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Determination of methylmercury in marine biota samples: Method validation

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ABSTRACT

Regulatory authorities are expected to measure concentration of contaminants in foodstuffs, but the simple determination of total amount cannot be sufficient for fully judging its impact on the human health. In particular, the methylation of metals generally increases their toxicity; therefore validated analytical methods producing reliable results for the assessment of methylated species are highly needed. Nowadays, there is no legal limit for methylmercury (MeHg) in food matrices. Hence, no standardized method for the determination of MeHg exists within the international jurisdiction. Contemplating the possibility of a future legislative limit, a method for low level determination of MeHg in marine biota matrixes, based on aqueous-phase ethylation followed by purge and trap and gas chromatography (GC) coupled to pyrolysis–atomic fluorescence spectrometry (Py–AFS) detection, has been developed and validated. Five different extraction procedures, namely acid and alkaline leaching assisted by microwave and conventional oven heating, as well as enzymatic digestion, were evaluated in terms of their efficiency to extract MeHg from Scallop soft tissue IAEA-452 Certified Reference Material. Alkaline extraction with 25% (w/w) KOH in methanol, microwave-assisted extraction (MAE) with 5 M HCl and enzymatic digestion with protease XIV yielded the highest extraction recoveries. Standard addition or the introduction of a dilution step were successfully applied to overcome the matrix effects observed when microwave-assisted extraction using 25% (w/w) KOH in methanol or 25% (w/v) aqueous TMAH were used. ISO 17025 and Eurachem guidelines were followed to perform the validation of the methodology. Accordingly, blanks, selectivity, calibration curve, linearity (0.9995), working range (1–800 pg), recovery (97%), precision, traceability, limit of detection (0.45 pg), limit of quantification (0.85 pg) and expanded uncertainty (15.86%, $k=2$) were assessed with Fish protein Dorm-3 Certified Reference Material. The major contributions to the expanded uncertainty, i.e. 86.1%, arose from the uncertainty associated with recovery, followed by the contribution from fluorescence signal. Additional validation of the methodology developed was effectuated by the comparison with the values reported for MeHg in the IAEA-452 inter-laboratory comparison exercise.

1. Introduction

Mercury (Hg) occurs naturally in the environment. However, over the last decades the biogeochemistry of Hg has raised considerable attention, mainly due to the extremely high toxicity of methylmercury (MeHg). The latter is an alkylmercury species capable to permeate through biological membranes, thus bioaccumulating and biomagnificating throughout the trophic chain [1]. The main exposure in humans to MeHg is through consumption of fish and shellfish, which is currently causing a widespread concern [2]. Indeed, the World Health Organization (WHO) rates mercury as one of the top ten chemicals of major public health concern [3]. Accordingly, the Global Legally Binding Treaty coordinated by the United Nations Environment Programme (UNEP) was launched in early 2013 [4]. International organizations responsible for providing leadership on global health matters, e.g. WHO [5] and legislative bodies such the US Environmental Protection Agency [6], and the European Commission [7] have regulated on the maximum level of total mercury (THg) threshold authorized in seafood for human consumption. Nevertheless, to date, no legislation establishing maximum levels of MeHg in seafood has been issued. The European Commission recently acknowledged the need for EU regulation on MeHg [8]. Future regulations on MeHg will require the existence of recommended procedures for quantitative determination of alkylmercury species in marine samples. The current analytical challenge faced is the development and validation of reliable and selective methods for routine determination of MeHg, at low concentration levels, in a variety of marine matrices.

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To address the high selectivity and sensitivity requirements for reliable speciation of trace and ultra-trace levels of MeHg in marine samples, a general analytical trend is the application of hyphenated techniques, which couple a powerful separation method, namely gas chromatography (GC) [9,10], high-performance liquid chromatography (HPLC) [11], and capillary zone electrophoresis (CZE) [12], to a selective and elemental sensitive detection system, particularly atomic absorption spectrometry (AAS) [13], atomic fluorescence spectrometry (AFS) [10,14], microwave-induced plasma atomic emission spectrometry (MIP-AES) [15], inductively coupled plasma optical emission spectrometry (ICP-OES) [16], inductively coupled plasma mass spectrometry (ICP-MS) [9,17] and furnace atomization plasma emission spectrometry (FAPES) [18]. Each of the aforementioned techniques, some of them very sophisticated, has their own merits and advantages. Due to its low cost in analytical instrumentation and its high sensitivity in detection, GC–AFS stands as one of the most used methodologies in analytical laboratories and it is the basis for the EPA method 1630 [14,19,20]. In the said method, the derivatization reagent $NaBEt_4$ is used to convert MeHg and Hg^{2+} into the volatile species EtMeHg and Et_2Hg, respectively. The volatile species are then purged out from the aqueous matrix, preconcentrated onto a trap, thermally desorbed and transferred to a packed GC column. After separation on the column, the alkylated Hg species are pyrolized and detected by AFS. Capillary GC columns provide better peak shape, separation time and higher resolution over packed GC columns [21]. The major hindrance of capillary GC is lower column capacity than packed GC, which limits the amount of sample that can be injected and makes it incompatible with purge and trap preconcentration. This hampers the attainment of low detection limits, as reported by Taylor et al. [22].

Despite the many improvements achieved in the selectivity and sensitivity provided by most of the analytical techniques commonly used for MeHg analysis, sample preparation remains as the crucial step for Hg speciation [23,24]. The extraction procedure must be robust, fast, efficient, lead to reliable results and, more importantly, it must preserve the integrity of the original chemical species [25,26]. The most widely utilized extraction procedures are alkaline digestion, either with potassium hydroxide or tetramethylammonium hydroxide [14,27,28] and acid leaching using HCl [29,30], CH_3COOH [31] and HNO_3 [32]. Since mercury exhibits high affinity to sulfhydryl groups, leaching solutions containing cysteine and 2-mercaptoethanol have also been used [26]. Conventional heating, microwave- or ultrasound-assisted extractions procedures at room- or elevated-temperature have been described to isolate MeHg from marine matrices [28,33–35]. Owing to the ability of enzymes to act on specific chemical bonds, thereby avoiding alteration of the chemical forms of mercury, and generally milder and environment friendly conditions of pressure, temperature and pH, enzymatic hydrolysis has been propounded as a promising technique for the extraction of mercury species [11,35]. In this regard, the use of protease XIV [23], trypsin [36] and lipase [29] has been reported.

