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Analytical Methods

Collaborative study on determination of mono methylmercury in seafood

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ABSTRACT

Eight laboratories participated in an inter-laboratory method-performance (collaborative) study of a method for the determination of mono methylmercury (MMHg) in foodstuffs of marine origin by gas chromatography inductively coupled plasma isotope dilution mass spectrometry (GC–ICP-IDMS) after dissolution, derivatisation and extraction of the species. The method was tested on seven seafood products covering both a wide concentration range and variations in the MMHg concentrations as well as matrix compositions. The samples were mussel tissue, squid muscle, crab claw meat, whale meat, cod muscle, Greenland halibut muscle and dogfish liver (NRCC DOLT-4), with MMHg concentrations ranging from 0.035 to 3.58 mg/kg (as Hg) dry weight. Repeatability relative standard deviations (RSD_r) for MMHg ranged from 2.1% to 8.7%. Reproducibility relative standard deviations (RSD_R) ranged from 5.8% to 42%. All samples showed HorRat value below 1.0, except for the sample with the lowest MMHg content, mussel tissue, with a HorRat value of 1.6.

1. Introduction

The purpose of the collaborative study described herein was to determine the performance characteristics of a potential future standard method for the determination of mono methylmercury (MMHg) in seafood.

Element 80 in the periodic table, mercury (Hg), is only formed naturally in the extreme temperatures and pressures of supernovas and is an element of low abundance in the earth crust. Natural and anthropogenic releases of mercury from the earth's crust has led to worldwide distribution of mercury through global transport of this heavy metal (Fitzgerald, Engstrom, Mason, & Nater, 1998; Schroeder & Munthe, 1998). Anthropogenic emissions of mercury include coal combustion, mining activities and commercial releases, whereas natural releases mainly are due to volcanoes (Horowitz, Jacob, Amos, Streets, & Sunderland, 2014).

When mercury is introduced in the environment and aquatic ecosystems it is oxidised to inorganic mercury and then methylated to form MMHg by various methylating microbes (Dizikes, Ridley, & Wood, 1978; Morel, Kraepiel, & Amyot, 1998). The MMHg is readily absorbed by organisms and biomagnified, with

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the result that predatory species in the food chain generally contain the highest levels of MMHg (Boening, 2000; Forsyth, Casey, Dabeka, & McKenzie, 2004; Mason, Laporte, & Andres, 2000). The teratogen MMHg is most dangerous to the developing fetus and continued elevated exposure to MMHg in the uterus may lead to central nervous system damage (Korbas et al., 2010). A major source of exposure to MMHg for humans is seafood which at the same time also provide important sources of energy, protein and a range of essential nutrients not easily found in other food (Kuntz, Ricco, Hill, & Anderko, 2010; Mozaffarian & Rimm, 2006).

The global trade of seafood requires international harmonisation of maximum limits for contaminants such as MMHg. The rules of global trade are dealt with by the World Trade Organization (WTO) whose agreements are signed by the majority of nations (WTO, 1994b). The Sanitary and Phytosanitary (SPS) agreement of the General Agreement on Tariffs and Trade (GATT) designates the Codex Alimentarius Commission (CAC) as the instrument for international standards, guidelines and recommendations for food safety (WTO, 1994a).

During the eighties it became evident that several different maximum limits for mercury in fish existed around the world (CCFAC, 1985). In 1987 the Codex Committee on Food Additives and Contaminants (CCFAC) recommended to establish a guideline level (GL) of 0.5 mg/kg for total mercury in fish except for predatory fish where the GL was set at 1.0 mg/kg (CCFAC, 1987). In 1988 the recommendation was changed from total mercury into MMHg by the CCFAC, but the GLs where not adopted by the CAC before 1991 and then only with the understanding that the GLs would be kept under review by CCFAC and Codex Committee on Fish and Fishery Products (CCFFP) (CAC, 1991). While the Codex General Standard for Contaminants and Toxins in Feed and Food (GSCTFF) defines the ML as the maximum legally permitted concentration and the GL as giving some discretion to governments, the SPS agreement in GATT does not differentiate between GLs and MLs (CAC, 1995; CCEXEC, 1999). Codex Committee on Contaminants in Food (CCCF) is currently considering ML for MMHg in certain species of tuna (CCCF, 2015).

