RESIDUES AND TRACE ELEMENTS

IMEP-115: Determination of Methylmercury in Seafood by Elemental Mercury Analysis: Collaborative Study

Fernando Cordeiro
European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, Retieseweg 111, 2440, Geel, Belgium
Josep Calderón
Laboratori Agència Salut Pública de Barcelona, Barcelona, Spain
Susana Gonçalves and Maria Helena Lourenço
Instituto Português do Mar e da Atmosfera, Lisbon, Portugal
Piotr Robouch, Hakan Emteborg, Patrick Conneely, Marie-France Tumba-Tshilumba, and Maria Beatriz De la Calle
European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, Retieseweg 111, 2440, Geel, Belgium

A collaborative study IMEP-115 was organized by the European Union Reference Laboratory for Heavy Metals in Feed and Food (EURL-HM) to validate a method for the determination of methylmercury in seafood. The method was based on a liquid–liquid extraction with an organic solvent and with an aqueous cysteine solution. The final quantitation was done with an elemental mercury analyzer. Fifteen laboratories experienced in elemental mercury analyses, from 10 European countries, took part in the exercise. Five test items were selected to cover the concentration range from 0.013 to 5.12 mg/kg. All test items were reference materials certified for the methylmercury mass fraction: DOLT-4 (dogfish liver), TORT-2 (lobster hepatopancreas), SRM 2974a (mussel), SRM 1566b (oyster), and ERM CE-464 (tuna). Participants also received a bottle of ERM CE-463 (tuna) to test their analytical method before starting the collaborative study. Method validation showed adequate accuracy and acceptable precision for all test items, thus fitting its intended analytical purpose. The repeatability RSD ranged from 3.9 to 12.3%, while the reproducibility RSD ranged from 8.4 to 24.8%.

Mercury is an environmental contaminant present in fish and seafood mostly in the form of methylmercury. According to the Scientific Opinion of the European Food Safety Agency (EFSA) published in 2012 (1), the major source of methylmercury intake in humans is fish meat followed by fish by-products. Specifically, large predatory fish at the top of the food chain, such as swordfish and tuna, were reported to contain levels of methylmercury of the order of 1 mg/kg. However, methylmercury levels in fish muscle may exceed 10 mg/kg for species living in industrially contaminated waters (2), and this poses significant risk to human health. Methylmercury can accumulate 100-fold in fish muscle (compared with the respective environmental aquatic level), and this can lead to dangerously elevated levels of mercury in seafood even in regions with typical aquatic mercury levels (3).

According to EFSA (1), contrary to inorganic mercury, methylmercury is able to enter the hair follicle and cross the placenta, as well as the blood–brain and blood–cerebrospinal fluid barriers, allowing accumulation in hair, fetus, and brain. The exposure of young children to methylmercury is an intermediate case between fetus and adults because their nervous systems are still developing and they are more sensitive to these substances than adults (4).

In 2003, the Joint Food and Agriculture Organization of the United Nations/World Health Organization Expert Committee on Food Additives established a Provisional Tolerable Weekly Intake of 1.6 µg/kg body weight (1). The U.S. National Research Council established an intake limit of 0.7 µg/kg body weight per week (5). So far, no maximum limit has been introduced for methylmercury in the European legislation for contaminants although the European Commission recommends pregnant or breastfeeding women and children to limit their consumption of large predatory fish.

From an analytical point of view, methylmercury determination is frequently performed by coupling GC (6) or HPLC (7) to different detectors such as electron impact-MS (8), inductively coupled plasma-MS (9), microwave induced plasma-atomic emission spectrometry (10), cold vapor-atomic absorption spectrometry (CV-AAS; 11) or CV-atomic fluorescence spectrometry. When GC is used for the separation of the species, derivatization of methylmercury is needed to convert the compounds into volatile species. Grignard reagents (9), sodium tetraethylborate and sodium tetraphenylborate (12) are frequently used as derivatizing agents (13). Papers summarizing and discussing the different analytical approaches used to determine methylmercury have been published (12, 14). So far, three analytical methods have been standardized for the determination of methylmercury in seafood, all of them, based on the use of GC or LC (15).

