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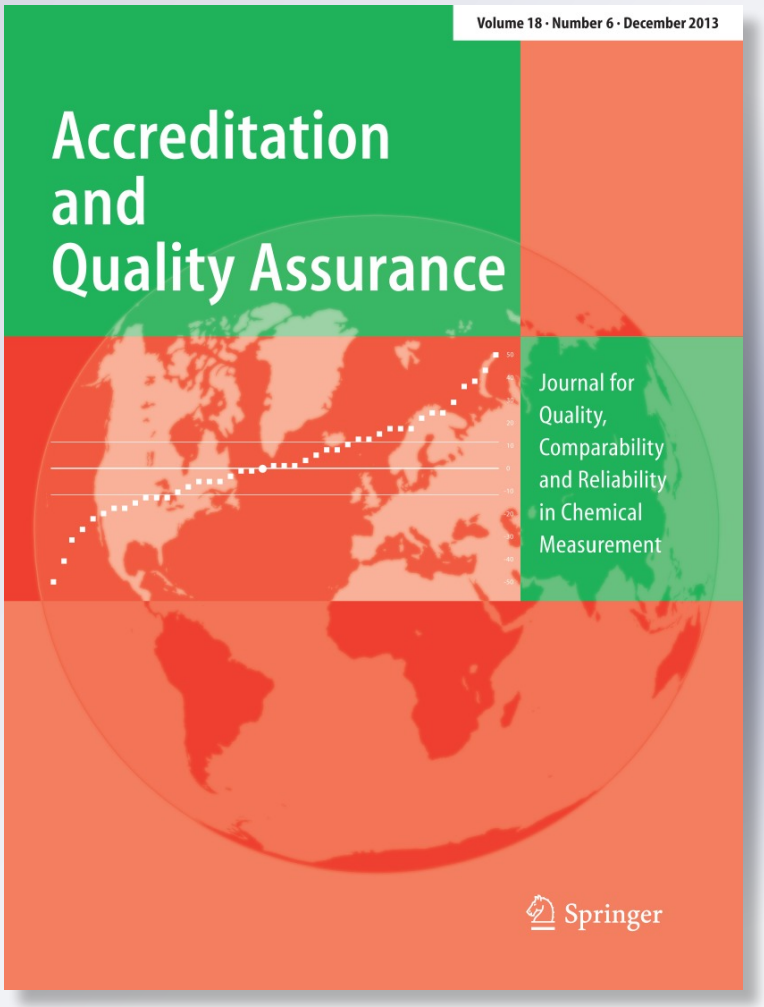

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PRACTITIONER'S REPORT

Preparation and certification of a reference material for the total mercury and methylmercury mass fractions in fish

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Abstract Certified reference materials (CRMs) play an important role in tracing results to the International System of Units through an unbroken chain of comparisons. Demand for new certified reference materials is steadily increasing in all areas. In Brazil, the demand for CRMs exceeds their availability by far, and the needs of the scientific community are not met. Food production is one area where CRMs are required, and they play an important role for export and local products. This paper describes the preparation and certification of a reference material for the content of mercury and methylmercury in fish samples. The material selection, preparation, homogeneity and stability studies, and characterization are described. Certification was performed by flow injection analysis–cold vapor atomic absorption spectrometry (FIA–CV-AAS) and isotope dilution–inductively coupled plasma mass spectrometry (ID–ICP-MS), which is a primary method. The standards ISO 30 (ABNT 30–34) and ISO Guide 35 were used as a basis for the preparation and characterization of the material. The material was certified for the mass fractions of total mercury w(total Hg) = (0.271 ± 0.059) μg g^{-1} and methylmercury w(MeHg) = (0.245 ± 0.053) μg g^{-1}.

Keywords Certified reference material · Fish · Mercury · Methylmercury

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Introduction

Mercury contamination of the marine environment is a serious environmental concern. Substantial concentrations of mercury accumulate in fish, and this can represent a major source of mercury in the human diet. Among the different mercury species, methylmercury (MeHg) is of particular concern because of the toxicity even at low concentration and its ability to bioaccumulate in fish, where it represents approximately 90 % of total mercury (total Hg). This is troubling because fish is an important part of the diet in several communities around the world. As a consequence, mercury can be a public health problem, particularly for women who are or may become pregnant, nursing mothers, and young children. Consequently, much effort has been devoted to developing methods for mercury determination in environmental and biological samples [1–3].