Method validation is an essential component of the measurement process that should be implemented to attain accurate, reliable, and comparable over time and space results. Some of the guidelines that exist for the validation of the measurement procedure are the ISO 17025 standard on “General requirements for the competence of testing and calibration laboratories” [37], the Eurachem Guide “Fitness for purpose of analytical methods” [38], the International Union for Pure and Applied Chemistry (IUPAC) guide on “Single method validation” [39] and the European Commission Decision on “Method validation for contaminants” [40]. Method validation, metrological traceability and measurement uncertainty are the three milestones to assess the quality of measurement results and the key concepts in the measurement science – metrology in chemistry. Uncertainty and traceability concepts are interlinked, as demonstrated by the definition of “metrological traceability” as the “property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty” [41]. This definition clearly shows that only results obtained with validated measurement procedure and having stated uncertainty, can be traceable to the common system of reference.

The uncertainty of the measures is often calculated considering the standard deviation of several repeated determinations. In this fashion, only uncertainty components arising from random effects are considered, thus leading to an underestimation of uncertainty. The overall analytical uncertainty is much larger and includes uncertainty components arising from systematic effects, such as components associated with corrections and reference standards. According to The Joint Committee for Guides in Metrology (JCGM) “Guide to the expression of uncertainty in measurement”, the uncertainty may be estimated from its components by using the rules for propagation of errors in order to combine them into total uncertainty [42]. Nonetheless, studies on measurement uncertainty associated to the determination of MeHg in marine samples have seldom been reported [43–46]. Particularly, to the best of our knowledge, no studies have been published performing the validation, according to the guidelines, of a method for the determination of MeHg, based on GC–Py–AFS.

Within this context, we present the method validation of a “fit-for-purpose” analytical procedure for the determination of MeHg in marine biota samples, which is based on alkaline extraction followed by aqueous phase ethylation, separation and detection by hyphenated gas chromatography interfaced to atomic fluorescence spectrometry via a pyrolyzer (GC–Py–AFS). Utmost care was placed on the full method validation. Accordingly, selectivity, linearity and working range of the calibration curve, limit of detection, limit of quantification, repeatability and reproducibility, as well as recovery and trueness (using a certified reference material, CRM) were systematically assessed. In addition, estimations of the individual uncertainty contributions of each parameter as well as the final expanded uncertainty have been performed. Demonstration of traceability of measurement results is also provided.

Moreover, the efficiency of different sample preparation procedures for the extraction of MeHg from marine samples, namely acid and alkaline leaching assisted by microwave and conventional heating, as well as enzymatic digestion, have been estimated and compared. Since matrix effects are responsible for a loss of accuracy in analytical measurements [27], special attention was paid to the matrix effects that may occur during the course of the extraction.

2. Material and methods

2.1. Apparatus and software

The analysis were accomplished with a dual trap desorption module TDM II interfaced to an Atomic Fluorescent Spectrometer (AFS) model III detector via a Hg speciation GC & pyrolysis module (Py). All the three modules were supplied by Brooks Rand Labs (Seattle, WA, USA). Ar 5.0 grade gas (Air Liquide, Paris, France) was attached to gas ports of the purge and trap unit via 1/8" Teflon tubing with in-line gold sands traps to remove Hg impurities in the gas. The dual trap desorption module controls both, the heating temperature (450–500 °C) and time (30 s) of Tenax traps (Brooks Rand Labs), as well as the carrier gas flow through the trap and detector. Species separation is accomplished with a packed column OV-3 (Brooks Rand Labs) kept in an isothermal heating oven at 36 °C. Thermal decomposition takes place in quartz packed pyrolytic column heated at approximately 750 °C. Argon 5.0 grade

gas was used as the carrier gas (17.3 mL/min). After thermal decomposition, mercury species are introduced to the detector as elementary mercury (Hg^0) and quantified. The AFS system is an extremely sensitive detector where the Hg^0 atoms, in an inert carrier gas stream, are excited by a source of UV radiation. Excitation and fluorescence occurs at a wavelength of 253.5 nm. Finally, data were acquired and processed by Mercury Guru Software version 4.0 supplied with the instrument.

A MARS microwave laboratory system (CEM, Arsay, France) equipped with temperature and pressure feedback controls was used for the extraction procedures. An optical fiber, which is introduced inside one sample vessel, regulates microwave power output to maintain a selected temperature parameter. The MARS system can extract up to twelve samples simultaneously. 1500 PSI vessels were used. A centrifuge (Jouan C4.12 Bench-model Centrifuge, Thermo Fisher Scientific, Villebon sur Yvette, France) and an oven (Memmert, Schwabach, Germany) were utilized for the sample preparation.

2.2. Chemicals and reagents

High quality deionized water (>18 MΩ cm) from Milli-Q Element system (Millipore, Bedford, MA, USA) was used throughout this work. Ultra-pure nitric and hydrochloric acids (Suprapur) and potassium hydroxide (pro analysi) were obtained from Merck (Darmstadt, Germany). Methanol for gas chromatography and an aqueous solution of 25% tetramethylammonium hydroxide (TMAH, traceselect ultra) were purchased from Fluka (Sigma-Aldrich, Steinheim, Germany). Reagent grade Protease XIV (from *Streptomyces griseus*) was obtained from Sigma-Aldrich. Phosphate buffer pH 7.2 was purchased from SCP Science (Courtaboeuf, France). A buffer solution (0.2 M) at pH 4.5 was prepared by mixing extra pure potassium acetate anhydrous (Merck) and acetic acid (plasma pure plus, SCP Science) in water. Certified reference material (CRM) DORM-3 (fish protein, certified value: 0.355 ± 0.056 mg/kg) was supplied by the National Research Council of Canada (NRCC, Ottawa, Ontario, Canada). CRM IAEA-452 (scallop soft tissue, certified value: 0.022 ± 0.004 mg/kg) produced in the IAEA Environment Laboratories (Monaco), was also used in the method validation process.

