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# DEVELOPMENT OF REFERENCE MATERIAL FOR PROFICIENCY TESTS: ARSENIC IN FISH TISSUE

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## ABSTRACT

Proficiency tests (PT) are extensively used to evaluate the analytical competence of laboratories, and are also used as a part of accreditation processes. For this reason are important tool for quality control of laboratories including laboratories that act directly with food exporting companies. In Brazil there are no providers of proficiency testing for toxic metals, such as arsenic in fish tissue. This study presents a protocol to produce reference material to be used in proficiency test for arsenic in fish tissue following the recommendations of the ISO Guide 35. The preparation scheme consisted of: selecting of individuals, cleaning of scale and skin, trituration, homogenization, and spiking with arsenic at two levels of concentration. The mixture was then irradiated in a cyclotron Cyclone 30 Applications ion beam with cobalt 60 at 10.00 ± 1.05 KGy, before being packed into sachets. To verify the efficacy of the irradiation procedure, 26 (randomly selected) irradiated sachets and 26 non-irradiated sachets were assessed for homogeneity and stability. The results indicate that irradiation with cobalt 60 is crucial for ensuring the preservation of the integrity of the material, providing stable material at room temperature for 2 months. The samples can therefore be transported at room temperature.

## 1. INTRODUCTION

Arsenic is hazardous substance and potentially carcinogenic to humans. Prolonged exposure to arsenic causes a series of diseases such as conjunctivitis, cardiovascular disease, disturbances of the central nervous system and peripheral vascular, skin cancer and gangrene. The main sources of contamination are contaminated water or food (mainly fish). Toxic levels are 3 to 30 mg / kg / body weight (oral doses above 60 mg / kg / body weight are lethal), while approximately 1mg $g^{-1}$ is considered a tolerable dose for consumption [1-5].

Fish products are especially prone to arsenic contamination, and there are strict sanitary standards for food quality that apply to national and international commerce. In Brazil, the level of arsenic in fish tissue is regulated by Resolution n° 685/1998 (ANVISA-Agency National of Sanitary Vigilance) and the permitted maximum limit is 1.0 µg $g^{-1}$. In order to comply with this regulation several private laboratories and National Regulatory agencies provide analytical quality control, all of which require accreditation by external certification bodies in conformity with the requirements of ABNT: NBR ISO/IEC 17025. One of the main criteria for accreditation is a demonstration of the technical capability of the laboratory through regular participation in proficiency testing [6].

Proficiency tests are inter-laboratory exercises in which the results of a particular laboratory are compared to a reference value or with the results obtained from similar laboratories. A proficiency test item is required for the effective execution of these tests, which can be: a sample product or device, reference material, equipment, a standard set of data or other information [7].

In Brazil there are currently no providers of proficiency testing for toxic metals such as arsenic in fish tissues. Therefore, this study describes a new protocol for the production of reference materials for use in proficiency testing for arsenic in fish tissue. The reference materials produced for proficiency testing should be homogeneous and stable over the duration of the test, and must follow all the requirements of international quality standards used in the preparation of certified reference materials [7, 8]. Homogeneity and stability studies are therefore essential for ensuring that the differences in results between laboratories are not caused by the instability or heterogeneity of the samples provided [9, 10].

## 2. METHODOLOGY

The proposed protocol for the preparation of the material was performed in accordance with the ISO 30 standards series and was based on known food production protocols and the international literature on the production of certified reference materials (Fig.1).

