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The determination of methylmercury in biological samples by HPLC coupled to ICP-MS detection

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The use of high-performance liquid chromatography (HPLC) coupled to inductively coupled plasma mass spectrometry (ICP-MS) for the determination of methylmercury ($MeHg^+$) in fish tissue and hair samples is described. Analysis of these sample types is required when carrying out biomonitoring studies to determine human dietary exposure to this toxic mercurial compound. The developed method used a mobile phase containing an organic modifier and a sulfydryl compound (1 : 1 v/v methanol : water containing 0.01% v/v 2-mercaptoethanol) to limit peak tailing and aid separation. The chromatographic separation was coupled to the ICP-MS detector via a short piece of PEEK tubing, attached to the nebulizer. A cooled spraychamber and oxygen addition post-nebulization were required to limit the solvent loading on the plasma and reduce carbon build-up on the cones, respectively. The sample preparation procedure employed a drying step followed by digestion of the sample using tetramethylammonium hydroxide (TMAH) and heating in an open vessel microwave system. Two fish tissue certified reference materials (CRM), tuna fish CRM 463 and 464 (BCR, Brussels), a tuna fish proficiency test sample, IMEP-20 (IRMM, Geel, Belgium) and a hair CRM NIES no. 13 (National Institute of Environmental Science, Japan), were used to evaluate the method. The recovery of $MeHg^+$ for these four materials was between 83 and 100%, with precisions better than 6% for three separate extractions of the different materials. The limit of quantitation for $MeHg^+$ using the developed protocol was 0.5 μg Hg g^{-1}. The stability of $MeHg^+$ in the fish sample extracts was also assessed and losses of 14–16% were observed after storage of the extracts in a refrigerator at 5 °C, in high-density polypropylene tubes, for 6 months. The developed protocol has been used previously with atmospheric pressure ionization mass spectrometry (API-MS) to provide structural characterization and also with calibration via isotope dilution (IDMS) to provide high accuracy quantitation.

KEYWORDS: biomonitoring; elemental speciation; methylmercury; hair; fish tissue; HPLC-ICP-MS

INTRODUCTION

Elemental speciation has been defined by IUPAC[1] as the distribution of defined chemical species of an element in a particular system and speciation analysis as the measurement of the quantities of one or more individual chemical species in a sample. The importance of determining specific chemical forms of an element becomes apparent when considering its toxicity and environmental bioavailability. This is particularly true for the heavy metal mercury because of the acute toxicity of all the chemical species, which has resulted in a number of fatal poisoning instances throughout the world.[2,3] Methylmercury ($MeHg^+$) is notable in this respect, because it is readily formed from inorganic mercury (Hg^{2+}) by natural environmental processes, can be absorbed across the gastrointestinal tract, cross the blood–brain barrier and can pass between mother and fetus. These characteristics can lead to an accumulation of $MeHg^+$ in fetal blood, resulting in neurological impairment of the newborn.[4]

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The main source of non-acutely toxic $MeHg^+$ in the general population is the dietary intake of certain species of fish and other marine life,[5] most notably those at the top of the ecological food chain. The monitoring of mercury in the tissue of edible fish is important because consumption of contaminated fish has caused serious neurological damage in vulnerable groups.[3] As a result of this potential risk, the US Food and Drug Administration (FDA) currently advise pregnant women, nursing mothers and young children to avoid the consumption of this foodstuff.[6] The monitoring of marine animals and biomonitoring of vulnerable groups is required to determine the long-term effects of exposure to mercury compounds and this requires the analysis of fish tissue and human samples.

The US Environmental Protection Agency (US EPA) provides guidance on the intake of $MeHg^+$ in the form of reference dose values (RfD). The current value for $MeHg^+$ is 0.1 $\mu g\,kg^{-1}$ body weight day^{-1}, which is derived from an epidemiological study on pregnant women from the Faroe Islands, whose diet contained large quantities of whale meat and fish.[7] The WHO also provides a guideline value of 0.47 $\mu g\,kg^{-1}$ body weight day^{-1} for $MeHg^+$ exposure. To derive dose–response relationships from epidemiological data, quantitative exposure assessments for the population under study are necessary. Three different measures of $MeHg^+$ exposure have been used, including dietary assessment, hair analysis and blood analysis, and these matrices can be considered to be the most toxicologically appropriate to study. More specifically, the measurement of $MeHg^+$ in predatory fish, maternal hair, maternal blood, fetal blood or fetal cord blood is thought to provide the best estimate of human exposure. However, the acquisition of hair samples for the assessment of environmental exposure is considerably easier than blood samples because of its non-invasive nature.