The demand for new certified reference materials (CRMs) for assessing the accuracy and reproducibility of experimental data is increasing, and CRMs for traceability of total Hg and MeHg results need to be developed. In the last years, several CRMs have been produced, such as Dorm-2, Dorm-4, Dolt-2, Dolt-3 (NRCC, Ottawa, Ontario, Canada), and CRM-463 (IRMM, Geel, Belgium), neither produced in Brazil.

The Laboratório de Caracterização Química of the Instituto de Pesquisas Energéticas e Nucleares (IPEN, São Paulo, Brazil) has produced Dourada-1, which is a CRM containing total Hg and MeHg in a fish matrix, following the principles of ISO Guides 34 [4] and 35 [5]. This work describes the production of the Dourada-1 CRM, including material processing, results of tests performed to assess its homogeneity and stability, and reference values concerning total Hg and MeHg.

Materials and methods

Collection of the candidate material

Ecomar Industria de Pesca S/A (Vigia, Pará, Brazil) collected the candidate reference material in Pará, Brazil. The reference material was produced from Dourada fish (*Brachyplatystoma Flavicans*). Circa 18 kg of Dourada fish, comprising nine fishes, was frozen (−20 °C) and transported to our laboratory. The individuals were cut and minced using a domestic mincer (head and tail despised), then freeze-dried (MLW-LG05, Leipzig, Germany), ground by hand in a mortar and pestle, and sieved using a domestic polypropylene sieve with 250 μm. The determination of relative moisture was measured, and the result was 9 %. All equipments used in the preparation were previously cleaned and decontaminated with special detergent (Extran®, Merck KgaA, Darmstadt, Germany) and nitric acid and then washed with Milli-Q water (18 MΩ cm, Millipore Corporation, Billerica, MA, USA) before use.

Homogenization and bottling

The freeze-dried material was transferred to a polyethylene container and sent to the Instituto Tecnológico de Alimentos (Campinas, Brazil) where it was homogenized in a mixing drum (type "V," TE200/10, Tecnal, Piracicaba, Brazil) and then bottled in brown borosilicate glass bottles. Eighty bottles, each containing 15 g of material, were produced for the candidate reference material. Before bottling, the material was sterilized using gamma irradiation (4.9 kGy) at the Centro de Tecnologia das Radiações at IPEN. After bottling, the samples were stored at room temperature.

Homogeneity

Between-bottle homogeneity was verified by determination of total Hg and MeHg contents in ten bottles. Three portions of approximately 0.4 g of sample from each bottle were tested for total Hg and two portions of 0.5 g for MeHg. Each portion was analyzed three times.

For total Hg determination [6], the samples were digested with an acid mixture containing 2 mL sulfuric, 1 mL nitric, and 1 mL perchloric concentrated acids, plus 1 mL demineralized water (D.P.L. 5.000 FB, Deion, S. Paulo, Brazil). The mixture was heated at 100 °C for 30 min and then cooled, and it was diluted to 20 g with demineralized water. The method utilized is a modification of the one described by Akagi et al. [7]. The final determination was performed by flow injection analysis–cold vapor atomic absorption spectrometry (SpectrAA220-FS, Varian Medical Systems Australasia Pty Ltd., Belrose, Australia) [8]. This method is accredited by CGCRE/INMETRO [9]. For MeHg determination, organic Hg and inorganic Hg were leached from the 0.5 g sample with 10 mL of 6 mol L^{-1} HCl solution and then separated on anion exchange resin (Dowex 1×8 100–200 mesh) [10, 11]. MeHg eluted (not fixed in resin) was decomposed to inorganic Hg II by UV irradiation during 12 h. The solution was diluted to 30 g with demineralized water, and determination was performed by flow injection analysis–cold vapor atomic absorption spectrometry (FIA–CV-AAS). Before analysis, the equipment was calibrated with Hg standard solutions. The method used, for both measurands, has a limit of quantification of 2 μg kg^{-1} (calculated as 10 times the standard deviation of blank measurement results), a repeatability standard deviation of 2–7 %, and recovery of 90–95 % (total Hg) and 80 % (MeHg).

One-way analysis of variance (ANOVA) of the results and uncertainty for the homogeneity study were performed as described in ISO Guide 35 [5]. The uncertainty component due to batch homogeneity, u_{bb}, was accounted for using the following equation:

$$u_{bb} = \sqrt{\frac{MS_{within}}{n}}\sqrt[4]{\frac{2}{v(MS_{within})}} \quad (1)$$

where MS_{within} is the "within mean square" obtained from the ANOVA, n is the number of observations for each sample, and $v(MS_{within})$ is the number of degrees of freedom for MS_{within}.