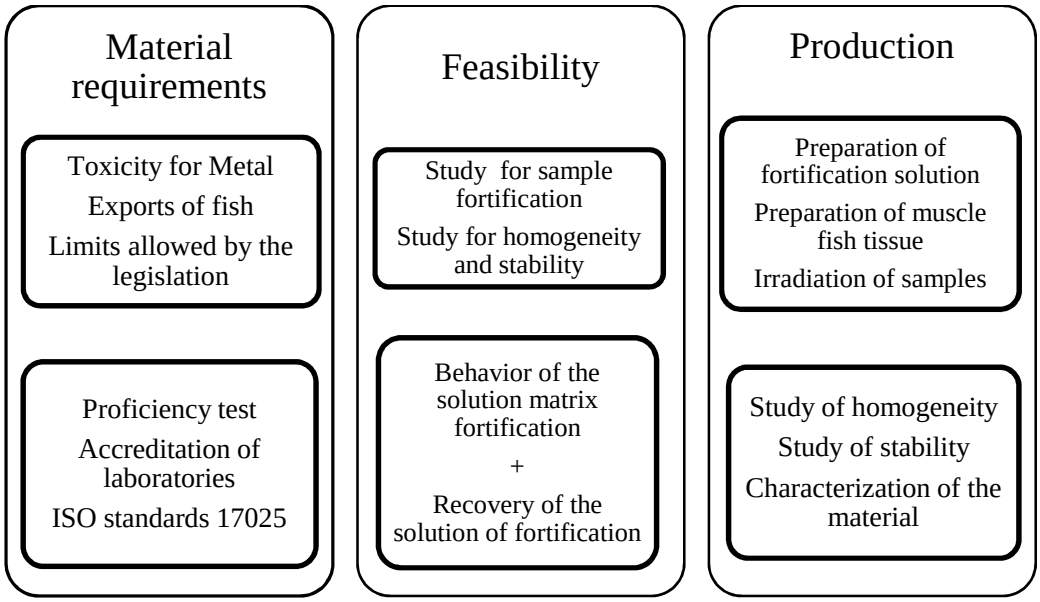


Fig. 1 Steps involved in the production of the reference material

### 2.1. Preparation of fortification solution

Solutions were prepared with two levels of arsenic concentration, 1.0 µg $g^{-1}$ and 1.5 µg $g^{-1}$, from a standard stock solution of 999.89 µg $g^{-1}$ of arsenic in 2 % nitric acid.

## 2.2. Preparation of samples

Four individuals (of approximately 660g per fish) of Peacock Bass (*Cichla sp.*) from the Tapajos River were purchased in the local market of Itaituba city in the central-east Amazon region. The specimens were cleaned to remove the skin, viscera's and scale using a stainless steel knife. The muscle tissue was then triturated using a domestic multiprocessor (Wallita ®) knives and homogenized for 5 minutes in domestic mixer beaters (drop-shaped) type "fouet" (Wallita ®), resulting in two portion of 1300 g and 1100 g respectively. The first portion of 1300 g was immersed in the 1.5 $\mu g\ g^{-1}$ arsenic solution and the second portion of 1100 g in the 1.0 $\mu g\ g^{-1}$ arsenic solution. Each portion was then homogenized for 5 minutes.

The resulting material was packed into 164 sachets, each containing 15 g of material. The sachets were separated and randomly placed in four groups of 41 units. Sachets from each group were irradiated with 10.0 ± 1.05 kGy, using a cobalt 60 ($^{60}Co$) source. The other sachets were preserved intact until the beginning of the homogeneity and stability analysis.

All procedures were performed in a clean room in laminar flow hood, using appropriate precautions such as gloves, cap, lab coat and mask (Fig.1). All materials were decontaminated with 5% nitric acid and rinsed with water (Milli-Q) with resistivity of 18 Ω before analysis.

## 2.3. Irradiation of samples

The samples were irradiated with cobalt-60 ($^{60}Co$) (at IPEN in a cyclotron Cyclone Applications ion beam 30 MeV) using a dose of 10.0 ± 1.05 kGy at 23 ± 4 °C. Irradiation was performed for the purpose of sterilization, allowing long-term stability during transportation at room temperature.

## 2.4. Determination of Arsenic

The portion of 0.5 g of the higher concentration material and 1.0 g of the lower concentration material in triplicate was placed in a volumetric flask. To this solution, 3 mL of concentrated $HNO_3$ was added and left for approximately 50 minutes to allow digestion. Then, 0.5 mL of $H_2SO_4$ and 1 mL $HClO_4$ was added and the mixture was placed into a heated plate at 250 °C. After 12 hs a 2 mL solution of concentrated HCl was added, and the resulting solution was diluted to 20 mL with Milli-Q water (18 Ω). In the first reduction, 2 g of reducing solution of KI + ascorbic acid-L 5 % (v / v) was added to 6 g of the digested sample and diluted to 10 g of dough final solution of 10 % HCl PA (v / v). The sample was then placed in a water bath at 80 °C for 30 minutes, allowed to cool, and analyzed [12].