The most effective instrumental based techniques for chemical speciation analysis rely on the use of chromatography coupled to an element specific detector. The established chromatographic modes for mercury speciation analysis include: capillary gas chromatography (GC) and high-performance liquid chromatography (HPLC). A variety of detectors have been used with these techniques, including atomic absorption spectroscopy (AAS) and atomic fluorescence spectrometry (AFS), usually with cold vapour (CV) generation. Other detection systems, based on excitation or ion formation in an inductively coupled plasma, include atomic emission spectroscopy (ICP-AES) or mass spectrometry (ICP-MS). One of the most versatile means to carry out mercury speciation analysis is by coupling chromatography to ICP-MS detection, because this can offer multi-isotopic analysis, over a wide range of concentration magnitudes (this may be limited by the chromatography) and the ability to accept both liquid or gaseous sample introduction. For the determination of $MeHg^+$ both GC and HPLC separations can be used; however with the GC approach the analyte requires derivatization to confer thermal stability, whereas with HPLC separations this step is not required. In the current work HPLC-ICP-MS was used because it allows for the direct analysis of $MeHg^+$ without conversion to another compound and therefore a more simplified analytical strategy can be employed. The speciation of Hg^{2+} and organomercury compounds including $MeHg^+$ by using HPLC has been reviewed for the period 1986–1999.[8]

Analytical methods for the determination of $MeHg^+$ in difficult matrices with the lowest possible uncertainty value[9] are one of the main requirements for determining fit-for-purpose dose values for $MeHg^+$ and hence the most reliable risk assessment for human exposure studies. We report a method for the determination of $MeHg^+$ in the toxicologically relevant materials fish tissue and human hair by HPLC coupled to ICP-MS. As part of the validation process for the method the stability of the analyte in the extraction medium over a 6-month time period was investigated.

EXPERIMENTAL

Reagents, standard solutions and samples

Deionized water >18 MΩ (Elga, Marlow, UK), tetramethylammonium hydroxide pentahydrate as an aqueous solution (25% w/v; GPR, Aldrich, Dorset, UK) or a commercially available solution (25% w/v) was used for the sample digestions. Methanol (HPLC grade, Fisher Scientific, Loughborough, UK) and 2-mercaptoethanol (Aldrich, Dorset, UK) were used for the HPLC eluent (1 : 1 v/v methanol : water containing 0.01% v/v 2-mercaptoethanol). A stock solution of methylmercury chloride (Pestanal Grade, Riedel-de Haen, Germany) was prepared in methanol. Two fish tissue CRM, tuna fish CRM 463 and 464 (BCR, Brussels), a tuna fish proficiency test sample, IMEP-20 (IRMM, Geel, Belgium) and a hair CRM NIES no. 13 (National Institute of Environmental Science, Ibaraki, Japan) were used to evaluate the method.

Instrumentation

The measurements were performed using reversed-phase high-performance liquid chromatography (HPLC) coupled to an Elan 5000A ICP-MS instrument (Perkin-Elmer, Beaconsfield, UK) for the three different tuna fish samples, or an Agilent 7500 instrument (Agilent Technologies Ltd, UK) for the NIES no.13 hair CRM. The stored fish tissue extracts, originally analysed using the Elan 5000A, were re-analysed after 6 months' storage using the Agilent 7500. The instrumental conditions for both instruments are given in Table 1.

Sample preparation

The moisture content of each sample was rigorously determined on a separate sub-sample of each material used in this work. In the case of the two tuna fish CRM and the hair CRM, drying instructions were provided with the certificate data. This was not the case with IMEP-20, so the moisture content was estimated in the same way as the two tuna CRM.