Stock calibration standard of methylmercury chloride (CH_3HgCl, 1 mg/L in 0.5% acetic acid, 0.2% hydrochloric acid) was obtained from Brooks Rand Labs. Working standard solutions were prepared gravimetrically weekly by diluting, in pre-cleaned Teflon vials, the stock calibration solution with a solution containing 0.5% (w/w) acetic acid, 0.2% (w/w) hydrochloric acid and Milli-Q water to a range of 0.1–100 μg/L, calculated as Hg. Working solutions were protected from light. The ethylating reagent was prepared by mixing 2 mL of 1.33 M sodium tetraethylborate ($NaBEt_4$) in tetrahydrofuran (THF) and 38 mL of 2% potassium hydroxide (KOH), both packed with an argon atmosphere and provided by Brooks Rand Labs, in order to obtain 1% $NaBEt_4$ solution in 2% potassium hydroxide. The ethylating reagent was divided into several 4 mL vials and frozen immediately. Vials were kept at -18 °C for short-term storage (up to 1 month) and thawed immediately prior to usage.

2.3. Sample treatment, extraction and clean-up

2.3.1. Extraction procedures

Five extraction procedures (herein denoted 1–5) were performed. Three subsamples of CRM IAEA-452 were prepared in each extraction procedure. Two procedural blanks were prepared along with the samples for quality assurance purposes. Samples were analyzed immediately after the extraction, in the same day.

In order to avoid risks of memory effects from previous experiments, digestion vessels were submitted to a supplementary hot cleaning procedure: 10 mL of concentrated HNO_3 were added to each vessel, a subsequent microwave treatment at 350 W for 15 min was performed and finally the vessels were thoroughly rinsed with Milli-Q water. All sample processing steps were performed in a clean bench class 100.

Procedure 1 for alkaline extraction with 25% (w/w) KOH in methanol [47]. A 0.25 g portion of IAEA-452 was accurately weighed in a pre-cleaned 30 mL Teflon vessel. Then, 10 mL of 25% (w/w) KOH in methanol solution were added. The Teflon vessels were immediately closed and shaken. The mixture was heated in an oven at 75 °C for 180 min. After the extraction, the resulting extracts were allowed to cool to room temperature and transferred to a 50 mL Teflon vessel. The final volume was adjusted to 50 mL with Milli-Q water. Finally, the vessels were manually shaken again and the mixture was allowed for sedimentation of un-dissolved particles. A 20 μL aliquot was taken from the upper layer of the solution.

Procedure 2 for microwave-assisted extraction (MAE) with 25% (w/w) KOH in methanol, modified from [48]. A 0.15 g portion of IAEA-452 and 6 mL of 25% (w/w) KOH in methanol were placed in a pre-cleaned 100 mL Teflon microwave vessel. The microwave vessels were sealed and irradiated at 70 °C for 8 min. A 3 min ramping time was used to reach the desired temperature of 70 °C. After microwave heating, the resulting extracts were allowed to cool to room temperature and then centrifuged at 3000 rpm for 20 min. A 30 μL aliquot was taken from the upper layer of the solution.

Procedure 3 for microwave-assisted extraction with aqueous 25% (w/v) TMAH [25]. A 0.1 g portion of IAEA-452 was accurately weighed into a pre-cleaned 100 mL Teflon microwave vessel. Then, 4 mL of 25% (w/v) TMAH were added to each sample vessel. The microwave and centrifugation conditions were the same as procedure 2. A 30 μL was taken from the upper layer of the solution.

Procedure 4 for microwave-assisted extraction with 5 M HCl [30]. A 0.5 g portion of IAEA-452 was accurately weighed in a pre-cleaned 100 mL Teflon microwave vessel. 10 mL of 5 M HCl and 0.25 M NaCl were added gravimetrically to each sample vessel. The microwave vessels were sealed and irradiated at 60 °C for 10 min. A 3 min ramping time was used to reach the desired temperature of 60 °C. After microwave heating, the resulting extracts were allowed to cool to room temperature and then centrifuged at 3000 rpm for 20 min. A 20 μL aliquot was taken from the upper layer of the solution.

Procedure 5 for enzymatic digestion with protease XIV, modified from [23]. A 0.2 g portion of IAEA-452 was accurately weighed in a pre-cleaned 30 mL Teflon vessel with 0.02 g of protease type XIV and 8 mL of 0.1 M phosphate buffer (pH 7.2) containing 0.05% (w/v) cysteine. The mixture was heated in an oven at 37 °C for 240 min while being agitated. After the digestion, the samples were manually shaken and allowed to cool to room temperature. A 20 μL aliquot was taken from the upper layer of the solution.

To address the validation of the analytical procedure, the described extraction procedures were also applied with CRM DORM-3.

2.4. GC–Py–AFS analysis

Instrumental analysis of MeHg with hyphenated GC–Py–AFS technique consists of the following steps.

2.4.1. Purge and trap sampling

An aliquot (10–30 μL) of the digested IAEA-452 was transferred to a 60 mL Teflon bubbler with of 20 mL Hg-free deionized water. The sample was buffered to pH 4.5 with 200 μL of 0.2 M acetic-acetate buffer solution. Then, 50 μL of 1% aqueous solution $NaBEt_4$ were added and the bubbler was immediately closed. The mixture

was allowed to react without bubbling for 15 min. The ethylation reaction results in the formation of volatile ethylmethylmercury and diethylmercury from reactive MeHg and inorganic mercury, respectively. After the reaction time, the solution was purged for 15 min at a flow-rate of 250 mL/min with Hg-free, N_2 5.0 grade gas (Air Liquide, Paris, France). The outflowing gas stream was passed through a Tenax trap, which adsorbs the volatile organomercury species. After the sample was purged, dry nitrogen is flushed through the Tenax to remove traces of condensed water vapor, which interfere the following chromatographic elution and AFS detection.