After pre-reduction, As concentration in both samples was performed by hydride generation (HG-AAS) atomic absorption spectrometry (AA-220 Varian FS) equipped with a Flow Analysis Injection (FIA), operated according to the manufacturer's recommendations. A total of 500 µL of digested sample was injected into the system, carried in a solution of 10 % HCl (v / v) (10 mL $min^{-1}$). The $As^{3+}$ in the samples was reduced with $BH_4Na$ 0.4 % (v / v) [12].

DORM-2 - "Dogfish" certified reference materials for trace metals provided by the National Research Council of Canada (NRCC) was used for validation of the analytical methodology.

The certificate concentration was used to generate the expanded uncertainty for arsenic (total of 18.0 ± 1.1 µg $g^{-1}$).

### 2.5. Homogeneity

Ten sachets were selected randomly from each material. The results were analyzed by one way ANOVA to investigate the level of heterogeneity of the material and to assess whether the irradiation procedure had changed the concentration of arsenic [8, 10, 13, 14].

### 2.6. Stability

The stability study was carried out for 2 months following the isochronous model. A sub-sample of 16 sachets was randomly selected at four different temperatures of 5 °C, 23 °C, 40 °C and 60 °C for a period of 0, 7, 15, 30 and 45 days, respectively. The purpose of this step was to evaluate sample degradation during the transport and distribution of materials to the laboratories participating in the proficiency testing [8, 9, 14].

## 3. RESULTS AND DISCUSSIONS

### 3.1 Protocol for homogeneity study

The homogeneity test was conducted within the sachet or between sachets to assess possible differences of concentration which can be displayed during the production stage [10].

The concentration of arsenic and expanded uncertainty obtained for the DOMR-2 in the study were, respectively, 17.8 ± 0.56 µg $g^{-1}$ and 14%.

The results obtained for the homogeneity test for arsenic at higher and lower concentrations of irradiation are shown in table 1 and the non-irradiated samples in table 2.

TABLE 1- Average values obtained in the determination of total arsenic in µg $g^{-1}$ by FIA-HG-AAS in higher and lower irradiation concentrations (wet basis) for the study of homogeneity (n = 30)

| Concentration average As (µg $g^{-1}$) | | | | |
|---|---|---|---|---|
| Temperature 23 ± 0.3°C | | lower | | higher |
| *Average ±S.D.* | | 0.64 ± 0.05 | | 1.09 ± 0.02 |
| *Variance* | | 0.0024 | | 0.001 |
| *RSD %* | | **7.6** | | **1.8** |
| $F_{calc}$ | | **2.27** | | **1.04** |
| $F_{crit}$ | | 3.88 | | 3.88 |
| | *gl(total)=14* | | *gl(total)=14* | |
| *valor-P (X1)* | | 0.98 | | 0.06 |
| $u_{bb,}$ µg $g^{-1}$ (%) | | 0.045 (6.5 %) | | 0.045 (4.5) |

RSD-relative standard deviation, F-value obtained for Fisher's test, P (X1) P-value for variable X1 (slope *b*1), gl-degree of freedom, As-total arsenic; F-calc –calculated, F-crit –critical.

TABLE 2- Average values obtained in the determination of total arsenic in µg $g^{-1}$ by FIA-HG-AAS in higher and lower irradiation concentrations (wet basis) for the study of homogeneity (n = 30)

| Concentration average As (µg $g^{-1}$) | | | | |
|---|---|---|---|---|
| Temperature 23 ± 0.3 °C | | Lower | | higher |
| *Aveerage ± S.D.* | | 0.72 ± 0.06 | | 1.30 ± 0.10 |
| *Variance* | | 0.0032 | | 0.0092 |
| *RSD %* | | **7.8** | | **7.4** |
| $F_{calc}$ | | **0.73** | | **2.55** |
| $F_{crit}$ | | 3.88 | | 3.88 |
| | *gl(total)=14* | | *gl(total)=14* | |
| *valor-P (b1)* | | 0.25 | | 0.09 |
| $u_{bb}$ , µg $g^{-1}$ *(%)* | | 0.06 (6.0 %) | | 0.04 (4.0) |

RSD-relative standard deviation, F-value obtained for Fisher's test, P (X1) P-value for variable X1 (slope *b*1), gl-degree of freedom, As-total arsenic, F-calc –calculated, F-crit –critical.