The minimum sample intake was determined by evaluating the within-bottle homogeneity using ten replicate determinations of the content of one bottle.

Stability

Stability tests were carried out following ISO Guide 35 [5] and the protocol developed at the BCR [12].

The stabilities of the total Hg and MeHg contents were tested to determine the suitability of this material as a CRM. Bottles were kept at +8, +20, and +40 °C for 12 months and analyzed at regular intervals (35, 70, 150, and 365 days) during the storage period. Samples were analyzed using the same procedure as for homogeneity study. The measurands were determined in five bottles. Two portions from each bottle were digested, and three replicate analyses were performed for each portion. Room temperature was chosen as the reference temperature.

The long-term stability standard uncertainty was evaluated according to ISO Guide 35 [5] as follows:

$$u_{lts} = s_b t \quad (2)$$

where u_{lts} is the long-term stability standard uncertainty, s_b is the slope of the standard deviation with time, calculated by regression analysis, and t is the storage time.

Certified values

Isotope dilution mass spectrometry (IDMS) was used to characterize the sample employing a spike solution enriched with ^{202}Hg (IRMM 640).

Isotope ratio measurements were performed by inductively coupled plasma mass spectrometry (ICP-MS) (ICP-sector field-MS, Finnigan MAT Element 1, Thermo Fisher Scientific, Waltham, MA, USA). The main conditions used in the analysis are presented in Table 1. Sample introduction into the plasma is managed by a self-aspirating Meinhard nebulizer, mounted outside the torch box (Fassel torch), using a peristaltic pump with a typical sample uptake rate of 0.5 mL min^{-1}. Memory effect in isotope ratio measurements was not detected after washing procedure with solution of 10 % (by volume) nitric acid for 120 s.

Good accuracy on the isotope ratio determinations (^{200}Hg/^{202}Hg) was ensured using a mass discrimination correction factor of the observed isotope ratios. The mass discrimination correction factor ($f = \rho_{\text{true}}/\rho_{\text{measured}}$), where ρ_{true} is the true value of the ratio and ρ_{measured} is the measured value of the ratio, was estimated daily obtaining an average value equal to $f = 1.0064$ (number of repetitions $n = 100$) by analysis of mercury standard solutions (IRMM 639).

The mass fraction of the measurands was calculated using the following equation [13]:

$$w_{\text{s}} = w_{\text{sp}} \frac{m_{\text{sp}}}{m_{\text{s}}} \frac{r_{\text{s}}}{r_{\text{sp}}} \frac{A_{\text{sp}}}{A_{\text{s}}} \frac{(\rho_{\text{b}} - \rho_{\text{sp}})}{(1 - \rho_{\text{b}}\rho_{\text{s}})} \tag{3}$$

where w_{s} is the unknown mass fraction of the element in the original sample (s) and w_{sp} is the mass fraction of the element in the spiked sample (sp); m_{s} and m_{sp} are the masses of the original and spiked samples, respectively; r_{s} and r_{sp} are the relative atomic masses in the original and spiked samples, respectively; A_{sp} is the isotope abundance (in atomic percent, at. %) of the reference isotope in the spiked sample; A_{s} is the isotope abundance of the reference isotope in the original sample; ρ_{b} is the isotope ratio in the mixture (isotope sample/isotope spike); ρ_{sp} is the isotope ratio in the spike (isotope sample/isotope spike); and ρ_{s} is the isotope ratio (isotope spike/isotope sample) in the original sample.

The combined standard uncertainty (u_{c}) from the characterization of the total Hg was obtained using the equation:

$$u_{\text{c}}(w_{\text{s}}) = w_{\text{s}} \sqrt{\left(\frac{u(w_{\text{sp}})}{w_{\text{sp}}}\right)^2 + \left(\frac{u(m_{\text{sp}})}{m_{\text{sp}}}\right)^2 + \left(\frac{u(m_{\text{s}})}{m_{\text{s}}}\right)^2 + \left(\frac{u(r_{\text{s}})}{r_{\text{s}}}\right)^2 + \left(\frac{u(r_{\text{sp}})}{r_{\text{sp}}}\right)^2 + \left(\frac{u(A_{\text{sp}})}{A_{\text{sp}}}\right)^2 + \left(\frac{u(A_{\text{s}})}{A_{\text{s}}}\right)^2 + \left(\frac{u(\rho_{\text{b}})}{\rho_{\text{b}}}\right)^2 + \left(\frac{u(\rho_{\text{sp}})}{\rho_{\text{sp}}}\right)^2 + \left(\frac{u(\rho_{\text{s}})}{\rho_{\text{s}}}\right)^2} \tag{4}$$

In the above equation, $u(i)$ is the standard uncertainty for the input quantity i.