2.4.2. Chromatographic separation, pyrolysis and detection steps

The Hg species adsorbed onto the Tenax were released by thermal desorption into the GC column. Under a flow of argon, the eluted Hg species were converted into Hg^0 by thermal decomposition at 800 °C and then detected by AFS. Each sample was analyzed three times to enable statistical evaluation of the samples ($n=3\times3$). Quantification was based on peak areas by external calibration and standard addition.

2.5. Determination of the moisture content

Moisture content in samples is to some extent "operationally defined" measurant. Correction for moisture content was obtained from 3 biota subsamples with minimum sample mass 1.0 g. The drying procedure at 85 ± 2 °C was established after experimental evaluation of stability and reproducibility of results. The material was dried for 24 h in a ventilated oven at a temperature of 85 ± 2 °C . Then weighing and repeated drying was performed until constant mass was attained (0.0002 g difference between two successive weights). Each weighing had to be carried out after the sample reached thermal equilibrium at room temperature in a desiccator. The loss of mass corresponds to the "dry mass correction factor", which was applied for the estimation of the combined uncertainty.

2.6. Experimental set up of the method validation

Different experiments were set up in order to investigate the performance characteristics of the method in terms of linearity, repeatability, intermediate precision, limit of detection (LOD), limit of quantification (LOQ), trueness and stability of the extracts, procedural and instrumental blanks.

The validation experiments were performed on six different days. Independent samples were prepared on each single day. Some of the experiments were used in the estimation of different parameters. The calculation of the analyte mass fractions in the sample was based on Eq.(1), Table 3 and the response factors applied were determined using the average values. Analyses were carried out in a class-100 clean chemical laboratory, under the same conditions by a single analyst. Indeed, reagents and sample material were kept constant in the assessment. The sequence of work was as follows:

The procedural blank was prepared along with the samples in order to check cross contaminations during the validation study. It underwent the same analytical procedure as the biota samples without adding biota matrix. Instrumental (including trap's contribution) and procedural blanks were conducted on every single day during the validation study. Blank measurements (instrumental and procedural blanks) represented at least 10% of all determinations.

The calibration curve was prepared with ten different mass quantities, namely 1, 5, 10, 20, 40, 80, 160, 200, 400 and 800 pg. In order to achieve such low mass levels, the stock standard solution of 1 mg/L was diluted 3 times, up to 0.1 μg/L. They were measured at the beginning of the sequence, followed by the procedural blanks, and then the samples. The standard with the lowest MeHg content was randomly re-measured during the sequence to monitor for instrumental drift. A common approach to overcome the hindrance owing to nonlinearity is the application of the bracketing technique (Eq.(2), Table 3). First, a preliminary estimate of the analyte concentration in the test sample is obtained. Second, two calibration standards are then prepared at levels that bracket the sample concentration as closely as possible, i.e. the two calibrant mass fraction value (C_D) differs from the sample mass fraction value (C_{meas}) in a factor no greater than 20% and the mean response factors, derived from replicate determinations at each standard, does not vary in more than a $\pm20\%$

The repeatability was tested by analyzing six samples of the CRM Dorm-3 in the same day, whereas the intermediate precision (within-laboratory reproducibility) was evaluated by analyzing 3 samples on three different days. All the experiments were performed in triplicate. The absolute limit of detection (LOD) was calculated as the mean background noise in a procedural blank plus three times the standard deviation of the background in twelve replicates. The absolute limit of quantitation (LOQ) was calculated as the mean background noise in a procedural blank plus ten times the standard deviation of the background in twelve replicates.

The standard addition method was accomplished by spiking the samples with two and four times, i.e. 0.02 mg/kg and 0.04 mg/kg, the expected analyte concentration of the sample solution. CRMs were analyzed in three replicates measurements during three different days.

2.7. Evaluation of the measurement uncertainty

Combined standard uncertainties were obtained by propagating together individual uncertainty components according to the ISO GUM guide [42]. In practice, a dedicated software program was used [49], based on the numerical method of differentiation described by Kragten [50]. All uncertainties indicated in the final results are expanded uncertainties $U=ku_c$, where u_c is the combined standard uncertainty and k is a coverage factor equal to 2.

3. Results and discussion

3.1. Evaluation of the different sample preparation procedures

Five different procedures were evaluated in terms of their extraction efficiency for mercury speciation in marine biota matrices. The performance was appraised by the analysis of scallop soft tissue IAEA-452 certified for MeHg. As it has been mentioned before, reliable results are still mostly dependent on sample preparation. The most suitable extraction protocol should be capable to solubilize the mercury species from the matrix of interest without altering the original chemical form.

The extraction procedures assayed were somewhat independent and singular in their approaches to extract mercury species from the matrix (Table 1). Procedures 1–3 were based on the traditional alkaline saponification [51]. Particularly, 25% (w/w) KOH in methanol was utilized in procedures 1 and 2 with conventional oven heating and MAE, respectively. In procedure 3, microwave-assisted extraction with 25% (w/v) aqueous TMAH was used. Conversely, procedural 4 was based on acid leaching using 5 M HCl and MAE technique. Owing to the formation of chloro-complexes, HCl is more efficient than nitric or acetic acid to release protein-bound mercury species [52]. Consequently, HCl was the acid selected. Finally, procedure 5 consisted in extracting the mercury species by enzymatic digestion with protease XIV. The

Table 1
The extraction procedures evaluated.

