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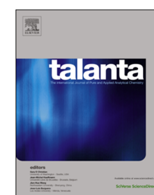

# Determination of methylmercury in marine biota samples: Method validation

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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.

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## 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

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