For total Hg, a 0.4-g sample was weighed and added to 0.20 g of a spike solution [w(Hg) = 0.013 μg g^{-1}] in a Teflon® container. Then, concentrated HNO_3 (2 mL) was added and the mixture shaken manually. The container was sealed with a Teflon cap, and Teflon tape was placed at the junction between the container and cap. The capped container was transferred to a hot plate, and digestion proceeded for 100 min at 100 °C. Finally, after cooling, the digested sample was transferred to a 15-mL plastic tube and diluted to a final mass of 10 g. Then, isotope ratio determinations were performed by ICP-MS.

For MeHg, a 0.5-g sample was weighed, separated on anion exchange resin, irradiated with UV light to be decomposed to inorganic Hg, and added to 0.20 g of the spike solution [w(Hg) = 0.013 μg g^{-1}], and the isotope ratio determinations were performed by ICP-MS. A spike solution of MeHg was not available for this experiment so the Hg spike IRMM 640 was used for the determination of total Hg. The procedure, particularly the extraction efficiency, was confirmed by analysis of the NRCC CRMs Dorm-2 (Dogfish Muscle) and Dolt-2 (Dogfish Liver).

Table 1 ICP-MS conditions

Rf power	1250 W
Cool gas flow rate	16 L min^{-1}
Auxiliary Ar gas flow rate	0.6 L min^{-1}
Sample Ar gas flow rate	1.1 L min^{-1}
Lens voltage	Focus: −1065/−776.2 V;
	x deflection: −7.6/−1.0 V;
	y deflection: 5.8/9.2 V; shape: 137.0 V
Resolution	300
Data acquisition	E-scan, 10 runs×10 passes; 5 % mass window, 0.001 s settling time, 0.01 s sample time

The combined standard uncertainty (u_c) of the MeHg content was obtained considering all potential sources of uncertainty. First, the influence on the result of the efficiency of the leaching and the extraction in the sample prior to blending with the isotopically enriched spike was assessed. Thus, the influence on the result of the efficiency was verified by estimation of the uncertainty associated with recovery (u_R), by the use of CRM, as described by Barwick and Ellison [14]:

$$u_R = R\sqrt{\frac{s_{obs}^2}{nC_{obs}^2} + \left(\frac{u(C_{CRM})}{C_{CRM}}\right)^2 + \left(\frac{u_c(\text{bal})}{m_{CRM}}\right)^2} \quad (5)$$

where R is the mean of several determinations of a CRM; s_{obs} is the standard deviation of the results from replicate analysis of the CRM; n is the number of replicates; C_{obs} is the certified value for the CRM; $u(C_{CRM})$ is the standard uncertainty in the certified value for the CRM; u_c(bal) is the combined standard uncertainty of the balance used to preparing the aliquot of CRM purchased and m_{CRM} is the mass of the CRM used. The uncertainty associated with the balance is estimated, using the data from the calibration certificate (u_{cert}) and the repeatability of the instrument obtained in the laboratory (u_{repe}):

$$u_c(\text{bal}) = \sqrt{u_{cert}^2 + u_{repe}^2} \quad (6)$$

where u_{cert} is the expanded uncertainty (U) from the calibration certificate divided by the stated coverage factor $k = 2$ and u_{repe} is the standard deviation of replicate experiments with a certified weight.

According to the guides Eurachem QUAM [15] and ISO GUM [16], the square root in Eq. 4, called u_c(IDMS), and Eq. 5 were combined to obtain u_c(MeHg):

$$u_c(\text{MeHg}) = w_s\sqrt{u_c^2(\text{IDMS}) + u_R^2} \quad (7)$$

The uncertainty associated with the certified value, for both measurands, of a candidate CRM can be expressed as follows [5]:

$$u_{CRM} = \sqrt{u_{char}^2 + u_{bb}^2 + u_{lts}^2 + u_{sts}^2} \quad (8)$$

where u_{char} is standard uncertainty from characterization (using Eq. 4 for total Hg and Eq. 7 for MeHg), u_{bb} is the uncertainty component due to batch homogeneity (Eq. 1), u_{lts} is the long-term stability standard uncertainty (Eq. 2), and u_{sts} is the standard uncertainty of short-term stability.