The European Commission's General Directorate for Health and Consumers requested the European Union Reference Laboratory for Heavy Metals in Feed and Food (EURL-HM) to validate a method that could be used by laboratories performing many methylmercury analyses per year without requiring the use of sophisticated hyphenated techniques.

The EURL-HM chose to validate an existing method based on a selective extraction of organic mercury species with hydrobromic acid followed by a double liquid–liquid extraction,

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Received July 8, 2013. Accepted by AK September 17, 2013.
Corresponding author's e-mail: Fernando.cordeiro-raposo@ec.europa.eu
DOI: 10.5740/jaoacint.13-235

Table 1. Statistical data evaluation (scrutinizing for outlier identification)

Sample	Laboratory	No. of outliers (replicates)	Outlier type
SRM 1566b	L04[a]	6	Cochran
	L06[a]	6	Cochran
	L12[b]	6	
SRM 2974a	L06[a]	6	Cochran
	L11[a]	1	Grubbs internal (lowest)[c]
	L12[b]	6	
TORT-2	L06[a]	1	Grubbs internal (lowest)[c]
	L12[a]	6	Cochran and Grubbs
DOLT-4	L12[a]	6	Cochran and Grubbs
ERM CE464	L06[a]	6	Cochran and Grubbs

[a] The test statistic is greater than its 1% critical value and the laboratory (or the single replicate value) is considered as an outlier.

[b] L12 reported < LOQ.

[c] Grubbs internal outlier refers to a single replicate being statistically significantly different ($p < 0.05$) from the other replicates within the same laboratory.

first with an organic solvent and then with a cysteine solution. The final quantitation is performed using an elemental mercury analyzer (16). The method is a single purpose AAS for the direct determination of mercury in solid and liquid samples. It is based on sample drying and thermal decomposition followed by an electro-thermal atomization of mercury. A gold amalgamator selectively traps and pre-concentrates the mercury, which is detected by the AAS at a specific wavelength (253.7 nm).

This analytical method was successfully implemented by the Portuguese National Reference Laboratory for the analysis of heavy metals in fish (Instituto Português do Mar e da Atmosfera; 17), and is currently included in the scope of accreditation of the Laboratori Agència Salut Pública de Barcelona. This paper presents the outcome of the collaborative study IMEP-115, organized by the EURL-HM, to validate the previously mentioned method.

Collaborative Study

Preparation, Packaging and Storage of the Test Items

Five reference materials certified for their methylmercury mass fraction (CRM) were used as test items in IMEP-115 to cover a wide concentration range in food samples: DOLT-4 [dogfish liver, National Research Council Canada (NRCC), Ottawa, Ontario, Canada]; TORT-2 (lobster hepatopancreas, NRCC); two standard reference materials, SRM 2974a [mussel tissue, National Institute of Standards and Technology (NIST), Gaithersburg, MD], and SRM 1566b (oyster tissue, NIST); and a European Reference Material, ERM CE-464 (tuna, EC-JRC-IRMM). The corresponding certified methylmercury mass fractions are listed in Table 1.

All test items included in the study were processed before dispatch. Each CRM was homogenized and bottled after thorough cleaning of the whole equipment to avoid any cross-contamination from the previous CRM. The supplied units of each CRM were opened, pooled into a 5 L acid-washed plastic drum and placed in a 3D-mixer for 30 min (Dynamix CM200, WAB, Basel, Switzerland) for careful mixing and re-homogenization. A handful of Teflon balls were added during mixing of the TORT-2 material to break up agglomerates, because the material was found to be severely clogged upon delivery. Each CRM was then refilled into vials using a vibrating feeder and a weight balance and using a high-efficiency particulate air filter clean-cell. Vials were relabeled to avoid easy identification by the participants. Vials containing 2.5 g of SRM 2974a or 5 g of all other CRMs were then dispatched to participants.

Before starting the collaborative trial, a "pre-test" item was sent to the registered participants to allow them to test and properly implement the method to be validated. The pre-test material consisted of the ERM EC-463 (EC-JRC-IRMM) reference material certified for the methylmercury mass fraction in tuna fish, a matrix similar to the other CRMs included in the collaborative study. The pre-test item was not processed but the bottles were relabeled to avoid any identification by the participants.