Procedure	Extraction reagent	Extraction technique and temperature (°C)	Total procedure time (min)	References
1	25% (w/w) KOH in MeOH	Oven – 75	180	[47]
2	25% (w/w) KOH in MeOH	Microwave – 70	46	[48]
3	25% (w/v) aqueous TMAH	Microwave – 70	46	[25]
4	5 M HCl	Microwave – 60	48	[30]
5	Protease XIV	Oven – 37	240	[23]

Table 2
MeHg mass fraction, recovery and calibration methods in scallop soft tissue CRM IAEA-452 (MeHg as Hg: 0.022 ± 0.004 mg/kg) measured by GC–Py–AFS. EC: external calibration. BA: bracketing approach. SA: standard addition.

Procedure	MeHg (as Hg) (mg/kg)	Recovery (%)	quantification method
1	0.021 ± 0.001	97 ± 7	EC with BA
2	0.011 ± 0.001	49 ± 10	EC with BA
	0.020 ± 0.003	93 ± 14	SA
3	0.004 ± 0.0002	20 ± 2	EC with BA
	0.020 ± 0.002	92 ± 8	SA
4	0.021 ± 0.004	94 ± 10	EC with BA
5	0.022 ± 0.001	99 ± 3	EC with BA

enzymes offer the advantage, over traditional chemical reagents, of operating only on specific chemical sites, thus minimizing species interconversion [35]. The enzymatic digestion is critically dependent on the enzyme–substrate contact time. Accordingly, the extraction of MeHg was evaluated by digesting IAEA-452 for 2, 3 and 4 h with protease XIV at 37 °C, pH 7.2. The highest extraction yield, i.e. 99 ± 3%, was obtained after a 4 h incubation period.

Among the procedures, the use of MAE, either with 25% (w/w) KOH in methanol, aqueous 25% (w/v) TMAH or 5 M HCl (procedures 2, 3 and 4, respectively) resulted in the fastest methods (about 45 min), compared with traditional oven heating using 25% (w/w) KOH in methanol and enzymatic digestion (procedures 1 and 5, respectively), which require 3 and 4 h, respectively.

The analyses were carried out by GC–Py–AFS hyphenated technique. The results for the determination of MeHg concentration in IAEA-452 are summarized in Table 2. The results are provided as averages of three different triplicates $\pm$ standard deviation. To assess MeHg recovery, the experimental values were compared to those corresponding to the certified value (0.022 ± 0.004 mg/kg).

ANOVA one-way test revealed statistically significant differences in the results obtained by the five different procedures ($p < 0.05$). In order to study more deeply the differences between the five procedures, preliminary tests for the equality of variances were performed, followed by two-sample *t*-tests. Performing multiple *t*-tests increases the probability of finding an incorrect significance. To correct for this hindrance, the *p*-values for each of the pair-wise comparisons were multiplied by the number of comparisons (Bonferroni adjustment). Significant differences in MeHg recoveries were obtained for all the pair-wise comparisons, with the exception of those values obtained with procedures 1, 4 and 5 (alkaline extraction with 25% (w/w) KOH in methanol, MAE with 5 M HCl and enzymatic digestion with protease XIV, respectively).

The highest extraction recoveries, i.e. 99 ± 3%, 97 ± 7% and 94 ± 10%, were attained with procedures 5, 1 and 4, respectively. There was not a significant difference between these high recoveries and the certified value. Contrarily, procedures 2 and 3 (MAE using 25% (w/w) KOH in methanol or 25% (w/v) aqueous TMAH) yielded the lowest extraction recoveries, namely 49 ± 10% and 20 ± 2%, respectively. For procedures 2 and 3, significant differences were observed between them, as well as when compared with the certified value.

Similar studies have been conducted with fish tissue samples and HPLC–CP-MS or GC–AFS determination [23,28]. Reyes and colleagues [23] reported the best recoveries (99 ± 3%) with alkaline saponification procedures assisted by ultrasound. On the contrary, the highest extraction efficiency (97%) recounted by Cabenero-Ortiz et al., [28] was attained with 5 M HCl leaching. With respect to enzymatic digestion using protease XIV, the recovery of MeHg presented in this study is higher than the 86 ± 3% described in tuna fish tissue certified reference material (ERM-CE464) [23] and similar to that (95 ± 1%) reported by Rai and colleagues in dogfish muscle tissue (NRCC-Dorm2) [35]. To the best of our knowledge, the evaluation of different sample pre-treatment with scallop soft tissue as a common sample and aqueous ethylation-purge trap and GC–Py–AFS determination has never been reported.

Taking into account the evidence of no significant differences among the recoveries yielded by procedures 1, 4 and 5, as well as the appropriate time and easy experimental manipulation of oven heating-alkaline extraction with 25% (w/w) KOH in methanol, the latter was the procedure selected to carry out the validation and further use in our analytical practice.

3.2. Method validation

According to the ISO 17025 guideline, validation is "the confirmation by examination and the provision of objective evidence that the particular requirements for a specific intended use are fulfilled" [37]. In the present study the five possible approaches for method validation, recommended by the ISO 17025 standard were followed: systematic assessment of the factors influencing the final result; calibration using reference standards; comparison of results obtained with other methods; assessment of the uncertainty of the results based on scientific understanding of the theoretical principles of the method and practical experience and participation in inter-laboratory comparisons.

3.2.1. Instrumental and procedural blanks

The instrumental blank is critical to the reliable determination of MeHg at low levels. In this sense, besides the procedural blanks commonly performed in every validation process, special attention should be paid to trap blanks. During the course of the analyses carried out in this study, the presence of a peak at the beginning of the chromatogram (1.2 min) was detected (Fig. 1). Grinberg et al., [53] suggested that it corresponds to Hg^0 arising from the decomposition of other Hg species. Needless to say that, in this experiment, such peak was also present when MeHg was not loaded onto the traps (trap blank). To identify the source of the aforementioned peak, a number of successive trap blanks were effectuated. It was observed that the area of the peak decreased over time, returning again when MeHg was loaded onto the traps. We therefore postulate that the studied peak, effectively, corresponds to Hg^0 arising not only from the decomposition of other Hg species, but also, from inorganic Hg adsorbed onto the trap from the lab atmosphere. This does not impair the accuracy of the technique as the Hg^0 peak area is kept constant during the course of the analyses. Correction for instrumental blank was carried out at intensity (fluorescence signal) level before other calculations were performed.