The short-term stability is associated with packaging and transport of the samples. As given in ISO Guide 35, no uncertainty contribution for short-term stability needs to be included in the certification if restrictive conditions for transport apply.

The expanded uncertainty (U_{CRM}) of a certified value of the measurand mass fraction was calculated using Eq. 9.

$$U_{CRM} = u_{CRM}k \quad (9)$$

The coverage factor was chosen to be $k = 2$ for approximately 95 % confidence.

Results and discussion

Method used for total Hg and MeHg determination

FIA–CV-AAS and IDMS used in the certification process require many steps of the sample manipulation and different instrumental that can insert errors associated with sample contamination or measurand loss. The main difficulty, mainly in IDMS, is the addition of the spike and finding the best proportion between the original sample and the spike. For MeHg determination by IDMS, the important step is after the extraction and addition of the spike because, if the extraction is not so good, the result of the isotope ratio is not correct. The accuracy of the proposed procedure was checked by the analysis of two CRMs.

Homogeneity

In the homogeneity study for total Hg and MeHg, no heterogeneity was detected in the material. Table 2 shows the results of ANOVA for total Hg, and Table 3 shows the results for MeHg which were obtained by the Fearn and Thompson method [17], called "sufficient homogeneity." The data of MS_{within} and the number of degrees of freedom (v) were used to calculate the uncertainty of the homogeneity of the material (Table 6).

Table 2 Data of ANOVA of the homogeneity study for total Hg

MS_{bb}	MS_{within}	$v(MS_{within})$	$F_{calculated}$	p value	F_{critic}
0.000711	0.001092	20	0.65	0.74	2.39

Table 3 Data of the homogeneity study for MeHg

Analytical variance[a] $s_{an}^2 = MS_{within}$	$v(MS_{within})$	Sampling variance[b] s_{sam}^2	Critical value for the test[c], c	Criterion for the test
0.00153	10	0.001912	0.002025	$s_{sam}^2 < c$

[a] According to Ref. [17], the analytical variance (s_{an}^2) is the "within mean square" (MS_{within}) obtained from the ANOVA

[b] The sampling variance, $s_{sam}^2 = (MS_{bb} - MS_{within})/2$ where MS_{bb} is "between mean square" obtained from the ANOVA

[c] The critical value is $c = F_1\ \sigma_{all}^2 + F_2\ s_{an}^2$, where F_1 and F_2 are constants, σ_{all}^2 is allowable between-sample variance calculated by $(0.3\sigma_p)^2$ where σ_p is the target standard deviation, obtained by the Horwitz function

As can be seen from Table 2, $F_{calculated}$ is smaller than the critical value F_{critic} and $p > 0.05$, two criteria indicative of homogeneity.

Using Eq. 1, the uncertainty component due to batch homogeneity, u_{bb}:

$$u_{bb}(\text{total Hg}) = \sqrt{\frac{0.001092}{9}}\,\mu g\,g^{-1}\sqrt{4}\frac{2}{20} = 0.006194\,\mu g\,g^{-1}$$

$$u_{bb}(\text{MeHg}) = \sqrt{\frac{0.00153}{6}}\,\mu g\,g^{-1}\sqrt{4}\frac{2}{10} = 0.01067\,\mu g\,g^{-1}$$

The within-bottle homogeneity study, cited under "Homogeneity" in the section "Materials and methods", was used to calculate a minimum sample mass of 0.2 g. In the present study, we used a mass of 0.4 g for convenience.

Stability

The data from the stability study were evaluated for some trends. No trends were detected, and the data were stable for the duration of the experiment studied.

Using Eq. 2 and the data uncertainty associated with the slope, calculated by regression analysis, the uncertainty contributions from long-term stability are showed in Table 4.

Based on these results, it was concluded that the material is stable. The expected shelf life is 12 months. The material will be monitored at regular intervals in future to confirm this. This material should be kept in the tightly closed original bottle and stored in a refrigerator or a temperature range of 20–40 °C.

