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ESTIMATE OF UNCERTAINTY OF MEASUREMENT IN THE DETERMINATION OF Zn, Cu, Hg AND Pb IN FISH MUSCLE

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ABSTRACT

Fish muscles are often used as biomarkers for metal pollution, providing important information about the environmental quality. For this reason the determination of metals in fish tissues requires the use of validated methods which demonstrating their robustness and reliability. In this context the estimate of uncertainty is an important tool allowing the identification the influence of each step of the analytical protocol in the overall quality of the results. In this paper, it will be discussed the estimate of uncertainty during the measurement of metals in fish tissue by using three different techniques: mercury by cold vapour atomic absorption spectrometry, Zn and Cu by atomic absorption spectrometry in flame mode and lead by high resolution inductively coupled plasma mass spectrometry. The total uncertainty for Hg , Zn , Cu and Pb were 11.4% ,7.2% respectively. The recovery of the certified reference material (about 70%) was the main contribution of the total uncertainty for AAS measurements. In the case of HR-ICPMS the main contribution was the sample dilution steps. In this paper, the concentration of Hg, Zn, Cu found in fish muscle using Cold Vapour Atomic Absorption Spectrometry (FIA-CV-AAS), and Atomic Absorption Spectrometry by flame mode was 7.70 ± 0.88 $\mu g\ g^{-1}$, 2.58 ± 0.30 $\mu g.g^{-1}$, 3.13 ± 0.37 $\mu g.g^{-1}$, respectively. The concentration of Pb determined by high resolution inductively plasma coupled mass spectrometer (HR ICP-MS) was 2.32 ± 0.17 $\mu g.g^{-1}$.

Keywords: uncertainty, fish tissue, mercury, FIA-CV-AAS

1. INTRODUCTION

Fish muscles are often used as biomarkers for metal pollution, providing important information about the environmental quality. This information can be used not only by biomonitoring programs, as well as, to identify anthropogenic sources of pollution. Thus, the determination of metals in fish samples requires the use of robust, reliable and validated methodologies.

Method validation is a set of procedures which confirms if an analytical procedure is in conformity for its intended use. Among several parameters, the estimate of uncertainty plays an important role in method validation. The uncertainty of measurement is a doubt about the result of any measurement, expressing its quality [1].

The estimate of uncertainty is generally conducted in accordance with the principles established by the Guide to the Expression of Uncertainty in Measurement-ISO GUM (GUM). The procedure is based on the identification, and quantification, of the influence of each analytical parameter used during the process of measurement provided by the change in the quantification of uncertainty process of measurement [2].

Since each parameter has its own uncertainty, the final estimate of uncertainty of the measurement takes in consideration the contribution of all of them and is expressed as the combined standard uncertainty [2], [3].

This paper aimed to describe the estimate of uncertainty for Hg measured by Cold Vapour Atomic Absorption Spectrometry, Zn and Cu concentrations by flame mode and Pb by HR ICP-MS in the fish tissue.

2. MATERIALS AND METHODS

2.1 Instrumentation

The Hg, Zn and Cu analysis were performed using a Fast-Sequential Atomic Absorption Spectroscope Varian, model Spectr-AAS-220-FS. Lead was determinate by using high resolution inductively coupled plasma mass spectrometer, (HR ICP-MS), ELEMENT 1, Thermo Finnigan. The samples were digested by using a microwave system (CEM Corporation, Mars 5).

2.2 Sample Preparation

Circa of 1.0g of muscle tissue was weighed directly in 100mL Teflon tubes and added 5 ml of concentrated HNO_3, 3ml H_2O_2 and 2 ml H_2O with a resistivity of 18 Ω . Samples digestions were carried out in a microwave system using the following conditions: 600 W, temperature ramp 10 min, 145 PSI, final temperature: 145°C and hold time: 5 min. After digestion, the solutions were diluted adding milli-Q water until 20 mL for Cu and Zn analysis. For Pb determination, the samples were diluted again (1:2) and 5 ppb Indium was used as internal standard for analysis by HR-ICP-MS [4]. For Hg analysis, tissues were digested in acid mixture (HNO_3 1 mL, H_2SO_4 2 mL and $HClO_4$ 1 mL) in glass flasks for 30 minutes in hot plate at 110 ° C, following the method described by Lima et al [5].