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Table 1. The developed instrumental HPLC-ICP-MS conditions used during the study. The Elan 5000A was used for the tuna fish tissue samples and the Agilent instrument for the aged fish tissue extracts and the hair analysis

Elan 5000A ICP-MS	
RF power (W)	1100
Gas flow (l min^{-1})	Plasma 16, auxiliary 0.8, nebulizer 0.92.
Isotopes monitored (m/z)	199, 200, 201 and 202
Interface	Cross-flow nebulizer, cooled spraychamber (−15 °C) and oxygen addition (2.6%), post-nebulisation.
HPLC pump	HP 1050 isocratic pump
Mobile phase	1 : 1 methanol: water (v/v) containing 0.01% 2-mercaptoethanol pumped at 0.4 ml min^{-1}
Injector	Rheodyne all PEEK biocompatible valve model 7125 (Cotati, California, USA) with 20 µl PEEK injection loop.
Column	Advanced Chromatography Technologies ACE 3 C_{18}, 15 cm × 3.2 mm, 3 µm, 100 Å, equipped with a C_{18} guard cartridge (Phenomenex, Maccelsfield, UK), at room temperature
Agilent 7500 ICP-MS	
RF power (W)	1550
Gas flow (l min^{-1})	Plasma 15, auxiliary 0.28, nebulizer 0.85
Isotopes monitored (m/z)	199, 200 and 202
Interface	Microflow PFA concentric nebuliser, cooled spraychamber (−5 °C) and 5% oxygen addition, post-nebulization
HPLC pump	Agilent Technologies 1100
Mobile phase	As for the Elan
Injector	20 µl using autosampler
Column and temperature	Column as for the Elan, though at 30 °C

In brief the materials were repeatly heated in an oven at 90 °C, with a cooling phase in a vacuum desiccator, until a constant weight value was achieved. The human hair CRM was dried at 85 °C for 4 h followed by 30 min cooling in a desiccator. This particular step in the analytical procedure is particularly important when high accuracy is required, because of the difficulty in removing all of the water from biological samples.

Sub-samples (~0.2–0.25 g) were accurately weighed into the glass digestion vessels and digested using tetramethylammonium hydroxide (TMAH) (5 ml aqueous 25% solution) by heating in an open vessel microwave system (Prolabo A301, Fontanay-sous-bois, France) according to the method of Tseng *et al*.[10] This involved heating the sample vessels for 4 min using 40 W of focused microwave power. The clear, dark brown solutions generated were neutralized with glacial acetic acid (900 µl), centrifuged for 5 min at 10 000 rpm (MSE, Micro Centaur) and the supernatant filtered through a 0.45 µm syringe filter (Qm_X Laboratories Ltd, Thaxted, Essex, UK). The filtered supernatant was subsequently made up to ~20 g with an aliquot of the mobile phase in 50 ml high-density polypropylene centrifuge tubes (HDPP, Sarstedt, Leicester, UK). Each sample was prepared in triplicate and the procedure was carried out on a gravimetric basis.

Sample storage studies

Once prepared the tissue sample extracts were analysed and then stored in 50 ml HDPP centrifuge tubes, in a refrigerator at 5 °C, in the dark (covered with aluminium foil), for 6 months. The samples were then re-analysed to determine the stability of the mercury species over this time period.

Instrumental analysis

The chromatographic conditions used during the study are detailed in Table 1 and were adapted from those of Harrington *et al*.[11] previously developed for the analysis of methylmercury in fish tissue by liquid chromatography coupled to atmospheric pressure chemical ionization mass spectrometry (HPLC-APCI-MS). Each of the three sample replicates for the IMEP sample, the tuna fish CRM and the hair CRM were analysed interspersed with injections of the mobile phase and the reagent blank solution, to eliminate the possibility of analyte carry over between samples. No sign of carry-over was observed during any of the sample runs.

The raw data from the analysis using the Elan 5000A, in the form of comma separated variable files, were exported into Excel spreadsheets to determine the peak area and height of each peak after baseline subtraction, Excel was also used to calculate the final concentrations in the samples. Three $MeHg^+$ standard solutions with concentrations (as mercury) in the range 5–60 ng g^{-1} were used to form linear calibration plots with r^2 values better than 0.99. For the hair CRM analysed using the Agilent instrument the data generated was processed using the ChemStation software supplied with the instrument.

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The lowest $MeHg^+$ standard had a concentration of 5.6 ng Hg g^{-1}. Using this standard solution the ratio of the intensity of the ^{202}Hg isotope found at the $MeHg^+$ peak apex to that of the noise, estimated using the average baseline counts after the chromatographic peak, had a value of 10, so this was used to determine the instrumental LOQ value of 5.6 ng Hg g^{-1}. Therefore the average dilution factor of 90 gave a method LOQ of 0.5 μg Hg g^{-1}, which is about an order of magnitude lower than the $MeHg^+$ content of the samples analysed.