The procedural blank was effectuated in identical way as the analysis of unknown samples. The correction for procedural blanks was carried out at concentration level, according to Eq. (3), Table 3. Procedural blank, which is an important parameter in the case of trace elements analysis, was found to be 0.00002% (below 1 pg) of the corresponding certified amount content for MeHg.

3.2.2. Selectivity

Selectivity is defined as the extent to which other substances interfere with the determination of a substance according to a given procedure [54]. Fig. 1 shows typical chromatograms of (a) MeHg standard, (b) CRM Dorm-3, and (c) procedural blank. Three peaks, corresponding to Hg^0 (t_R=1.2 min), MeHg (t_R=2.4 min) and inorganic mercury as Et_2Hg (t_R=4.9 min) species, are observed in all the three chromatograms. As can be seen, MeHg is the most prominent peak. Values for the separation factor (α) and resolution (R_s) were: Hg^0/MeHg (α=1.6; R_s=1.4), MeHg/Et_2Hg (α=2.1; R_s=1.9). Furthermore, it is important to note the absence of interfering peaks in the chromatogram corresponding to the CRM DORM-3 sample. This results show the selectivity of the analytical methodology.

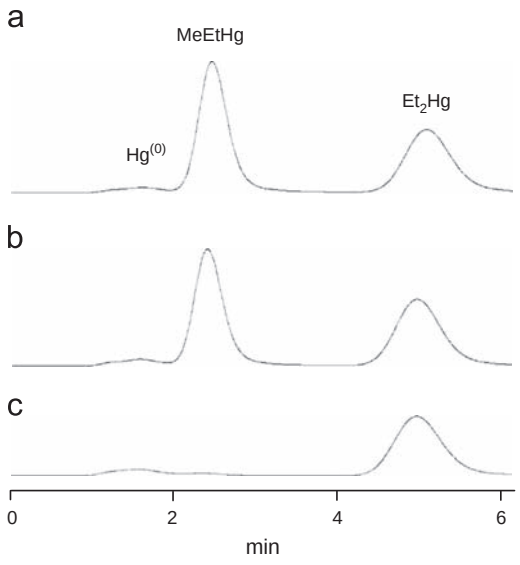

Fig. 1. GC–Py–AFS chromatograms obtained for (a) MeHg standard, (b) CRM Dorm-3 (fish protein), and (c) procedural blank.

3.2.3. Linearity and working range

For any quantitative method, it is necessary to determine the range of analyte concentrations or property values over which the method may be applied. This refers to the range of concentrations in the solutions actually measured rather than in the original samples. In the present study this test was performed with 10 different concentrations. At the lower end of the concentration range, the limiting factors are the values of the limits of detection and/or quantitation. At the upper end of the concentration range, limitations are imposed by various effects depending on the instrument response system. Within the working range a linear response range exists. The extent of the linear range of the calibration curve was established during the evaluation of the working range. The regression calculations on their own are insufficient to establish linearity. Additionally visual inspection of the line and residual analysis were also performed.

The working range was between 1 and 800 pg as introduced absolute mass. The external calibration linearity (R^2) was found to be 0.9995.

The heteroscedasticity of variance was evaluated by checking the increase of variance between the lower and the higher concentration levels of the calibration range using a one-tailed *F*-test for a confidence level of 95%. The ten calibration curves collected over the time were used to develop models of the variation of the standard deviation (s_i) of the concentration of MeHg with its concentration (γ_i) (s_i vs. γ_i) further used to define weighing factor of the weighed regressions. Approximately linear relations between s_i and γ_i were observed for the determination of MeHg. After this validation, calibration of GC–AFS was performed with duplicate indications of ten calibration standards and prior models of s_i vs. γ_i to define weighing factors.

3.2.4. Recovery and matrix effects

As stated above, procedures 1 and 2 are both based on alkaline extraction of the MeHg species from the matrix, using 25% (w/w) KOH in methanol. However, the application of oven heating or MAE led to significant differences in MeHg recoveries, with the former one

Table 3
Modeling approach for the calculation of combined uncertainty of MeHg mass fraction in biota sample.

Preparation of calibration standards

$$C_{D\_i} = C_M \times \frac{m_M}{(m_M + m_{d\_1})_1} \times \frac{m_1}{(m_1 + m_{d\_2})_2} \times \ldots \times \frac{m_{(i-1)}}{m_{(i-1)} + m_{d\_i\,i}} \quad (1)$$

Sample bracketing calibration and other corrections

$$C_{meas} = \frac{C_{D\_(i+1)} \times (A_S - A_i) + C_{D\_i} \times (A_{(i+1)} - A_S)}{(A_{(i+1)} - A_i)} \quad (2)$$

$$\overline{C}_{Blk} = \frac{1}{q} \times \sum_1^q C_{Blk} \quad (3)$$

$$C_{corr} = C_{meas} - \overline{C}_{Blk} \quad (4)$$

$$\overline{R} = \frac{1}{n} \times \sum_1^n \frac{[C_{CRM\_corr}]_n}{C_{CRM\_cert}} \quad (5)$$

$$C_{rec} = \frac{1}{p} \times \sum_1^p \frac{[C_{corr}]_p}{\overline{R}} \quad (6)$$