The certified concentration, and its uncertainty associated, for Hg is (4.64 ± 0.26) $\mu g.g^{-1}$ in the reference material; For Cu the concentration of certified is (2.34 ± 0.16) $\mu g.g^{-1}$ and for Zn is (25.6 ± 2.3) $\mu g.g^{-1}$ in the reference material certified Dorm-2 (NRCC). The concentration

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of Pb and associated uncertainty in this material certified Oyster tissue (NIST) is (0.308 ± 0.009) $\mu g.g^{-1.}$

2.3 Cause-effect diagrams

The cause-effect diagram (also known as an Ishikawa or fishbone diagram) consists of a hierarchical structure culminating in a single outcome. It is used with the objective to facilitate the identification of the main sources of uncertainty associated with each step of the measurement protocol of the metals of interest. The central vector represents the measurand and the ramifications are the contributions of different factors that affect the analysis results. The cause effect diagram for AAS and the cause effect diagram for HR ICP-MS measurements are presented in the figures 1 and 2 [6].

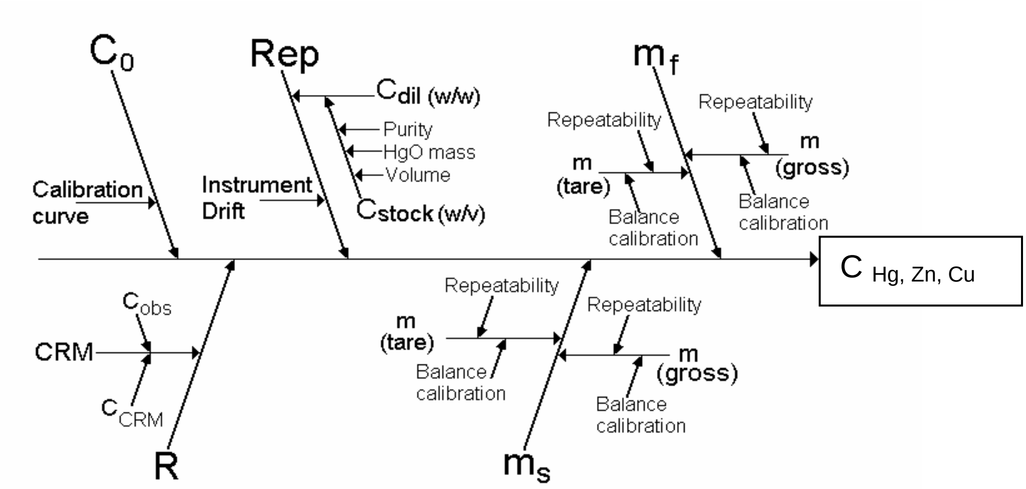

Figure 1. Cause-effect diagram for the analysis by Atomic Absorption

Where: C_o - Concentration of aliquot sample; R – Recovery of reference material; m_f - The final dilution mass; m_s – Sample mass; Rep - Precision associated with the dilution of calibration solutions (C_{dill} stock solution).