The ANOVA evaluates the value of the result $F_{critical}$ and $F_{calculated}$ for a confidence level of 95%, so it is assumed that the sample is homogenous when the value of $F_{calculated} < F_{critical}$ because there is no significant difference between the sample values. The linear regression analysis also revealed no significant trend in the results (P>0.05).

The calculation of uncertainties for the homogeneity analysis was based on the results obtained from the ANOVA, using the following equation 1 [8,14]:

$$u_{bb} = \sqrt{\frac{MQ_{between}}{n}} \sqrt[4]{\frac{2}{\nu_{MQ_{between}}}} \quad (1)$$

Where: $u_{bb}$ = uncertainty for homogeneity; $MQ_{between}$ = average quadratic between sachets, n = number of sachets and $\nu_{MQ_{between}}$ = degrees of freedom for average quadratic between sachets.

### 3.2 Protocol for stability study

The stability study was performed in different temperature ranges (5-60 °C) for a period of 0-45 days using an isochronous model. The results were obtained in the simultaneous measurements of the samples, under good conditions of repeatability [14].

The results were submitted to Grubbs test for the presence of outliers and were then submitted to linear regression analysis and significance testing. The results are shown in Tables 4 and 5 for samples exposed to higher and lower irradiation concentrations and Tables 6 and 7 for samples for non-irradiated samples.

The significance of the linear regression slope was evaluated by the "regression analysis" program Windows / Excel ® at a level of 95%. In this case, the slope (| *b*1 |) of the regression line was considered to be insignificant. Table 3-6 [8, 9, 10, 14].

TABLE 3 - Average values obtained in the determination of total arsenic in µg $g^{-1}$ by FIA-HG-AAS in lower irradiation concentrations (wet basis) for 0, 7, 15, 30, 45 days

| | Freezer (5 °C) | Room (23 °C) | Warm house (40 °C) | Warm house (60 °C) |
|---|---|---|---|---|
| *Average ±S.D.* | 0.69 ± 0.02 | 0.70 ± 0.04 | 0.69 ± 0.04 | 0.81 ± 0.09 |
| | | | | |
| *RSD %* | **2.9** | **5.9** | **5.4** | **11.5** |
| *\|b1\|* | 0.0009 | 0.0023 | 0.0020 | 0.0059 |
| *s(b1)* | 0.0004 | 0.0007 | 0.0001 | 0.0012 |
| | | | | |
| *[a]\|b1\|<t(0,95,n-2) x s(b1)* | 0.19 | 0.20 | 0.20 | 0.29 |
| *$u_{sts}$ µg $g^{-1}$, (%)* | 0.001 (0.1 %) | 0.001 (0.1 %) | 0.001 (0.1 %) | 0.002 (0.2 %) |

$b_1$: slope; s($_{b1}$): standard deviation in $b_1$; $u_{sts}$: uncertainty of short-term stability; t0,95, n-2 x s(b1) the Student's t-factor at the degree of freedom of n-2 and a confidence level of 95 %.