RESULTS AND DISCUSSION

Mercury speciation

The use of capillary GC-ICP-MS for the determination of mercury speciation in the type of biomonitoring samples discussed here has been widely reported in the literature. This could be because it offers lower limits of detection[12] than using HPLC-ICP-MS and can be more easily used for multi-elemental speciation analysis. The GC-ICP-MS approach requires an extra sample preparation step to derivatize the analytes to a thermally stable species to facilitate analysis. However, the degradation of $MeHg^+$ to Hg^0 during the GC sample derivatization procedure has been reported for biological tissues[13] and seawater samples.[14] The mechanism for this effect has not been fully elucidated, but it can be controlled by careful sample handling during the preparation steps. Alternatively, the most reliable approach to overcome this is to use isotopically enriched standards if they are available or can be synthesized,[15] because these can compensate for any analyte redistribution occurring during the sample preparation procedure. However, this involves additional costs and requires a good understanding of the IDMS approach for success.[16] The use of HPLC-ICP-MS has not been shown to suffer from artefactual species formation during the measurement step, because it does not require derivatization of the analyte. HPLC is also easier to couple to ICP-MS detection compared with GC, because a heated transfer line is not required, making for a less complex chromatographic interface. However, HPLC hyphenation requires an additional mass-flow controller and a cooled spray-chamber. The choice of speciation system to use will be determined by the preference and experience of the analyst with each approach and the requirement to establish fit-for-purpose protocols.

The original HPLC protocol[17] upon which the current one is based used a mobile phase composed of an ion-pairing agent (tetrabutylammonium bromide, 10 mM) a sulfur-containing compound (2-mercaptoethanol, 0.01% v/v) and an organic modifier (methanol, 50% v/v), with detection by ICP-MS. The inclusion of the organic modifier and the sulfur ligand was shown to overcome a number of significant problems related to the adsorption of inorganic mercury (Hg^{2+}) and methylmercury ($MeHg^+$) in the system, which caused poor peak shapes, retention of Hg^{2+} within the chromatographic system and detection limits inadequate for biomonitoring studies. With further development the ion-pairing reagent was removed from the eluent so that detection by a molecular mass spectrometry technique, atmospheric pressure chemical ionization mass spectrometry (APCI), could be used for the detection of the mercury species.[11] Removal of the ion-pair reagent had no significant effect on the resolution of the different mercury species. The current system uses the same conditions as for the APCI system, but detection by ICP-MS, thus the developed methodology can be used with both molecular mass spectrometry for species identification and elemental detection for quantitation at the low μg g^{-1} concentration level. This allows for robust species validation, without relying solely on retention time matching or standard spiking. Other recent work by us and another group using our methodology, demonstrates the use of the methodology for applications requiring high accuracy quantitation, by using species-specific[18,19] and species-unspecific IDMS calibration.[18]

The chromatographic method described herein can separate a number of different mercury (II) containing compounds, including inorganic mercury, methylmercury, ethylmercury and phenylmercury (listed in elution order),[11] although with environmental and human biomonitoring samples the separation of Hg^{2+} and $MeHg^+$ is of greatest interest, because the other species are rarely seen in these matrices. The elution order is important with mercury speciation analysis because matrix effects can affect the Hg^{2+} retention in reversed phase separations to a greater extent than the other species.[17] In the current system any matrix effects causing the inorganic Hg^{2+} species to elute earlier than expected will not result in co-elution with another mercury-containing species because the inorganic Hg^2 species elutes first from the column, followed by other species in order of increasing aliphatic chain length.

Some studies using HPLC-ICP-MS for the analysis of mercury speciation in biological matrices such as fish and shellfish have highlighted mercury species transformations, but these were shown to occur during sample pre-treatment with TMAH and not during the measurement step. The two most important papers in this regard[20,21] used species containing isotopically enriched mercury to follow the interconversions occurring. By spiking various fish tissue materials with Hg^{2+} and $MeHg^+$ and then treating with TMAH followed by pH adjustment, up to 12% of the added Hg^{2+} was methylated and 6% of the $MeHg^+$ was demethylated.[21] However, in the current study described herein the fish tissue materials contained only very low levels of Hg^{2+} and no mercury species were spiked into the samples, so it is unlikely that this process is occurring to the same extent. Hence the method of correction described in the earlier work[21] was not applied to any of the results obtained in the current study.