In 2006 the joint FAO/WHO Expert Committee on Food Additives (JECFA) confirmed the PTWI of 1.6 μg/kg bw for MMHg and CCFAC requested for a joint expert consultation on the risks and benefits of fish consumption (FAO/WHO, 2006). In 2010 such a consultation on fish was carried out by the FAO with the contaminants MMHg and dioxins and long-chain omega-3 fatty acids as nutrients (FAO/WHO, 2010). The conclusion of the consultation was that consumption of particularly fatty fish lowers the risk of mortality from coronary heart disease. The neurodevelopmental risks of not eating fish was found to exceed the risk of eating fish with MMHg in most circumstances, but the benefits are reduced by exposure to MMHg. Reduction of anthropogenic contamination of fish would hence increase the beneficial effects of fish consumption. In 2012 EFSA further reduced the tolerable weekly intake (TWI) for MMHg to 1.3 μg/kg bw/week expressed as mercury (EFSA, 2012).

Standardised methods are an indispensable element in guaranteeing high level of food and feed safety in international trade, and the same year as the expert consultation was carried out by the FAO, the European Commission issued standardisation mandate number M/422 rev. 1 through the European Standardization Committee (CEN). This mandate called for the development of a standardised method for the determination of MMHg in food of marine origin. In the autumn 2012 and spring 2013 the pre-trial and collaborative study was carried out with eight participating laboratories and the report was delivered to CEN in the late spring of 2013. The method got 100% agreement within the CEN enquiry and will now be sent to CEN TC 275 for voting. The method has also been listed as a potential standard method in codex (CCCF, 2013).

Several methods have been published on the determination of MMHg using different analytical techniques. Often a hyphenated technique is used, although simpler approaches are possible by *e.g.* using a direct mercury analyser (DMA). A collaborative study has recently been performed by JRC-IRMM for the DMA determination of MMHg (Cordeiro et al., 2014). For hyphenated techniques, it is common to use either GC or LC for the separation, although the former requires derivatisation/alkylation of the MMHg into volatile mercury species. Among the more common detectors used are MS, ICP-MS, MIP-AES, CV-AAS and CV-AFS (Ferreira et al., 2015; Uria & Sanz-Medel, 1998).

The method evaluated in this study uses GC–ICP-IDMS, which offers superior sensitivity and selectivity. Although the GC requires the MMHg to be derivatised, GC is much less prone to matrix effects due to co-eluting organic compounds compared to LC. The use of ICP as ionisation technique offers element-specific determinations and avoids the need for mathematical correction of the M + 1 ^{13}C interference seen in regular GC–MS instruments. Isotope dilution is a highly precise method since the quantification depends solely on measuring the isotope ratios of the analyte and does not involve the use of external standards (Inagaki et al., 2008; Yoshinaga & Morita, 1997).

2. Collaborative study

Participants in the collaborative study were recruited using different channels such as the NMKL newsletter and contact with instrument vendors, as well as through analytical networks and direct contact with potential participants. Twelve laboratories agreed to participate in the study, nine laboratories reported the results within the timeframe of this study and eight participants followed the draft of the method. The countries represented in the data for the final evaluation study were France, Germany, Spain and Sweden. The laboratories represented commercial laboratories, academic institutions, and government agencies, as well as research and development laboratories.

Table 1
List of test materials used and expected concentrations of total and methylmercury (dry matter).

Sample type	Food product	Total mercury, mg/kg	MMHg, mg/kg	% MMHg
Reference material[a]	Oyster tissue (NIST SRM 1566b)	0.0358–0.0384	0.0125–0.0139	36
Reference material[a]	Tuna fish muscle (ERM-CE464)	5.140–5.340	4.959–5.275	98
Reference material	Dogfish liver (NRCC DOLT-4)	2.360–2.800	1.210–1.450	52
Monitoring sample	Cod muscle	1.518–2.276	1.659–2.583	≈100
Industrial powder	Squid muscle	0.142–0.214	0.162–0.200	≈100
Industrial powder	Mussel tissue	0.044–0.066	0.0161–0.0393	≈50
Monitoring sample	Whale meat	1.148–1.722	1.456–1.532	≈100
Industrial powder	Crab claw meat	0.257–0.385	0.291–0.359	≈100
Monitoring sample	Greenland halibut muscle	2.702–4.052	3.206–4.506	≈100

[a] Samples used in pre-trail.