Corrections on signal intensities

$$A_{Std\_corr} = A_{Std\_meas} - A_{Std\_Blk} \quad (7)$$

Parameter		Index	
C, $\overline{C}$	Mass fraction, average mass fraction (mg MeHg/kg)	D	Working calibration standard
m	Mass (kg)	M	Stock solution
A	Peak area	i	Dilution step after dilution 1 ($i \geq 2$)
$\overline{R}$	Recovery	S	Sample
		CRM	Certified Reference Material
		Std	Calibration standard
		meas	Measured
		cert	Certified
		n, p, q	Number of repeats
		Blk	Procedural blank
		rec	Correction for recovery
		corr	Correction for procedural blank (inc. instrumental background)

yielding better results (Table 2). The occurrence of matrix effects during the course of the extraction of MeHg species with KOH solutions has been previously observed and discussed. In this regard, in a previous publication [27], we suggested that matrix effects could arise from sulphur-containing amino acids in fish tissue. Alike, low recoveries, attributable to high content of cysteine, were observed in erythrocytes samples [47]. A strong interference was perceived when analyzing TORT-2 (lobster hepatopancreas certified reference material) [32,55]. Hintelmann and Nguyen [32] postulated that such interferences most likely come from the organic matrix remaining in the sample digest. The alkaline digestion process is described as saponification, i.e., breakdown of fats into their corresponding fatty acids. It seems that when oven heating is applied, a better solubilization of tissues is achieved, carrying MeHg to the solution. Nonetheless, the use of MAE may leave functional groups (such as sulphur-containing) intact in the matrix. Those groups can interfere with the determination of MeHg. A similar trend, i.e. low MeHg recovery, was exhibited by MAE with 25% (w/v) aqueous TMAH (procedure 3).

To overcome the low recoveries, attributable to matrix effects, yielded by procedures 2 and 3, two approaches were effectuated: standard addition and the introduction of a dilution step. Standard addition method is widely used when analyzing complex environmental matrices [27,55]. In our case of study, three subsamples of IAEA-452 were spiked with MeHg standards at different concentration levels as indicated in the experimental section, and subsequently subjected to MAE with 25% (w/w) KOH in methanol. The recovery was found to be $93 \pm 14\%$. It is noteworthy that the concentration of MeHg measured in IAEA-452 by applying procedure 2 and standard addition calibration was statistically not different from the certified value. A similar result, i.e. $92 \pm 8\%$ recovery, was obtained after applying standard addition on procedure 3 (MAE with 25% (w/v) TMAH). This finding is in agreement with other studies in which standard addition calibration has been used [27,55,56], and demonstrates its power in compensating for such interferences.

Consistent with previous studies [32,55], further dilution of the digest should improve the MeHg recovery when strong matrix effects occur. Accordingly, a 1 mL volume of samples extracted by procedures 2 and 3 were diluted to 5 mL with high quality deionized water. 30 μL aliquots were taken for the posterior ethylation-GC–Py–AFS determination. The obtained values of recovery of MeHg from the IAEA 452 matrix were $97 \pm 10\%$ and $95 \pm 12\%$, for procedures 2 and 3, respectively.

Considering that no matrix effects were observed when applying the extraction protocol based on oven heating and 25% (w/w) KOH in methanol, this procedure was selected to assess the recovery of MeHg in Dorm-3. The comparison between the experimental and certified values (0.355 ± 0.056 mg/kg) yielded a recovery of $97 \pm 7\%$ ($n=12$).

The exact matching of sample and calibration solutions was also applied, which allowed the avoidance of the influence of matrix effects on the final measurement step.

3.2.5. Repeatability and within-laboratory reproducibility

The repeatability was evaluated by the application of the described measurement procedure on 6 subsamples, the same day; whereas the intermediate precision (within-laboratory reproducibility) was assessed by analyzing 3 subsamples on three different days. The relative standard deviations (RSDs) were found to be 5% and 8%, respectively.

3.2.6. Limit of detection and quantification

The absolute limits of detection (LOD) and quantification (LOQ) of the method were 0.45 pg and 0.85 pg, respectively. A review of the literature suggests that the LOD provided with this procedure based on alkaline extraction, aqueous-phase ethylation followed by purge and trap and GC coupled to Py–AFS detection is lower than the 0.6–2.0 pg range reported in a number of studies in which GC–Py–AFS was used [33,57–59], as well than the 1 pg value reported by Hintelmann and colleagues [60] for GC–ICP–MS. Other studies performed with GC–ICP–MS attained lower LOD (0.05 pg) than our value [61]. However, it is important to highlight that albeit GC–ICP–MS could offer lower detection limit than GC–Py–AFS, the latter stands as the most cost effective alternative. Such low LOD allows the direct determination of MeHg levels incurred in marine biota samples using the proposed measurement procedure.

3.2.7. Estimation of the measurement uncertainty

The estimation of combined uncertainty of measurement results was done with the modeling approach recommended by ISO GUM [42]. The entire measurement process is described by the set of equations presented in Table 3.

Two calibration strategies were compared: ten points linear regression and two points bracketing. The latter type of calibration (Eq.(2), Table 3), compared to the former, is in theory more advantageous in term of uncertainty propagation as (i) the measurement cycle is fast and the instrumental drift is minimized and (ii) the effect of instrumental non-linearity is insignificant.

There was a ~2% difference on calculated results from both calibration strategies applied, while the difference on the associated combined uncertainty estimations was not significant. Therefore, the uncertainty coming from the calibration step of the measurement procedure was estimated according to the Eq. (2), Table 3, which describes the application of bracketing approach.

All standard peak areas were corrected for the instrumental blank according to Eq. (4), Table 3. Each set of samples consisted of 3 procedural blanks and 3 biota samples. Calculations of the average procedural blank and the correction for it were carried out following Eqs. (3) and (4). Finally, the correction for recovery was applied as described in Eq. (5). Typically, the relative expanded uncertainty on the MeHg content in biota sample was found to be 15.86% ($k=2$). As expected, this uncertainty estimation was at least equal to (in fact larger than) the experimental standard deviation of the mean of all six replicate measurements. As illustrated in Fig. 2, the main uncertainty component (86.1%) was originated from the uncertainty coming from recovery, followed by the repeatability of the fluorescence signal (9.2%). The uncertainty of the reference value of the MeHg stock solution contributed with 2.2%. Finally, uncertainties coming from other factors and moisture determination accounted for 1.3 and 1.2%, respectively.