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The quantification of methylmercury in fish tissue by HPLC-ICP-MS

The results for using conventional external calibration to determine the concentration of $MeHg^+$ in the IMEP-20 tuna sample and the two tuna CRM materials are given in Table 2. A sample chromatogram showing the separation achieved for the mercury species present in the IMEP-20 tuna fish TMAH extract is shown in Fig. 1. The retention times are 2.70 and 3.08 min for Hg^{2+} and $MeHg^+$, respectively. The data in Table 2 show good agreement with the certified values for $MeHg^+$ in the tuna CRM materials[22] and the IMEP-20 sample.[23] Precision values for $MeHg^+$ in the fish tissue samples were good, with coefficients of variance of<6% for three replicate determinations and an uncertainty value of 12.4% for the IMEP sample (coverage factor $k = 2$).

Inorganic mercury was not determined using this methodology, although this would be possible. The current fish tissue materials analysed contained very low quantities of Hg^{2+}, which is in agreement with most studies on fish samples, where the majority of the total mercury is present as $MeHg^+$.[24] A possible high accuracy approach to quantitative mercury speciation in fish tissue containing $MeHg^+$ and only low concentrations of Hg^{2+} would involve the initial determination of the total mercury content after a total digest, using analysis by IDMS.[25] The current methodology using HPLC-ICP-MS with species-specific ID-MS calibration[18,19] could then be used to determine the $MeHg^+$ concentration. The difference between the $MeHg^+$ and total mercury concentrations would then give a reliable value for the Hg^{2+} content by difference, because the amounts being measured would be much larger. This type of approach, but using detection by cold vapour atomic absorption spectrometry and conventional calibration methods, has been reported in the literature.[26]

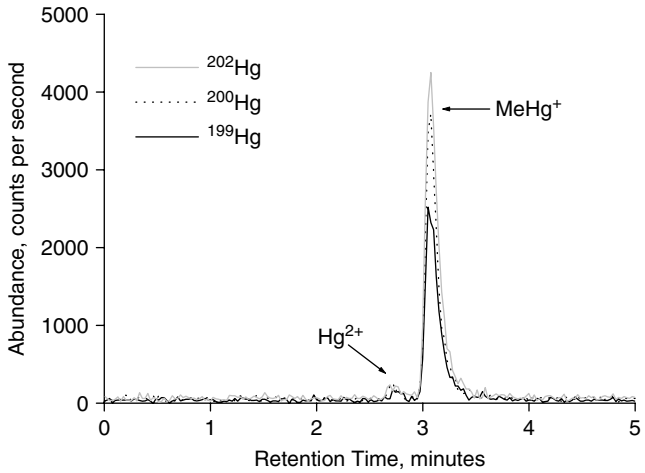

Figure 1. HPLC-ICP-MS chromatogram of the IMEP-20 tuna fish TMAH extract. The ^{202}Hg, ^{200}Hg and ^{199}Hg isotopes are shown and retention times are 2.70 and 3.08 min for inorganic mercury and methylmercury, respectively.

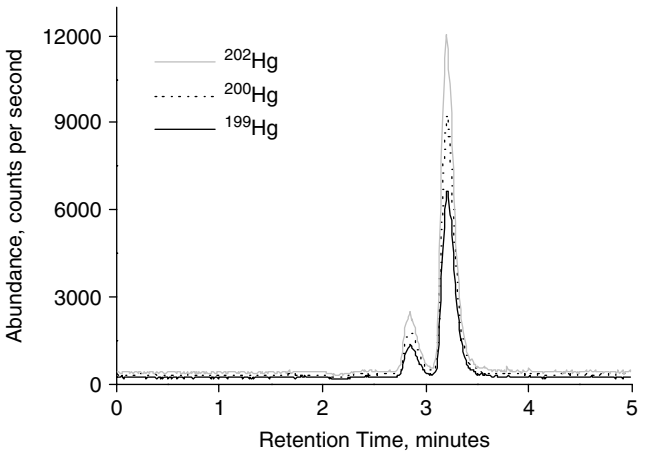

Figure 2. HPLC-ICP-MS chromatogram of the NIES no.13 human hair TMAH extract showing ^{202}Hg, ^{200}Hg and ^{199}Hg isotopes. Peak identification as in Fig. 1.