2.1. Study materials

The samples used in this study were selected to cover a wide range of MMHg concentrations including variations in percentage MMHg relative to total mercury, as well as differences in matrix compositions found in seafood (Table 1). No samples were spiked so the MMHg concentrations were incorporated in the samples as it would be in ordinary seafood. The test samples used in this study including the pre-trial were a mixture of monitoring samples from NIFES, certified reference materials, standard reference materials and industrial powders of seafood kindly donated by Seagarden (Seagarden ASA, Husøyvegen 278, Karmsund Fiskerihavn, 4262 Avaldsnes, NORWAY, www.seagarden.no). The certified reference materials and standard reference materials used were NIST SRM 1566b (oyster tissue), ERM-CE464 (tuna fish muscle) and NRCC CRM DOLT-4 (dogfish liver). The monitoring samples from NIFES (*i.e.* whale meat, cod muscle and Greenland halibut muscle) were homogenised and freeze-dried at NIFES according to an ISO 17025 accredited procedure. Certified reference materials and the industrial powders were homogenised. The test materials used in the collaborative study and pre-trial were all based on dry weight. For the samples of whale meat, cod muscle and Greenland halibut muscle about 0.3–0.9-g portions were weighed into PP cryotubes, and for the remaining samples 0.3–1.8-g portions were weighed into PP cryotubes. The cryotubes were sealed with parafilm before the cryotubes were put in another PP-tube, which was also sealed with parafilm and forwarded to the participants. All samples were analysed as replicates, and two of the samples (DOLT-4) were analysed as blind duplicates. All concentrations of MMHg in this study are given as Hg.

2.2. Statistical analysis

Twenty portions of each sample were selected for homogeneity testing. The homogeneity study was performed and evaluated according to IUPAC Harmonised protocol for Proficiency testing and demonstrated that the samples were homogeneous (Thompson, Ellison, & Wood, 2006). The method performance statistics and detection of outliers were performed according to the harmonised guideline for method performance studies by IUPAC (Horwitz, 1995) using an Excel program provided by AOAC; "International Interlaboratory Study Workbook", version 2.0 (2006). The excel program provided by AOAC calculates with the modified HorRat formula (Eq. (1)) (McClure & Lee, 2003):

$$\text{HorRatvalue} = \frac{1}{(10^{-k})^{-0.1505}} \left(\frac{S_R}{0.02\bar{X}^{0.8495}} \right) \quad (1)$$

where k is the is the exponent raising power to which 10 must be raised to obtain the mass fraction, S_R is the reproducibility standard deviation and $\bar{X}$ is the mean concentration. This expression is a modified version of the original expression suggested by Horwitz (Horwitz, 1995; McClure & Lee, 2003) and known as the Horwitz equation, an empirical formula to estimate RSD_R in an acceptable method performance study. The HorRat value should not exceed 2 (Horwitz, 1995). Cochran tests (repeatability within a laboratory) and Grubbs tests (reproducibility among laboratories) were used to identify outliers. Outliers were not included in the final statistical calculations.

2.3. Protocol

Prior to the collaborative study, the participants were given the opportunity to become familiar with the method in a pre-trial. The method has previously been validated in-house and more information including chromatograms produced using this method can be found there (Valdersnes, Maage, Fliegel, & Julshamn, 2012). The materials for the pre-trial consisted of two certified reference materials (Table 1). Oyster tissue was purchased from National Institute of Standards and Technology (NIST) SRM 1566b, and tuna fish muscle was purchased from Institute for Reference Materials and Measurement (IRMM) ERM-CE464. The oyster tissue contained MMHg at a certified concentration of 0.0132 mg/kg, and the tuna fish muscle had a certified value of 5.12 mg/kg. In addition to the two samples, the laboratories received a draft of the method, an ampoule of commercially available spike solution from ISC (Innovative Solutions in Chemistry S.L., Edificio Científico-Tecnoló gico, Campus de "El Cristo", 33006, Oviedo, SPAIN www.isc-science.com), and an estimate of the concentrations of total mercury in the samples. The results were reported to the organiser and evaluated before the test samples in the collaborative study were sent out. Seven laboratories reported results for the pre-trial. One laboratory reported problems to calculate the results using the isotope dilution equation, so it was decided to include a Microsoft® Excel® template for calculation when the test samples were sent out to the participants. One laboratory did not manage to quantify the oyster tissue due to sensitivity problems. All laboratories that reported results, managed to quantify the tuna muscle pre-trial sample with high content of MMHg. Some participants reported that the method was easy to read and straightforward to use. The method seemed to be manageable by both experienced technicians and technicians new to the isotope dilution technique. A critical point in the determination affecting the sensitivity of the method was found to be the concentration of the nitric acid used. Since concentrated nitric acid may vary between 65% and $\geqslant$69%, the amount of acid needed to adjust the pH to 5 before derivatisation must be calculated based on the actual acid strength of the concentrated nitric acid used. This comment was included in the revised draft of the method sent to the participants when the samples were sent out. The results were reported using a template sent out to the participants and in addition to the analytical results the participants were asked to give detailed information about the following: (1) any modification of the method, (2) sample preparation (*e.g.* buffer, acid, etc.), (3) GC-instrument, (4) ICP-MS instrument, (5) tuning procedure, and (6) analytical control system in use. Five of the laboratories were ISO 17025 accredited, one laboratory was ISO 9001 accredited and two laboratories were research laboratories and not accredited.