The transparency provided throughout this mathematical model, associated to the thorough estimation of combined uncertainties, contributes once again to the validation of the analytical protocols developed for this analytical procedure.

3.2.8. Traceability

A principal requirement exists in ISO 17025 [37] for laboratories to produce measurements that are traceable to a common system of measurement, SI system in this case, to ensure comparability of measurement results. A typical chemical measurement involves a number of steps as illustrated in Table 3. The way to demonstrate traceability is to use an uncertainty budget, where all the parameters influencing the final result are systematically assessed. Key steps in the attainment of traceability were as follows:

1. The analytical method used was properly selected and validated, both in terms of matrix composition and analyte concentration.
2. The unbroken chain linking the MeHg mass fraction to SI unit was described with the mathematical model. Table 3 presents the mathematical model of the analytical procedure, which is

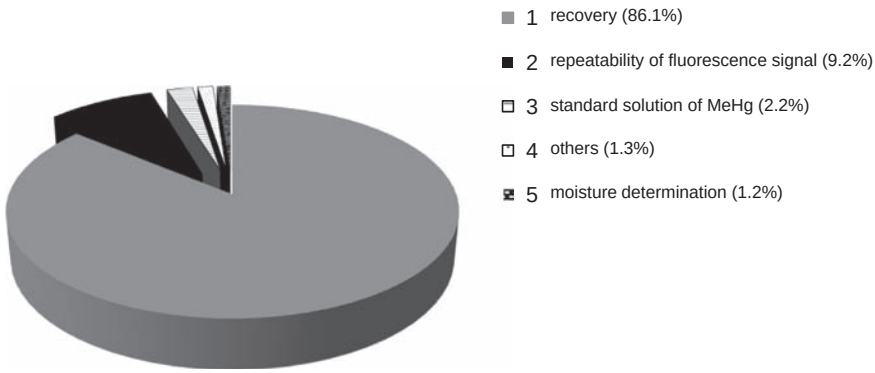

Fig. 2. Relative uncertainty contributions for each input quantity to the combined uncertainty.

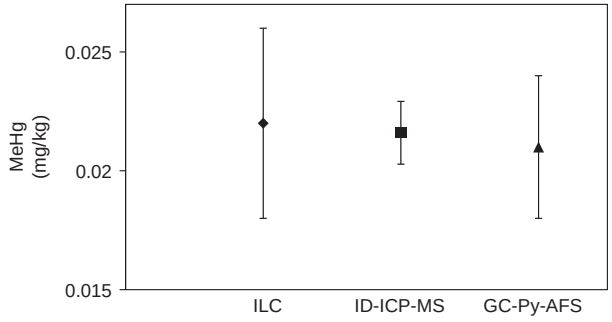

Fig. 3. Comparison of the MeHg mass fractions in CRM IAEA-452 obtained from: the participation in the IAEA worldwide interlaboratory comparison (ILC), the application of an independent method isotope-dilution inductively coupled plasma mass spectrometry (ID-ICP-MS) and the validated method proposed in this study (GC–Py–AFS).

completely understood. This model, together with sub-calculations or references to certified values, relates each of the input quantity to SI units of the mol or the kilogram.

3. The use of CRM for calibration and bias (recovery) estimation is the way to link MeHg mass fraction to the common system of reference SI. CRM with similar matrix was used also in the validation of sample preparation step.
4. The use of gravimetrical operation in the preparation of all working and standard solutions is a way to link the final measurement result to the kilogram.

3.2.9. Comparison with IAEA-452 assigned value

The last approach for method validation recommended by ISO 17025 was as well applied on our validation scheme. Accordingly, the results acquired by GC–Py–AFS were compared firstly with the assigned value assessed by the IAEA worldwide inter-laboratory comparison on trace elements and MeHg in biota [62], and secondly, with that attained by species specific ID-ICP-MS.

As demonstrated in Fig. 3, there was an excellent agreement within stated uncertainties between the above results, which is contributing for the further validation of the described analytical procedure for GC–Py–AFS determination of MeHg in biota sample based on external calibration.

In addition to this, two prospect IAEA CRMs for marine biota matrix, namely oyster and scallop, with reference values obtained with the primary method of measurement species specific ID-ICP-MS: 0.0048 ± 0.0002 mg/kg and 0.062 ± 0.002 mg/kg, respectively, were also analyzed. The MeHg concentration found by GC–Py–AFS in the said matrices was 0.0042 ± 0.0007 mg/kg and 0.066 ± 0.010 mg/kg, respectively, which was in good agreement to the reference values.

4. Conclusions

A method for the determination of MeHg in marine biota samples, based on aqueous ethylation derivatization, GC separation and Py–AFS detection has been developed. Five different sample preparation procedures were evaluated on the basis of the recovery yielded in the extraction of MeHg from CRM IAEA-452. Digestion with protease, alkaline saponification with 25% (w/w) KOH in methanol and microwave-assisted extraction with 5 M HCl resulted in the highest extraction efficiencies, namely 99, 97 and 94%, respectively. Matrix effects are a major problem when biological samples are analyzed. In the performance of this study, the occurrence of said effects was observed when microwave-assisted extraction using whether 25% (w/w) KOH in methanol or 25% (w/v) aqueous TMAH were utilized. Both, standard addition calibration and the introduction of a dilution step, proved to be successful tools to correct for the matrix effects. The validation of the methodology was effectuated according to the ISO 17025 and Eurachem guidelines. An uncertainty budget based on modeling ISO GUM approach was build, allowing also the quantification of the relative uncertainty contributions for each parameter in the measurement procedure and the determination of their relative contributions to the final combined uncertainty. The uncertainty budget was dominated by the uncertainty coming from recovery, reinforcing the importance of proper selection of the method of sample preparation. The proposed methodology could ultimately be a fit-for-purpose routine analytical method for the determination of MeHg incurred in marine biota samples.

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