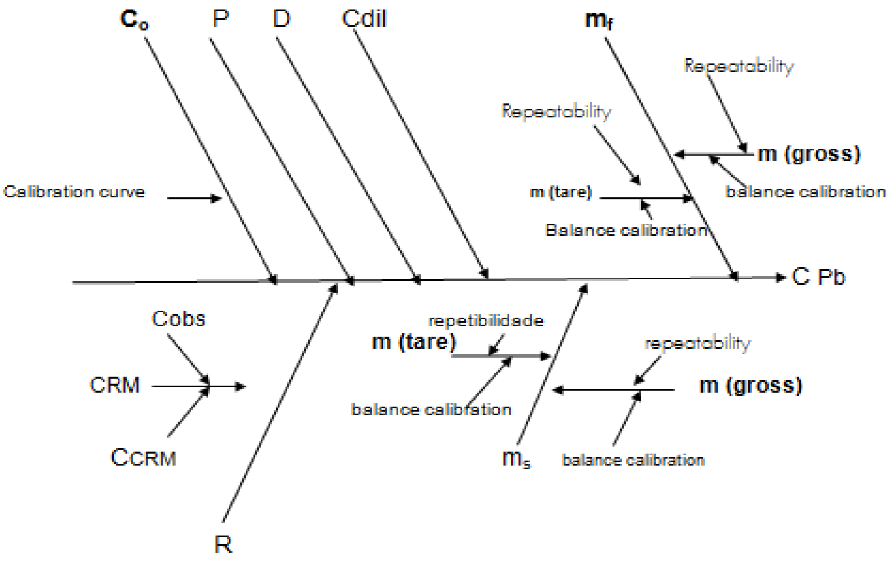

Figure 2. Cause-effect diagram for the analysis by HR-ICPMS

Where: C_o – Concentration in the analyzed aliquot; P – precision; D – Dilution; C_{dil} – Dilute concentration; m_f – The final dilution mass; R – Recovery of reference material; m_s – Sample mass.

3. RESULTS AND DISCUSSION

The determination of metals by AAS technique used the same protocol. In this case only mercury will be presented the calculation step by step.

3.1. m_f - The final dilution mass

The mass of sample in the experiment were 1g. The uncertainty associated with standard deviation of control chart is 0.001g and uncertainty associated with calibration certificated is 0.0005g.

$$u(m) = \sqrt{(u_1)^2 + (u_2)^2} \qquad (1)$$

Where: μ_1 is uncertainty of certified of balance; μ_2 is standard deviation obtained in weighing.

$$u(m) = \sqrt{(0.0005)^2 + (0.001)^2}$$

$u(m_f)= 0.001$

3.2. C_{dill} - Uncertainty of the preparation of solution standards

The analytical curve was constructed from a stock solution of 100µg.Kg^{-1}, prepared with six different concentration of metals from SPEX -Certprep standards with 1000 µg.mL^{-1}, the uncertainty of preparation of a solution $C_{dil}{}^{1}$ was obtained by the equation 2.

$$\mu_{C_{dil\_100}} = C_{dil\_100}\, x \sqrt{\left(\frac{\mu_{C_{dil\_10}}}{C_{dil\_10}}\right)^2 + \left(\frac{\mu_{m_t}}{m_t}\right)^2 + \left(\frac{\mu_{m_f}}{m_f}\right)^2} \tag{2}$$

Where: C_{dill_100} is concentration of 100µg.Kg^{-1}; $\mu_{Cdill\ 10}$ is uncertainty of solution of 10 µg.g^{-1} ; μ_{mt} is uncertainty of initial mass of solution (10 µg.g^{-1}); m_t is initial mass of solution (10 µg.g^{-1}); μ_{mf} is uncertainty of final mass of solution (10 µg.g^{-1}); m_f is final mass of solution (10 µg.g^{-1}).

$$\mu_{C_{dil\_100}} = 100.007 x \sqrt{\left(\frac{0.0428718}{10.001}\right)^2 + \left(\frac{0.00118}{1.018}\right)^2 + \left(\frac{0.00118}{100.007}\right)^2}$$

$\mu_{cdil\_100} = 0.443$

3.3. C_o - Concentration of aliquot sample

After the digestion procedure the metals were determined using an analytical curve based in a equation of 1° degree (y = ax+b), and the uncertainty is represented by u (C_o).

$$u(c_o) = \frac{S}{B_1} x \sqrt{\frac{1}{p} + \frac{1}{n} + \frac{(c_o - \bar{c})^2}{Q_{xx}}} \tag{3}$$

Where: S is the residual standard deviation; B_1 is the slope of the calibration curve; p is the number of measurements to determine C_o; n is the number of measurements for the calibration; $\underline{c}$ is the mean value of the different calibration standards; j is the index for the number of measurements to obtain the calibration curve.

$$u(c_o) = \frac{0.011594}{0.0288} x \sqrt{\frac{1}{3} + \frac{1}{18} + \frac{(408.52 - 13.9)^2}{1655.2213}}$$