Homogeneity and Stability Tests

The homogeneity and stability of the five CRMs documented in the respective certificates were considered suitable for the purpose of the collaborative study. No additional homogeneity and stability studies were conducted.

Organization of the Collaborative Study

This validation exercise was announced via the International Measurement Evaluation Program web page (18), and to the National Reference Laboratories belonging to the EURL-HM network. Fifteen participants from 10 different EU member states registered for the exercise.

Each participant received two bottles for each CRM, one bottle of the pre-test item, together with a "sample accompanying letter," a "confirmation of receipt" form, and a copy of the standard operating procedure (SOP) to be strictly implemented and followed.

The sample accompanying letter described the measurand, the number of independent replicates required (three independent measurements per bottle, under repeatability conditions, on two different days; one bottle/day), and detailed instructions for the moisture determination and how to report results. Results should have been reported referring to dry mass, thus corrected for the moisture content.

Participants received an individual code to access the on-line reporting interface, to report their measurement results and to complete a questionnaire. The questionnaire was used to extract all relevant information related to the measurements and to the laboratory expertise.

At first, participants were asked to analyze the pre-test item and to report the results obtained. If the reported result was in agreement with the certified value (2.83 ± 0.32, $k = 2$, in mg/kg), the laboratory was entitled to start the analysis for the collaborative study. If a significantly biased value was reported,

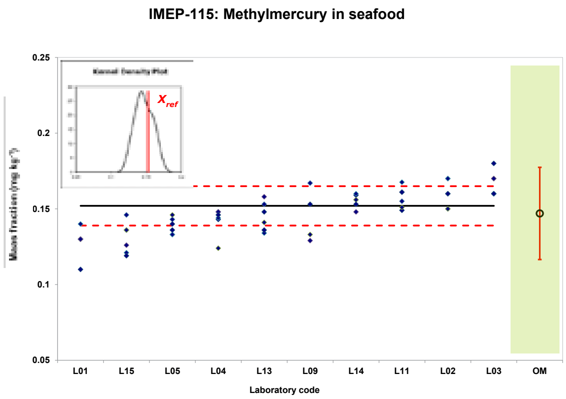

Figure 1. Overall performance for TORT-2 for methylmercury in seafood (IMEP-115). All reported results for each independent replicate are presented. The solid line and the dashed lines refer to the certified range (0.152 ± 0.013 mg/kg, k = 2). OM refers to the overall mean (0.147 ± 0.030 mg/kg).

the laboratory was requested to initiate a root cause analysis investigation and take proper corrective actions.

Standard Operating Procedures (SOP)

The SOP of the method under validation was drafted by the Laboratori Agència Salut Pública de Barcelona, based on a protocol proposed/developed by the Instituto Português do Mar e da Atmosfera. The final SOP provided to participants can be downloaded from the EURL-HM webpage. The SOP contained a comprehensive description of all operational procedures, including sample preparation (liquid and solid samples), and instrumental and method response calibration, for the internal quality control assurance.

Statistical Analysis

The statistical evaluation of the data was performed following the recommendations of the ISO 5725-2:1994 standard (19). AOAC INTERNATIONAL harmonized guidelines for collaborative study procedures to validate characteristics of the analysis methods (20, 21) were also followed as a cross-validation for the data evaluation. The following tests were performed:

(**a**) Analysis of variance, to confirm that no statistically significant difference existed, for any of the test items, between the two individual bottles provided to the participants, analyzed on different days. Because this was the case, all six replicated measurements were pooled for further statistical analysis.

(**b**) Check for outliers in the laboratory precision (variance), applying the Cochran test. This test compares the highest laboratory internal repeatability variance with the sum of reported variances from all the participants.

(**c**) Check for laboratory outliers within the series of independent replicates, applying the Grubbs-internal test (repeatability).

(**d**) Check for outliers in the laboratory mean, applying the Grubbs test. This test checks for laboratory means deviating significantly from the total mean calculated from data reported from all participants.