3. Method

3.1. Principle

The sample is spiked with an appropriate amount of Hg-isotope enriched MMHg and extracted using tetramethylammonium hydroxide (TMAH). After pH adjustment, derivatisation and extraction, the organic phase is analysed using GC–ICP-MS. The GC separates the different mercury species before MMHg is atomised and ionised in the high temperature of the ICP. The Hg-ions are extracted from the plasma by a set of sampler and skimmer cones and transferred to a mass spectrometer where the ions are separated by their mass/charge ratio and determined by a pulse-count and/or analogue detector. Calculation of the result is done using the areas found for the three Hg-isotopes 200, 201 and 202 and the isotope dilution equation.

3.2. Chemicals and reagents

The concentration of the trace elements in the reagents and water used must be low enough to not affect the results of the determination. Always, when using a multi-elemental method of

high sensitivity like ICP-MS, the control of the blank levels of water, acid and other reagents is very important. Generally ultrapure water complying with ISO 3696 grade 1 (*i.e.* electrical conductivity below 0.1 μS/cm at 25 °C) or ASTM D1193-91 (*i.e.* electrical resistivity above 18.0 MΩ cm at 25 °C) and acid of high purity, *e.g.* cleaned by sub-boil distillation, are recommended. Reagents should be of minimum *p.a.* quality where possible. Special facilities should be used in order to avoid contamination during the steps of preparation and measurement (*e.g.* uses of laminar flow benches or comparable clean room facilities). The following reagents and solutions are used in the protocol:

(a) MMHg stock solutions – Commercially available MMHg standard enriched in the ^{201}Hg-isotope with a concentration of 5.5 μg/g from ISC was used in this study. Other MMHg Hg-isotope enriched standards may also be available in suitable concentrations from other suppliers or may be prepared in-house and can be used by adjusting the method accordingly. The quality of the standards should be designed for use in isotope dilution methods. Stock solutions in diluted acid are preferred.
(b) Diluted MMHg stock solution – The concentration levels of the MMHg in the diluted stock solution may be chosen in relation to the type of samples analysed. It is important that all dilutions are done by weighing so that their accurate concentrations can be calculated. The following descriptions are given as an example.
  (i) MMHg ≈ 500 ng/g: Dilute 1 ml of the ^{201}Hg enriched MMHg stock solution from ISC with water to the mark of a 10 ml volumetric flask. Calculate the exact concentration using the concentration of the stock solution and the weights of the volumes used.
  (ii) MMHg ≈ 50 ng/g: Dilute 1 ml of the 500 ng/g ^{201}Hg enriched MMHg solution with water to the mark of a 10 ml volumetric flask. Calculate the exact concentration using the exact concentration of the 500 ng/g solution and the weights of the volumes used.
  (iii) MMHg ≈ 5 ng/g: Dilute 1 ml of the 50 ng/g ^{201}Hg enriched MMHg solution with water to the mark of a 10 ml volumetric flask. Calculate the exact concentration using the exact concentration of the 50 ng/g solution and the weights of the volumes used.
(c) Tetramethylammonium hydroxide (TMAH) – 25 wt% in water, minimum synthesis quality.
(d) Acetic acid – concentrated acetic acid with a density of 1.05 g/ml, minimum *p.a.* quality.
(e) Sodium hydroxide – minimum *p.a.* quality.
(f) Sodium hydroxide solution (0.1 mol/l) – Transfer 0.4 g sodium hydroxide to a 100 ml volumetric flask and add water to the mark.
(g) Sodium acetate – minimum *p.a.* quality.
(h) Sodium acetate/acetic acid buffer (pH 5) – Dissolve 41 g of sodium acetate in approximately 0.5 l of water. Adjust the pH of the solution to 5 by adding concentrated acetic acid dropwise while monitoring the pH of the solution with a pH-meter. Finally, dilute the solution to 1 l with water.
(i) Nitric acid – 65 %, with a density of approximately 1.4 g/ml. Suprapur quality. Other acid strengths may be used if the volume added to each tube is adjusted accordingly.
(j) Sodium tetraethylborate – minimum synthesis quality (98%).
(k) Sodium tetraethylborate solution (2%) – Dissolve 1 g sodium tetraethyl borate in 0.1 mol/l sodium hydroxide (solution f) *vide supra*, transfer to a volumetric flask and dilute to 50 ml with 0.1 mol/l sodium hydroxide.
(l) Hexane – minimum HPLC-quality.
(m) Optimising solution for the ICP-MS – Use the optimising solution for check and optimising procedures during set up of the ICP-MS.