Table 4 Data of the s_b, t, and results of the stability study for total Hg and MeHg

Measurand	Storage temperature (°C)	s_b (μg g^{-1} month^{-1})	t (month)	u_{lts} (μg g^{-1})
w(total Hg)	20	0.002356	12	0.028272
w(total Hg)	40	0.002875	12	0.034500
w(MeHg)	20	0.0014520	12	0.0174240
w(MeHg)	40	0.0027606	12	0.0331272

Certified values

The method was validated by analyzing two CRMs (Dorm-2 and Dolt-2). These materials were subjected to the same treatment that samples were subjected, as described in the subsection "Certified values" of the "Materials and methods" section. The results showed that the measured values are in good agreement with the certified ones, as shown in Table 5.

The estimation of the uncertainty associated with recovery of MeHg (u_R) was obtained by using Eq. 5. The CRM used was Dorm-2, where $R = 0.94$, $s_{obs} = 0.08$ μg g^{-1}, $n = 3$, $C_{obs} = 4.22$ μg g^{-1}, $u(C_{CRM}) = 0.32$ μg g^{-1}, $C_{CRM} = 4.47$ μg g^{-1}, and $u_c(\text{bal}) = 0.000028$ g and $m_{CRM} = 0.20362$ g. The result, u_R, was 0.068. Using Eq. 7 with $w_s = 0.245$ μg g^{-1} and $u_c(\text{IDMS}) = 0.0158785$, one arrives at u_{char} of w(MeHg).

Table 6 shows the results of the characterization of the material Dourada-1 by IDMS.

Thus, using Eq. 8 and the data of Table 6, the certified values and their expanded uncertainties (with $k = 2$ for a level of confidence of approximately 95 %) for total Hg and MeHg are given in Table 7.

Table 5 Analytical results for methylmercury w(MeHg) and total mercury w(total Hg) in CRMs obtained by IDMS and their expanded uncertainties ($k = 2$)

Reference material	Certified value (μg g^{-1})		Found value (μg g^{-1})		Recovery (%)	
	w(MeHg)	w(total Hg)	w(MeHg)	w(total Hg)	R(MeHg)	R(total Hg)
Dorm-2	4.47 ± 0.32	4.64 ± 0.26	4.22 ± 0.13	4.59 ± 0.14	94.4	98.9
Dolt-2	0.693 ± 0.053	1.99 ± 0.10	0.680 ± 0.022	1.96 ± 0.06	98.1	98.5

Table 6 Results of the characterization of the material Dourada-1 by IDMS analysis and the uncertainty contributions from characterization, homogeneity, and stability studies

	Results of characterization by IDMS analysis[a] (μg g^{-1})	Characterization standard uncertainty[b] (u_{char}) (μg g^{-1})	Homogeneity standard uncertainty (u_{bb}) (μg g^{-1})	Stability standard uncertainty (u_{sts}) (μg g^{-1})
w(total Hg)	0.271	0.00423	0.006194	0.028272
w(MeHg)	0.245	0.01711	0.01067	0.0174240

[a] Using Eq. 3

[b] Using Eq. 4 for total Hg and Eq. 7 for MeHg

Table 7 Certified values of Dourada-1 and their expanded uncertainties ($k = 2$) for the mass fractions, w, of total Hg and MeHg

Measurand	Certified value ($\mu g\ g^{-1}$)
w(total Hg)	0.271 ± 0.059
w(MeHg)	0.245 ± 0.053

Conclusions

In this work, a standard operational procedure was used to prepare a reference material for total Hg and MeHg content in fish, and the reference material was certified in compliance with ISO Guides 34 and 35 [4, 5]. The CRM "Dourada-1" was homogenous and stable under the test conditions.

Isotope dilution applied to mass spectrometry was used for the determination of both measurands. For MeHg, a spike was added after the extraction step and the result was satisfactory. ICP-MS provided precise and reliable isotope ratio measurements, and the contribution of the isotope ratio data to the overall uncertainty was approximately 1 %.

The mass fractions of both total Hg and MeHg were certified with an expanded relative uncertainty (expanded uncertainty by mass fraction) of approximately 22 %. The uncertainty was higher because the technique used (FIA–CV-AAS) to study homogeneity and stability had an expanded uncertainty of 12.5 % (total Hg) and 15 % (MeHg). The stability studies in classical layout caused the largest contribution (more than 40 %) to the combined standard uncertainty. Better results for the overall uncertainty could be obtained using, for example, isotope dilution mass spectrometry for the studies realized.

The material Dourada-1 is the first CRM for mercury compounds in fish produced in Brazil.

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