The quantification of methylmercury in human hair by HPLC-ICP-MS

The results for the analysis of the human hair CRM (Table 2) using the developed protocol gave excellent method recovery and precision values. This shows that the sample extraction method, using fast microwave heating and a weak alkaline extractant, was effective in solubilising both the fish and hair sample matrices, without destroying the $MeHg^+$ present. This is in contrast to studies[27] using a mixture of nitric

Table 2. Results for methylmercury in the two fish tissue CRM samples, a fish tissue proficiency test sample and a human hair CRM, as determined by HPLC-ICP-MS using external calibration

Material	Certified $MeHg^+$ content, mg kg^{-1} dry mass	Uncertainty[a]	Mean determined $MeHg^+$ content, mg kg^{-1} dry mass	RSD%, ($n = 3$)	Recovery, %
IMEP-20	4.24	0.27	4.26	5.1, 12.4[a]	100
BCR 463	3.04	0.16	2.52	4.6	83
BCR 464	5.50	0.17	5.37	5.6	98
NIES 13	3.80	0.40	3.80	0.3	100

[a] The percentage uncertainty values listed in the table are for a coverage factor 2 ($k = 2$).

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acid and hydrogen peroxide with closed vessel microwave heating to digest hair, where it was established that the $MeHg^+$ content of the sample was destroyed during the heating process. This effect was overcome by leaving the samples in the digestion medium overnight with no heating, which fully solubilized the sample matrices leading to quantitative recovery. The current method has a more rapid sample preparation procedure because the microwave-heating programme is complete in just 4 min, with no analyte decomposition observed with any of the samples analysed.

Calculation of uncertainty for the analytical method

Analysis of the IMEP sample was used to evaluate the level of uncertainty in the protocol and identify the areas with the greatest contribution to this value, so that possible improvements in the method could be made. The approach used to calculate the uncertainty value for the measurement of $MeHg^+$ by the developed method was based on the Eurochem guide[28] and the work of Barwick *et al*.,[29] adapted to speciation analysis from its initial use for total elemental measurements.

Initially, the main and lower level effects contributing to the uncertainty value were identified using a fishbone diagram, as described in the Eurochem guide.[28] The six main effects that required evaluation included analyte recovery, sample moisture content, dilution, measurement precision, conversion factor and analyte concentration. Various lower level effects contributed to these main effects and were used in the calculation of each individual contribution to the uncertainty value. A value for each of the effects was determined during the analysis of the IMEP sample using conventional analytical procedures and by inclusion of the tuna fish CRM BCR 464, which provided a value for the recovery effect. The largest magnitude effects contributing to the uncertainty value were determined to be the analyte recovery, the measurement precision and the purity of the $MeHg^+$ standard. The combined uncertainty value for the $MeHg^+$ mass fraction in the IMEP sample with a coverage factor of 2 was 0.53 mg kg^{-1}, which equates to 12% compared with the $MeHg^+$ mass fraction in the sample. This agrees very well with the relative expanded uncertainty value of 20% for a dog fish reference material (DORM-2) containing a similar concentration of $MeHg^+$ and analysed by species-specific IDMS with HPLC-ICP-MS analysis[19] and reasonably well with the uncertainty value of 6.3% determined for $MeHg^+$ in the IMEP-20 sample using species-specific IDMS and analysis by GC-ICP-MS.[30]

Stability of $MeHg^+$ in the fish tissue extracts

The stability of mercury species in sample extracts is an important consideration when validating an analytical protocol, because transformation and degradation processes have been shown to occur under a variety of storage conditions. It is therefore useful to know the time period for which the chosen storage conditions maintain the original chemical speciation. The factors affecting the stability of Hg^{2+} and $MeHg^+$ during sample storage have been reviewed.[31] Several possible mechanisms for the loss of Hg^{2+} were described, including adsorption onto the container walls volatilization of mercury and conversion of the mercury species, but none of the work reviewed dealt specifically with the loss mechanisms for $MeHg^+$. However, a similar range of mechanisms as described for Hg^{2+} would most likely be responsible for any loss of $MeHg^+$. A more recent paper discusses the analysis of mercury in environmental samples in detail and summarizes the current knowledge related to the problems encountered at each stage of the analytical procedure, including sample collection, pretreatment and measurement.[32] However, it is clear from this review that relatively little is known on the effect of extract storage on the stability of $MeHg^+$.