3.3. Apparatus, equipment and gasses

(a) Analytical balance – capable of accurately weighing to 0.5 mg.
(b) Rotator – capable of 0.04 g (20 U min^{-1}).
(c) Centrifuge – capable of 1 200 g (4 k U min^{-1}).
(d) pH-meter.
(e) Laboratory ware – volumetric flasks glass or polypropylene tubes (10 ml) for samples GC-vials, pH paper.
(f) Inductively Coupled Plasma Mass Spectrometer (ICP-MS) – Mass spectrometer with inductively coupled argon plasma operating in a mass range from 5 amu to 240 amu. Using routine settings the mass spectrometer shall be capable to resolve 1 amu peak width at 5% peak height or better (resolution 300) with sufficient sensitivity.
(g) Argon – purity min. 99.999%.
(h) Gas chromatograph (GC) – GC capable of injection, injector heating, programmable column heating and heating of transfer line to ICP-MS.
(i) GC-column – capillary or preparative column capable of separating ethylmethylmercury from other mercury species (*e.g.* 30 m × 0.32 mm id analytical column with HP-5 – 5% phenyl methyl siloxane; film thickness: 0.25 μm may be used).
(j) Helium – purity min. 99.999%, Helium with 1–2% added xenon may be used for tuning of the GC–ICP-MS interface. Other tuning setups (*e.g.* ^{36}Ar^{40}A or ^{202}Hg) may also be used provided they are able to produce sufficient sensitivity for the determination.

3.4. Procedure

(a) Calculation of optimal spike amount to be added – Estimate/calculate the amount of spike to be added to the sample in order to avoid overspiking/underspiking of the sample. This must be done to achieve approximate matching of the ^{200}Hg, ^{201}Hg and ^{202}Hg isotopes when the sample is analysed. For the specified ^{201}Hg enriched MMHg spike solution from ISC this approximate matching is achieved when the amount of spike to analyte is 1–7. The calculation of spike amount in *g*, m_{Sp}, to be added to the sample of mass, m_S in grams (g) from the spike solution with the concentration, C_{sp} in ng/g to a sample with estimated MMHg concentration, $\hat{C}_S$ in ng/g is given in Eq. (2):

Amount of spike to be added to the sample :

$$m_{Sp} = \frac{\left(\frac{\hat{C}_S \times m_S}{7}\right)}{C_{Sp}} \quad (2)$$

If another spike solution with a different isotope distribution is used the calculations have to be adjusted accordingly. A spike solution with concentration, C_{Sp} giving an amount of spike to be added in the range 0.1–1 g should be used in the calculation. If the sample has been analysed for total mercury this value could be used as an estimate of MMHg in the calculation with a correction factor depending on the amount of MMHg of the total Hg. To ensure that the measurement is within acceptable error limits the matching of the isotopes should be as close as possible (Catterick, Fairman, & Harrington, 1998).
(b) Sample preparation – Weigh accurately 0.2 g of sample to be analysed in a polypropylene tube and spike with the appropriate amount of ^{201}Hg enriched MMHg. Add 3 ml TMAH to

the tube and mix the contents by *e.g.* rotating the mixture overnight or until the sample is completely dissolved and a homogeneous mixture is formed. After digestion, add 1 ml pH 5 sodium acetate/acetic acid buffer followed by 600 µl nitric acid and mix the solution using a swirl mixer. If other nitric acid strengths are used the volume to be added must be adjusted accordingly so that the pH is 5 after acid addition and mixing. Add 1 ml hexane and then 500 µl sodium tetraethyl borate to derivatise the sample. Cap the tube and mix the contents by *e.g.* rotating for 10 min before centrifugation at 1200*g* for 5 min. Transfer the hexane layer to a GC-vial and analyse the sample on the GC–ICP-MS instrument.

(c) ICP-MS settings – Use the instrument parameters described in the manufacturers operating manual. Tune RF-Power, gas flows and *x*,*y*,*z*-position according the instrument being used. Make sure that integration time is short enough to provide enough points per chromatographic peak depending on your GC-column. It is recommended to collect 12–20 points per chromatographic peak since shorter or longer integration times on the isotopes may influence the sensitivity and accuracy of the determination.