TABLE 4 - Average values obtained in the determination of total arsenic in µg $g^{-1}$ by FIA-HG-AAS in higher irradiation concentrations (wet basis) for 0, 7, 15, 30, 45 days

| | Freezer (5 °C) | Room (23 °C) | Warm house (40 °C) | Warm house (60 °C) |
|---|---|---|---|---|
| *Average ±S.D.* | 0.95 ± 0.04 | 1.07 ± 0.07 | 1.11 ± 0.06 | 1.27 ± 0.14 |
| | | | | |
| *RSD %* | **3.6** | **6.7** | **5.6** | **10.8** |
| *\|b1\|* | -0.0001 | -0.0001 | -0.0002 | 0.008 |
| *s(b1)* | *0.00123* | *0.0022* | *0.00222* | *0.0013* |
| | | | | |
| *[a]\|b1\|<t(0,95,n-2) x s(b1)* | 0.42 | 0.55 | 0.61 | 0.83 |
| *$u_{sts}$ µg $g^{-1}$, (%)* | 0.002 (0.2 %) | 0.003 (0.3 %) | 0.002 (0.2 %) | 0.003 (0.3 %) |

$b_1$: slope; s($_{b1}$): standard deviation in $b_1$; $u_{sts}$: uncertainty of short-term stability; t0,95, n-2 x s(b1) the Student's t-factor at the degree of freedom of n-2 and a confidence level of 95 %.

The test of significance of the slope of the regression b1 was verified by a t test for n-2 degrees of freedom, with value t, corresponding to 95% confidence level. Thus, the result | b1 | <t0, 95, n -2 x s(b1) confirmed the stability of the material within the conditions provided, because the slope of the regression was insignificant [8, 9, 10, 14].

TABLE 5 - Average values obtained in the determination of total arsenic in µg $g^{-1}$ by FIA-HG-AAS in lower non-irradiation concentrations (wet basis) for 0, 7,15,30, 45 days

| | Freezer (5 °C) | Room (23 °C) | Warm house (40 °C) | Warm house (60 °C) |
|---|---|---|---|---|
| *Average ±S.D.* | 0.70 ± 0.04 | 0.70 ± 0.04 | 0.71 ± 0.05 | 0.81 ± 0.11 |
| | | | | |
| *RSD %* | **5.5** | **5.1** | **6.9** | **13.5** |
| \|*b*1\| | 0.0020 | -0.0013 | 0.003 | 0.007 |
| *s(b*1*)* | 0.001 | 0.0003 | 0.001 | 0.002 |
| | | | | |
| [a] $\|b_1\| < t(0,95,n-2) \times s(b1)$ | 0.20 | 0.20 | 0.20 | 0.29 |
| $u_{sts}$ µg $g^{-1}$, *(%)* | 0.002 (0.2 %) | 0.001 (0.1 %) | 0.002 (0.2 %) | 0.003 (0.3 %) |

$b_1$: slope; $s_{(b1)}$: standard deviation in $b_1$; $u_{sts}$: uncertainty of short-term stability; t0,95, n-2 x s(b1) the Student's t-factor at the degree of freedom of n-2 and a confidence level of 95 %.

TABLE 6 - Average values obtained in the determination of total arsenic in µg $g^{-1}$ by FIA-HG-AAS in higher non-irradiation concentrations (wet basis) for 0, 7, 15, 30, 45 days

| | Freezer (5 °C) | Room (23 °C) | Warm house (40 °C) | Warm house (60 °C) |
|---|---|---|---|---|
| *Average ±S.D.* | 0.93 ± 0.03 | 1.03 ± 0.06 | 1.15 ± 0.05 | 1.29 ± 0.15 |
| | | | | |
| *RSD %* | **3.2** | **5.9** | **4.4** | **11.3** |
| \|*b*1\| | -0.0013 | -0.003 | 0.002 | 0.01 |
| *s(b*1*)* | 0.0013 | 0.002 | 0.001 | 0.0013 |
| | | | | |
| [a] $\|b_1\| < t(0,95,n-2) \times s(b1)$ | 0.40 | 0.51 | 0.66 | 0.85 |
| $u_{sts}$ µg $g^{-1}$, *(%)* | 0.003 (0.3 %) | 0.004 (0.4 %) | 0.004 (0.4 %) | 0.002 (0.2 %) |

$b_1$: slope; $s_{(b1)}$: standard deviation in $b_1$; $u_{sts}$: uncertainty of short-term stability; t0,95, n-2 x s(b1) the Student's t-factor at the degree of freedom of n-2 and a confidence level of 95 %.