Studies have shown that, over a storage period of 5 days, plastic bottles and clear glass bottles perform similarly well for potable water samples.[33] Methanolic TMAH extracts of biological material stored in screw-capped polypropylene bottles have been shown to be stable for 1 year under normal conditions.[34] Methylmercury chloride (MeHgCl) stability in methylene chloride (used as a solvent in GC analysis) at various temperatures has been studied.[35] Approximately 50% of the MeHgCl in a standard solution stored in glass vials in the dark at a temperature approximating that of our stability study was lost over 15 days. However, the initial concentration levels in aged MeHgCl standards used in the study were significantly lower than those investigated here. The most relevant study[21] was performed by spiking different fish tissues with $MeHg^+$ and Hg^{2+} containing enriched isotopes of Hg. The tests showed that methylation of Hg^{2+} and demethylation of $MeHg^+$ was occurring during extraction using TMAH with ultrasonication followed by subsequent pH treatment and that this effect was greatest after 1 h, but significantly lower after 24 h, presumably indicating some equilibrium effect. The authors concluded that methylation of Hg^{2+} is insignificant during alkaline digestion and storage and is most probably taking place at a later stage of sample treatment, during and after pH adjustment. However, as stated earlier because of the much lower Hg^{2+} concentration in the current materials compared with those used by Qvarnstrom *et al*.,[21] it is unlikely that any significant methylation effects are occurring in the present work.

The stability of $MeHg^+$ in the tuna fish extracts over a 6-month storage period was investigated in the current study. The results for the analysis of the acid neutralized extract solutions for $MeHg^+$, after 6 months' storage in high-density polypropylene tubes in a refrigerator, with exclusion of light from the samples by using a cover, are given in Table 3. It can be seen that there was a consistent loss in $MeHg^+$ content over the study period of 15% from all three of the fish extracts, compared with the originally determined concentration. The most plausible possible explanations for this loss of $MeHg^+$ are formation of another mercury species, physical loss of $MeHg^+$ onto or through the container walls or a combination

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Table 3. Results for methylmercury determined by HPLC-ICP-MS with external calibration, after storage of the acid neutralized extracts at 5 °C in the dark for 6 months. Each sample analysed in triplicate. Certified contents for each CRM as detailed in Table 2. Also shows the change in peak area ratio ($MeHg^+$: Hg^{2+})

Material	Original mean $MeHg^+$ content, mg kg^{-1} dry mass (% RSD)	Mean peak area ratio, $MeHg^+$: Hg^{2+}	Mean $MeHg^+$ content after storage, mg kg^{-1} dry mass (% RSD)	Peak area ratio, $MeHg^+$: Hg^{2+}	Change in concentration[a] (change in ratio[b]), %
IMEP-20	4.26 (5.1)	6.67	3.57 (3.5)	19.9	−16 (+66)
BCR 463	2.52 (4.6)	27.8	2.12 (11.3)	70.5	−16 (+61)
BCR 464	5.37 (5.6)	13.0	4.60 (4.8)	24.8	−14 (+48)

[a] Percentage change in $MeHg^+$ concentration from the original value. [b] Percentage change in $MeHg^+$: Hg^{2+} peak area ratio from the original value.

of these effects. After formation of another species, e.g. demethylation, resulting in the formation of Hg^{2+} followed by reduction to Hg^0, or methylation to dimethylmercury (Me_2Hg), the $MeHg^+$ in the extract could be lost through volatilization. However, all of the extracts analysed after 6-months' storage looked similar to Fig. 1, showing no sign of either Hg^0 or Me_2Hg being formed. The ratio of the peak areas for $MeHg^+/Hg^{2+}$ increase over the study period, although the amount of $MeHg^+$ drops over the same time course (Table 3). This means that the amount of Hg^{2+} must also decrease, to a greater extent than the $MeHg^{2+}$ content. This provides some evidence that the loss of $MeHg^+$ is not due to demethylation to Hg^{2+} or the formation of Hg^0, Hg^{2+} or Me_2Hg.

CONCLUSIONS

A method based on the direct determination of mercury species, without the need for analyte derivatization, has been validated and shown to be applicable to the determination of $MeHg^+$ in sample matrices of relevance in human biomonitoring studies. Specifically, the protocol can be used to determine $MeHg^+$ in hair and fish tissue matrices down to the low μg g^{-1} concentration level.

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