For both statistical tests (Cochran and Grubbs), results were compared with their respective critical values at $1\%_{cv}$ (99% confidence level) and $5\%_{cv}$ (95% confidence level), as provided in ISO 5725-2. Three cases may happen:

$$\text{C or G} \leq 5\%_{cv} \rightarrow \text{the tested item is accepted;}$$

$$5\%_{cv} < \text{C or G} \leq 1\%_{cv} \rightarrow \text{the tested item is identified as a straggler;}$$

$$\text{C or G} > 1\%_{cv} \rightarrow \text{the tested item is identified as a statistical outlier.}$$

Method performance characteristics related to the method precision were estimated after the identification and elimination (when relevant) of outlier results.

Results and Discussion

Collaborative Study Results

Twelve laboratories submitted results to IMEP-115. They are presented in the report to participants EUR 25830 EN (18). Figure 1 shows the results submitted by the laboratories (excluding L06 and L12) for the determination of methylmercury in the TORT-2 samples.

Laboratory L12 submitted results that were identified as outliers for DOLT-4 and TORT-2 samples and reported "lower than X" values for the SRM 2974a and SRM 1566b samples (with X referring to its LOQ). Similarly, laboratory L06 was identified as an outlier for SRM 2974a, SRM 1566b, and ERM CE-464 samples and a single replicate value was identified as an outlier when compared to its respective average (Grubbs-internal) for TORT-2. Hence, the performance of these two laboratories was considered unsatisfactory and the corresponding results were not included in the final data treatment (18, 19).

Table 1 provides an overview of the identified outliers for all test samples. Laboratories having reported within-laboratory variability significantly larger than that of the remaining laboratories were identified as Cochran outliers (C). Laboratories for which their calculated mean (of its corresponding six replicates) was identified as a Grubbs outlier (G) with a 99% confidence level or, for which one single replicate was found as a "Grubbs internal" outlier (GI), were also identified. All identified outliers were excluded from the final data treatment. The remaining results were used to evaluate the performance characteristics of the method under investigation, related to accuracy and precision.

Table 2 summarizes the main performance characteristics of the investigated method:

(**a**) the number of laboratories used to assess the performance characteristics of the method (after outlier exclusion);

(**b**) the number of outlier laboratories and replicates;

(**c**) the certified values of the test items and their associated expanded uncertainties (X_{ref}, U_{ref});

(**d**) the overall observed mean (after the outlier rejection, X_{obs}) and their respective expanded uncertainty, expressed as the reproducibility standard deviation (S_R) multiplied by a coverage factor of 2, which approximates to a 95% confidence interval;

(**e**) the repeatability SD (S_r), the repeatability limit r computed as $2.8S_r$, and the repeatability relative standard deviation, or within-laboratory variability (RSD_r);

Table 2. Method performance characteristics computed according to ISO 5725-2[a]

Variable	Unit	SRM 1566b	SRM 2974a	TORT-2	DOLT-4	ERM CE464
No. of laboratories[b]	—	9	10	10	10	10
No. of outlier lab & test used	—	2C[c]	1C	1C/1G[d]	1C/1G	1C
No. of replicates excluded	—	12	6 + 1GI	6 + 6 (L06)	6 + 6 (L06)	6
$X_{ref} \pm U_{ref}$ ($k = 2$)	mg/kg	0.0132 ± 0.0007	0.069 ± 0.0008	0.152 ± 0.013	1.33 ± 0.12	5.12 ± 0.34
$X_{obs} \pm 2S_R$	mg/kg	0.019 ± 0.010	0.071 ± 0.016	0.147 ± 0.030	1.09 ± 0.38	4.47 ± 0.76
S_r	mg/kg	0.0023	0.0050	0.0092	0.061	0.18
r	mg/kg	0.0065	0.014	0.026	0.17	0.49
RSD_r	%	12.3	5.1	6.3	5.6	3.9
S_R	mg/kg	0.0047	0.0081	0.015	0.19	0.38
R	mg/kg	0.013	0.023	0.043	0.52	1.05
RSD_R	%	24.8	11.5	10.4	17.1	8.4
HorRat	—	0.85	0.48	0.49	1.08	0.66
E_N	—	0.6	0.1	–0.2	–0.6	–0.8
$R_A \pm 2u_R$	%	143.1 ± 71.4	103.3 ± 23.6	96.7 ± 21.7	82.3 ± 29.0	87.3 ± 15.8

[a] Reference 19.