(d) Set up procedures for the GC–ICP-MS – Before starting routine measurements the following set up procedure should be done: Warm up the ICP-MS in full running mode for a minimum 20–30 min. Check mass resolution, mass calibration, sensitivity and stability of the system by the use of a suitable optimising solution. Adjust the ICP-MS to achieve maximum ion signals and both low oxide rates (*e.g.* <1%) and low rates of doubly charged ions (*e.g.* <3%) with the optimising solution. Optimise the flow rate of the cell gas(es) if a collision- or reaction cell instrument is used, in order to ensure sufficient reduction of polyatomic interferences. Check mass calibration and sensitivity for every range of resolution if a high resolution mass spectrometer is used. Tune the interface after the GC is connected to the ICP-MS by tuning the plasma parameters such as; *x*,*y*,*z* position of the torch, carrier gas flow, plasma RF-power and auxiliary gas flow. This may be done by using one or more Xe-masses (*e.g.* ^{124}Xe, ^{129}Xe or ^{132}Xe) or other equivalent methods capable of optimising the interface. If a sufficient sensitivity cannot be achieved simply by tuning the plasma parameters, the ion lens parameters may also be tuned. Run a MMHg standard to check that the instrument sensitivity and the integrity of the system are satisfactory.

(e) Mass bias – May be checked by running a solution of MMHg with known isotopic composition. Compare experimental ratios with theoretical ratios of ^{200}Hg, ^{201}Hg and ^{202}Hg. Mass bias should typically be less than 1%.

(f) Dead time correction – Determine the dead time of the detector using the procedure in the manual of the ICP-MS. A typical value for the dead time would be around 15–100 ns. Set up the instrument for automatic dead time correction. Alternatively the dead time can be calculated using procedures found in the literature (Nelms, Quetel, Prohaska, Vogl, & Taylor, 2001).

(g) Polyatomic interferences – These interferences are caused by the plasma gas, reagents and matrix present in the plasma. Since the ICP is connected to a GC, these interferences are not very likely for Hg when analysing samples.

(h) Check of Hg-isotope enriched MMHg spike solution – A check of the spike solution to verify the integrity of the spike with respect to isotopic abundances and concentration may be performed. Prepare a sufficiently concentrated sample of the spike, derivatise it and analyse for the different isotopes using GC–ICP-MS to verify the isotopic abundances. Run a concentration check by doing a reverse isotope dilution using a MMHg standard of natural isotopic composition and known concentration. To correct for any mass bias and/or detector dead time use the procedures *vide supra* in e) and f).

(i) Determination of samples – Analyse a low level sample to check that the sensitivity of the instrument and chromatographic separation is sufficient for the series of samples to be determined. Analyse instrument blank, reagents blank and samples and determine the peak areas of ^{200}Hg, ^{201}Hg and ^{202}Hg. Check regularly within suitable short intervals (*e.g.* after 5 or 10 samples), the blank solution and one mass bias solution. Test the system for wash out times using a sample with high MMHg concentration. Check the memory effect by blank control measurements after high count rates of mercury. Since there is no sample cleanup (*i.e.* removal of fat) pay attention to the condition of the GC-liner when running multiple samples with high fat content.

(j) Analytical quality control – Analyse blank solutions and reference samples of comparable matrix having reliably known contents of the element to be determined in parallel with all the series of samples for analytical quality control. Subject the reference samples to all the steps in the method, starting from the extraction. Check trueness by analysing relevant reference materials (SRMs/CRMs) and by participating in proficiency tests.

(k) Calculation – Calculate the content, C_S, as mass fraction, of MMHg in ng/g of sample, using the isotope dilution equation (Eq. (3)) (Rodriguez-Gonzalez, Marchante-Gayon, Alonso, & Sanz-Medel, 2005). Other versions of the same formula may also be used for calculation (Fassett & Paulsen, 1989; Lambertsson, 2005). The ion counts may be corrected if significant mass bias is found during the experiment (Snell & Quetel, 2005).