The values of | b1 | <t0, 95, n-2 x s(*b*1) obtained for the analysis of both materials of higher and lower irradiation concentrations are within the limits of analytical uncertainty adopted (14%), even in presence of degradation temperatures (40 ° C and 60 ° C).

Short-term stability is very important to establish the conditions for transporting the material and uses a principle of calculation of uncertainty (it is assumed that the degradation is linear) equal to the long-term study, with smaller uncertainties due to the short period of time. The uncertainties are calculated by equation 2 [14].

$$u_{sts} = {}_{sb} \, . \, t \qquad (2)$$

The calculation of the uncertainty of the certified value of a reference material uses the uncertainty of homogeneity, long and short term stability and the combined uncertainty characterization. The use of combined standard uncertainty is recommended as it is takes into account all factors that contribute to the uncertainty associated with the property values of the MRC [14].

In the case of this particular material long-term uncertainty was not used. Equation 3 thereby expresses the uncertainty associated with the property value of the reference material for proficiency testing of arsenic in fish muscle tissue. It uses the uncertainty characterization provided by: i) calculating the uncertainty for the sample analyzed via FIA-HG-ASA in the laboratory; ii) the uncertainty due the homogeneity calculated previously, and; iii) the uncertainty in the short term stability at 23 ° C (transport temperature) (table 7):

Table 7 - Estimation of uncertainties in µg $g^{-1}$ for the element As-total determined by FIA-HG-AAS at lower and higher irradiation concentrations (wet basis)

| Element | $u_{char}$ | | $u_{bb}$ | | $u_{sts}$ | |
|---|---|---|---|---|---|---|
| | (µg $g^{-1}$) | % | (µg $g^{-1}$) | % | (µg $g^{-1}$) | % |
| As *(higher irrad.) | 0.21 | 19.3 | 0.04 | 3.7 | 0.004 | 0.37 |
| As **(higher non-irrad.) | 0.23 | 17.7 | 0.01 | 0.8 | 0.003 | 0.23 |
| As *(lower irrad.) | 0.15 | 23.3 | 0.03 | 4.7 | 0.001 | 0.15 |
| As **(lower non-irrad.) | 0.15 | 21.4 | 0.01 | 1.5 | 0.001 | 0.15 |

*Irrad-irradiated, **non-irradiated.

Using equation 3, the combined standard uncertainty for reference material ($k$ = 2) was calculated (table 8) [14].

$$U_{MR} = k\sqrt{u_{car}^2 + u_{bb}^2 + u_{sts}^2} \quad (3)$$

Table 8 - Estimated expanded uncertainty in µg $g^{-1}$ for As-total

| Element | [a]Average ± $U_{MR}$ | $U_{MR}$ |
|---|---|---|
| | (µg $g^{-1}$) | % |
| As* (higher irrad.) | 1.09 ± 0.42 | 39 |
| As** (higher non-irrad.) | 1.30 ± 0.45 | 35 |
| As* (lower irrad.) | 0.64 ± 0.30 | 46 |
| As** (lower non-irrad) | 0.70 ± 0.30 | 43 |

[a]Average-obtained in the characterization, represents the average of 45 replicates of each material; *Irrad-irradiated, **non-irradiated

The reference value of both materials corresponded to the value obtained in the characterization by analyzing 15 replicates of the material with three reads each, totaling 45 results.

## 4. CONCLUSIONS

The quality of the results obtained in the study of homogeneity and stability clearly demonstrate the adequacy of protocols - in both studies the relative standard deviation for the reference temperatures 5 ° C and 23 ° C was less than 10%. Irradiation of the material with cobalt 60 source ($^{60}$Co) to 10.00 ± 1.05 KGy guaranteed stability of the material. Between 5 ° C to about 23 ° C, the element showed high stability throughout the study period. Therefore, there is no need for freezing during transport.

Variations in the concentration values given in critical temperatures of 40 ° C and 60 ° C were considered suitable for a natural matrix with high moisture content (82 %), as they were within the limits of analytical uncertainty and expanded uncertainty (14 % and 40 % respectively).

The study of homogeneity indicated no significant variations that could affect the intended use of this material. Thus, the produced material is suitable to be used in a program of proficiency testing of laboratories.

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