$u(c_o) = 3.9$

3.4. R – Recovery

The estimate of uncertainty for recovery was calculated analyzing a certified reference material DORM-2 dogfish muscle. The concentration of Hg, in the aliquot sample of reference material obtained was 16.100.

$$\mu_{sol} = C_{sol} x \sqrt{\left(\frac{Inc_{s\tan dart}}{C_{s\tan dart}}\right)^2 + \left(Inc_{balance}\right)^2} \tag{4}$$

Where: C_{sol} is concentration of Hg in the aliquot solution prepared; $Inc_{standard}$ is uncertainty of standard solution; C_{standard} is concentration of solution standard prepared; Inc_{balance} is uncertainty of certified of balance.

$$\mu_{sol} = 15.467 x \sqrt{\left(\frac{0.26}{4.64}\right)^2 + (0.0011)^2}$$

$\mu(_{sol}) = 0.867$

R is calculated using Eq.

$$R_m = \frac{C_{obs}}{C_{MRC}} \tag{5}$$

Where: C_{obs} is the mean of the results obtained from the replicate analysis of the solution and certified reference material, this analysis R = 1.04.; C_{MRC} is concentration of reference material analysis.

$$R_m = \frac{16.1}{4.64}$$

$R_m = 1.04$

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$$\mu_{Rm} = R_m x \sqrt{\left(\frac{\mu_{sol}}{C_{sol}}\right)^2 + \frac{S_{obs}^2}{n \times C_{obs}^2}} \tag{6}$$

Where: μ_{sol} is uncertainty of solution of reference material analysis; C_{sol} is concentration of Hg in the aliquot solution prepared; S^2_{obs} is Standard deviation of replicate analysis results of the reference material certified; *n* is number of replicates; C^2_{obs} is concentration obtained from analysis of the reference material certified.

$$\mu_{Rm} = 1.04 x \sqrt{\left(\frac{0.867}{15.467}\right)^2 + \frac{0.05^2}{3 \times 16.1^2}}$$

μ_{Rm} = 0.06

Table 1 shows the values of standard uncertainties and relative standard uncertainty for the contributions of measurement uncertainty for the determination of Hg by the FIA-CV-AAS.

Table 1. Summary of contribuitions to the measurement uncertainty for the determination of Hg by FIA-CV-AAS.

Source of uncertainty	Values	standard uncertainty u(x)	Relative standard uncertainty u(x)/x
(Co) in the aliquot of sample (µg.Kg^{-1})	7.703	3.91	0.010
Recovery R	1.04	0.058	0.056
Final mass mf (g)	20.018	0.0011	0.0011
Sample mass ma (g)	1.0	0.0011	0.0011
(Cdil) stock solution (µg.Kg^{-1})	100.007	0.443	0.004

3.5. Calculation of expanded uncertainty

The measurement uncertainty was calculated from the given in Table 2 according to the rules set out in the Eurachem Guide [6].

$$u_{C_{Hg}} = C_{Hg} x \sqrt{\left(\frac{u(C_o)}{C_o}\right)^2 + \left(\frac{u(Sol_{100ppb})}{Sol_{100ppb}}\right)^2 + \left(\frac{u(m_f)}{m_f}\right)^2 + \left(\frac{u(R)}{R}\right)^2 + \left(\frac{u(m_a)}{m_a}\right)^2} \tag{7}$$