$$\text{Calculation of result}: \quad C_S = C_{Sp}\frac{m_{Sp}}{m_S}\frac{M_S}{M_{Sp}}\frac{A^b_{Sp}}{A^a_S}\left(\frac{R_m - R_{Sp}}{1 - R_m \cdot R_S}\right) \tag{3}$$

where C_{Sp} is the analyte concentration in spike solution, in ng/g, m_S is the mass of sample analysed, in g, m_{Sp} is the mass of added spike solution, in g, M_S is the molar mass of analyte in sample, in g/mol, M_{Sp} is the molar mass of analyte in spike solution, in g/mol, A^a_S is the percent of reference isotope a (^{200}Hg or ^{202}Hg) in sample, A^b_{Sp} is the percent of reference isotope *b* (^{201}Hg) in spike solution, R_S is the isotope ratio of Hg-isotope *a* and *b* in sample, see Eq. (4), R_{Sp} is the isotope ratio of Hg-isotope *a* and *b* in spike solution, see Eq. (5), R_m is the measured isotope ratio between isotope *a* and *b* in spiked sample, see Eq. (6):

$$R_S = \frac{A^b_S}{A^a_S} \tag{4}$$

$$R_{Sp} = \frac{A^a_{Sp}}{A^b_{Sp}} \tag{5}$$

$$R_m = \frac{N^a_m}{N^b_m} \tag{6}$$

Use both the ^{202}Hg/^{201}Hg and ^{200}Hg/^{201}Hg ratios measured for calculation and report the mean value of MMHg from these calculations. If the two values obtained are far apart, corrective measures should be taken (*e.g.* check the integration, mass bias and other potential sources of error).

Table 2
All results of the collaborative study for the determination of methylmercury (mg/kg dry matter).

Lab. No	Mussel tissue	Squid muscle	Crab claw meat	Dogfish liver[a] (NRCC DOLT-4)		Whale meat	Cod muscle	Greenland halibut muscle
1	0.021	0.185	0.313	1.998[b]	1.290	1.600	1.913	3.402
	0.017	0.177	0.306	1.294	1.254	1.380	1.994	3.343
2	0.030	0.165	0.296	1.384	1.295	1.460	2.010	3.583
	0.027	0.177	0.295	1.303	1.306	1.496	1.974	3.611
3	<LQ[c]	0.171	0.231	1.230	1.115	1.164	1.766	3.482
	<LQ[c]	0.180	0.263	1.351	1.107	1.340	1.640	3.449
4	0.043	0.180	0.318	1.388	1.420	1.560	2.104	3.705
	0.042	0.192	0.328	1.395	1.409	1.582	2.130	3.787
5	0.067	0.182	0.301	1.309	1.109	1.226	1.837	2.930
	0.059	0.203	0.267	1.273	1.150	1.291	1.919	2.687[b]
6	0.026	0.197	0.335	1.371	1.359	1.515	2.067	3.732
	0.027	0.183	0.337	1.326	1.418	1.558	1.986	3.629
7	0.030	0.186	0.346	1.451	1.421	1.588	2.012	3.845
	0.031	0.184	0.342	1.435	1.424	1.584	2.175	3.804
8	0.029	0.195	0.318	1.431	1.385	1.445	2.029	3.777
	0.036	0.201	0.313	1.427	1.359	1.477	2.066	3.540

[a] Blind duplicates of the same sample.
[b] Outliers according to Cochran's test.
[c] <LQ = below Limit of Quantification.

4. Results and discussion

4.1. Pre-trail and collaborative study

The mean result from the pre-trial on MMHg in oyster tissue from seven laboratories was 0.015 ± 0.002 mg/kg (1 SD; the certified value was 0.0132 ± 0.0007 mg/kg (95% uncertainty). The mean result for mercury in tuna muscle from the seven laboratories was 5.28 ± 0.44 mg/kg (1 SD); the certified value 5.12 ± 0.34 mg/kg (95% uncertainty). The results showed that the seven laboratories were well prepared to undertake the collaborative study.

The results of the collaborative study from the eight laboratories that followed the method are given in Table 2 and the method performance characteristics are given in Table 3. For mussel tissue, the sample with the lowest MMHg content, laboratory 3 did not manage to quantify the content due to sensitivity problems. After removal of this laboratory the HorRat value was calculated to 1.57, the highest HorRat in this study, but nevertheless lower than 2 which is the highest acceptable HorRat value. Squid muscle and crab claw meat gave HorRat values of 0.28 and 0.55, respectively, and no Cochran or Grubb outliers were detected for these samples. Statistical evaluation of the results for the dogfish liver (DOLT-4) revealed that the 1.998 mg/kg result from laboratory 1 was detected as a Cochran outlier and removal of this result gave a *z*-score of 0 (Table 4). Whale meat and cod muscle did not have any outliers and all results were included when calculating the method performance characteristics. This gave HorRat values of 0.64 and 0.50, respectively, for these two samples. For Greenland halibut muscle, which had the highest concentration of MMHg of the samples in this study (3.575 mg/kg (grand mean)), one result from laboratory 5 (2.687 mg/kg) was detected as Cochran outlier and removed. The HorRat value was then calculated to be 0.52. The relative standard deviation for repeatability (RSD_r) of the method for the determination of MMHg was estimated to be between 2.1% for Greenland halibut and 8.7% for mussel tissue. The relative standard deviation for reproducibility (RSD_R) was estimated to be between 5.8% for squid and 42% for mussel tissue.