[b] After outlier rejection.

[c] C = Cochran test.

[d] G = Grubbs test applied to laboratory means.

(**f**) the reproducibility SD (S_R), the reproducibility limit R computed as $2.8S_R$, and the reproducibility RSD (RSD_R);

(**g**) the HorRat ratio expressed as the ratio between the observed RSD_R divided by the estimated relative standard uncertainty for repeatability according to the Horwitz equation (22);

(**h**) the E_N number (23) computed as:

$$E_N = \frac{X_{obs} - X_{ref}}{\sqrt{(4S_R^2 + U_{ref}^2)}}$$

(**i**) the overall analytical recovery R_A, calculated as:

$$R_A = \frac{X_{obs}}{X_{ref}} \times 100$$

(**j**) the associated uncertainty of R_A (u_R) (24), estimated as:

$$u_R = R_A\sqrt{\left(\frac{S_R}{X_{obs}}\right)^2 + \left(\frac{u_{ref}}{X_{ref}}\right)^2}$$

Four observations can be drawn from the data provided in Table 2. The absolute values of the E_N numbers for all samples are always below one. This indicates no significant differences between the observed and certified distributions characterized by ($X_{obs} \pm U_{obs}$) and ($X_{ref} \pm U_{ref}$), respectively. Secondly, all the HorRat ratios are below 2, which proves the fit-for-purpose of the method investigated. All of the analytical recovery ranges presented with their corresponding 95% confidence intervals ($R_A \pm 2\ u_R$) bracket the theoretical values of 100%, indicating once more that no significant bias can be identified for any of the test items investigated. Moreover, the precision, expressed as S_r, shows a linear relationship versus the mass fraction (m) for the whole investigated range, and can be estimated as $S_r = 0.035$ m, with a coefficient of determination, r^2, of 0.995. Finally, the reason for the precision obtained at the lowest end of the investigated working concentration range (0.013 mg/kg) having a larger RSD_R of 24.8%, was due to the fact that this concentration level approaches the LOQ of the proposed analytical method (0.010 mg/kg), as established during preliminary single laboratory validation studies (18).

While most of the laboratories strictly implemented the SOP under investigation, few minor experimental modifications were reported to the ring-trail organizer (listed in the report to participants; 18). Laboratory L02 reported having used L-cysteine standard solutions for all samples except for the calibration standards. As no significant matrix effects were observed while measuring the DOLT-4 samples, the Advisory Board for the collaborative study decided not to exclude the results from this participant from the final evaluation of the performance characteristics of the method.

Conclusions

The method for the determination of methylmercury by elemental mercury analyzer after a double liquid–liquid extraction was validated for the analysis of different seafood samples. When applying the SOP, the method showed adequate accuracy and precision for the methylmercury mass fractions ranging from 0.013 to 5.12 mg/kg. This method complies with the requirements laid down in the European legislation.

Acknowledgments

Franz Ulberth and Yiannis Fiamegos, Institute for Reference Materials and Measurements (IRMM), Geel, Belgium, are thanked for revising the manuscript. Bibi Kortsen (IRMM) is acknowledged for the logistic support. The following laboratories that participated in this exercise are also acknowledged:

Centre d'Étude et de Recherches Vétérinaires et Agrochimiques, Tervuren, Belgium

Federal Agency for the Safety of the Food Chain, Gentbrugge, Belgium

State Veterinary Institute Olomouc, Kromeriz, Czech Republic

Central Institute for Supervising and Testing in Agriculture, Brno, Czech Republic

Laboratoire SCL de Bordeaux, Bordeaux, France

Service Commun des Laboratoires, Montpellier, France

Istituto Zooprofilattico Sperimentale, Turin, Italy

National Institute of Public Health—National Institute of Hygiene, Warsaw, Poland

Instituto Português do Mar e da Atmosfera, Lisbon, Portugal

Laboratorio Arbitral Agroalimentario, Madrid, Spain

Centro de Salud Publica de Alicante, Alicante, Spain

Laboratori Agència Salut Pública de Barcelona, Barcelona, Spain

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