC–ICPMS, was successfully applied. The use of two internal standards (one for sample pretreatment, one during the measurements) for the correction of procedural errors led to good results for lyophilized certified standard reference material. The same procedure showed good performance in its application for speciation in deep-frozen shark fillets, which were analysed after totally thawing out and homogenization. The results obtained were validated by an independent technique (AFS). With both techniques, comparable results for total mercury were obtained. In two samples (Shark 1 and Shark 3) the concentration of mercury exceeded the limit value set by the

European regulation. The concentration of the third sample (Shark 2) was in the range of this limit value. Expected high contributions of MeHg (≥94%) to total mercury were confirmed. The fact that mercury was mainly present as $MeHg^+$ shows that the European regulation is no longer realistic.

The speciation procedure has good potential for the analysis of various kinds of fish, also as fresh material. However, the handling of fresh fish material causes other effects and problems in addition to the handling of lyophilized fish material, e.g. increasing inhomogeneity and formation of fat/meat and water layers. These effects have been studied and will be discussed elsewhere.[28]

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