4.2. Collaborators' comments

Laboratory 2 reported that they used glass tubes instead of polypropylene tubes.

4.2.1. Response to comments from laboratory 2

This has no effect on the determination of MMHg, but in our experience glass tubes may give a higher background for inorganic mercury in the determination.

Laboratory 6 reported that 600 μl of nitric acid was replaced by 200 μl of 37% hydrochloric acid and 1 ml of hexane was replaced by 1 ml of isooctane.

4.2.2. Response to comments from laboratory 6

The type of acid used is not critical since this is only used to obtain the required pH optimum for alkylation of the MMHg cation and possible species transformations are automatically corrected for by the isotope dilution technique. In our experience

Table 3
Method performance characteristics from the collaborative method study.

	Mussel tissue	Squid muscle	Crab claw meat	Whale meat	Cod muscle	Greenland halibut muscle
No labs	8	8	8	8	8	8
No labs after outlier rejection	7	8	8	8	8	8
No outlier labs	1[a]	0	0	0	0	0
No replicates after outlier rejection	14	16	16	16	16	15
No replicates rejected	2	0	0	0	0	1
Overall mean, mg/kg	0.035	0.185	0.307	1.454	1.976	3.575
Repeatability s_r, mg/kg	0.003	0.008	0.012	0.074	0.064	0.076
Reproducibility S_R, mg/kg	0.014	0.011	0.032	0.140	0.142	0.244
RSD(r), %	8.67	4.53	3.97	5.11	3.24	2.14
RSD(R), %	41.73	5.75	10.54	9.66	7.17	6.90
HorRat	1.57	0.28	0.55	0.64	0.50	0.52

[a] One laboratory was not able to quantify the sample.

Table 4
Results for trueness of MMHg in the collaborative study based on NRCC DOLT-4 (dogfish liver) expressed as *z*-score.

Statistics	MMHg
Found mean level (mg/kg)	1.329
Certified value (mg/kg)	1.33
S_R (mg/kg)	0.104
z-Score	0

iso-octane does not impose a significant change on the determination of MMHg but iso-octane may lead to less co-extraction of fat and is more HSE friendly compared to hexane.

Laboratory 7 reported that they used iso-octane instead of hexane and 5 ml of acetate buffer solution (pH 4.5).

4.2.3. Response to comments from laboratory 7

The increased amount of buffer may lead to a small change in the extraction efficiency, provided that the partitioning coefficient is unchanged, of dialkylated mercury into iso-octane due to increased partitioning of MMHg into the water phase. The change from pH 5 to pH 4.5 for the buffer solution used should not influence the determination significantly since the most efficient pH for ethyl transfer to the MMHg cation has been found to be between 4 and 7, with a maximum around pH 5 (Bloom, 1989; Rapsomanikis, 1994).

5. Conclusion

This collaborative study has demonstrated the performance characteristics for the proposed GC–ICP-IDMS method for the determination of MMHg in food of marine origin. Evaluation of the results from the collaborative study showed that both experienced laboratories and laboratories new to the methodology managed to produce good results using the draft protocol. Preliminary training was conducted with SRMs/CRMs and the participants were offered the possibility to use a standard calculation template provided with the samples. A critical point in order to be able to determine samples with low concentrations of MMHg is the adjustment of pH before derivatisation and the sensitivity obtained after tuning the instrument. Therefore, extra attention has to be paid to the concentration of the nitric acid used and the pH after pH adjustment when determining samples with low content of MMHg.

The HorRat values were below 1.0 for all test samples, except the mussel tissue, which was the sample with the lowest MMHg concentration, with a HorRat value of 1.6. Due to the very low concentration in the mussel tissue (grand mean 0.035 mg/kg) such a high HorRat value is not unusual. The trueness as *z*-score was calculated to be 0 for the determination of MMHg in NRCC DOLT-4 (dogfish liver). The method was hence applicable in the concentration range from 0.035 to 3.6 mg/kg. These results shows that the method is fit for purpose for the determination of MMHg in food of marine origin and produce robust and reliable results.

Based on the results from this study we recommend that the method should be adopted as a standard method for the determination of MMHg in samples of marine origin after minor modifications and clarifications as discussed above.

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