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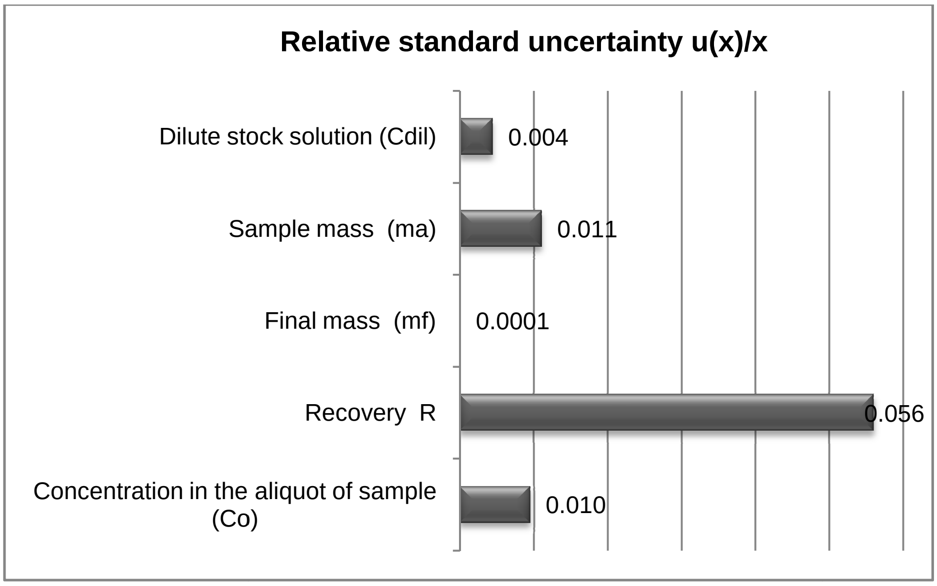
Where: C_{Hg} is concentration of Hg in the sample; $\mu(C_o)$ is uncertainty of concentration of Hg in the solution analysis; C_o is concentration of Hg in the solution analysis; μ(Sol) is uncertainty of solution of 100µg.g^{-1} prepared; Sol is concentration of solution of 100µg.g^{-1} prepared; $\mu(m_f)$ is uncertainty of associated with calibration of the balance and mass final of the sample; m_f is the mass final of the sample; μ(R) is uncertainty of recovery of reference material certified; R is the recovery of reference material certified; $\mu(m_a)$ is uncertainty of associated with the initial sample mass and calibration of the balance; (m_a) is initial mass of the sample.

$$u_{C_{Hg}} = 7.703x\sqrt{\left(\frac{3.9}{408.52}\right)^2 + \left(\frac{0.443}{100.007}\right)^2 + \left(\frac{0.001}{20.018}\right)^2 + \left(\frac{0.058}{1.04}\right)^2 + \left(\frac{0.001}{1.019}\right)^2}$$

u_{CHg} = 0.44 x 2 = U = 0.88 µg.g^{-1}

Based on this value, was calculated the uncertainty expanded using a coverage factor of two, which gives a level of confidence of approximate 95%, the $u(C_{Hg})$ = 0.88µg.g^{-1}.The contributions of the parameter and influence quantities to the measurements uncertainty are illustrated in Graphic 1.



Graph 1. Illustration of contributions to the uncertainty budget (µg.g^{-1}).

Next, was calculated step by step the estimate of uncertainty for the determination of lead.

3.6. m_a - Mass of sample

The mass of sample in the experiment were 1g. The uncertainty associated with standard deviation of control chart is 0.001g and uncertainty associated with calibration certificated was 0.0011g and repeatability of standard weight scale with mass close to the sample was 0.0007g

$$\mu_{M_a} = \sqrt{(\mu_1)^2 + (\mu_2)^2 + (\mu_3)^2} \tag{8}$$

Where: μ_{Ma} is uncertainty of certified of balance; μ_1, μ_2, μ_3 is standard deviation obtained in weighing.

$$\mu_{M_a} = \sqrt{\left(\frac{0.0011}{2}\right)^2 + (0.001)^2 + \left(\frac{0.0007}{1.002}\right)^2}$$

$\mu_{Ma} = 0.001$

3.7. D- DILUTION

The uncertainty of the dilution was obtained by the initial and final mass of the sample.

$$\mu_D = Dx\sqrt{\left(\frac{s_1}{m_1}\right)^2 + \left(\frac{s_2}{m_2}\right)^2} \tag{9}$$

Where: D_x is dilution; S_1 is standard deviation of successive weightings of the weight of mass m_1; m_1 is initial mass; S_2 is standard deviation of successive weightings of the weight of mass m_2; m_2 is final mass.

$$\mu_D = \frac{20.000}{0.2} x \sqrt{\left(\frac{0.00063}{19.999}\right)^2 + \left(\frac{0.0018}{0.19}\right)^2}$$

$\mu(D) = 0.95$

3.8. C_{dill} - Uncertainty of the preparation of a solution standards

The six standards of analytical curve were prepared from diluted of SPEX-Certprep solution of 1000 µg.mL^{-1} for Pb. The uncertainty was calculated by equation 10.

$$\mu_{C_{dil}} = C_{dil}\, x \sqrt{\left(\frac{\mu_{Cstock}}{C_{stock}}\right)^2 + \left(\mu_{equip}\right)^2} \tag{10}$$

Where: C_{dil} is Concentration of the diluted stock standard (ug/L); μ_{cstock} is uncertainty of provided by the manufacturer's stock solution (ug/mL); C_{stock} is concentration of solution stock; μ_{equip} is uncertainty of equipment (balance).

$$\mu_{C_{dil}} = 1.389 x \sqrt{\left(\frac{0.003}{1.0106}\right)^2 + \left(0.00041\right)^2}$$

μ_{cdil} = 0.004

3.9. P- Precision

The precision was calculated with the preparation of two solutions of different concentrations analyzed three times, the average of three measurements was: Solution 1 is y_s = 4.15745 and solution 2 is y =370.925.

$$\Pr ecision(X) = \frac{y_s}{y} \tag{11}$$

Result of average = P (x) = 0.015

3.10. C_o - Concentration of aliquot sample

The concentration of the Pb was estimated from calibration linear curve with equation of 1º degree (y = ax+b), and the uncertainty was represented by u (C_o).

$$\mu_{C_0} = \frac{S}{B_1} x \sqrt{\frac{1}{p} + \frac{1}{n} + \frac{(c_0 - \bar{c})^2}{Q_{xx}}} \qquad (12)$$

Where: S is the residual standard deviation; B_1 is the slope of the calibration curve; p is the number of measurements to determine C_o; n is the number of measurements for the calibration; $\bar{c}$ is the mean value of the different calibration standards; j is the index for the number of measurements to obtain the calibration curve.

$$u(c_o) = 0.534 x \sqrt{\frac{0.388}{2.125} + 0.027}$$

$u(_{Co}) = 0.162$

3.11. R – Recovery

The uncertainty of the method of recovery was calculated from the preparation of certified reference material Oyster Tissue for two solutions of 10 and 50 µg.Kg^{-1}. The concentration of Pb in this material certified was as 0.308 ± 0.009 with the uncertainty was quoted at the 95% confidence level, the average value of the solutions was:

$$\mu_{Rm_{pool}} = \sqrt{(\mu_{Rm_{SolA}})^2 + (\mu_{Rm_{SolB}})^2} \qquad (13)$$

Where: μ_{RmSolA} is uncertainty calculated for reference material certified of dilution A; μ_{RmSolB} is uncertainty calculated for reference material certified of dilution B.

$$\mu_{Rm_{pool}} = \sqrt{(0.3342)^2 + (0.3338)^2}$$

$$\mu_{Rm_{pool}} = 0.47$$

The table 2 shows the values of standard uncertainties and relative standard uncertainty for the contributions of measurement uncertainty for the determination of Pb by HR ICP-MS.

Table 2. Summary of contributions to the measurement uncertainty for the determination of Pb by HR ICP-MS.

Source of uncertainty	**Values x**	**standard uncertainty u(x)**	**Relative standard uncertainty u(x)/x**
Sample mass (m_a) (g)	0.200	0.001	0.006
Dilute concentration (C_{dil})	1.4	0.004	0.003
Recovery (R)	1.004	0.472	0.472
Precision (P)			0.015
Concentration in the analyzed aliquot (C_{aliq}) (µg.kg^{-1})	2.32	0.085	0.036
Dilution (D) (g)	100	0.947	0.009

3.12. Calculation of expanded uncertainty

The measurement uncertainty was calculated from the given in Table 3 according to the rules set out in the Eurachem Guide [4].

$$\mu_{C_{element}} = C_{element}\,x\sqrt{\left(\frac{\mu_{M_a}}{M_a}\right)^2+\left(\frac{\mu_{C_{dil}}}{C_{dil}}\right)^2+\left(\frac{\mu_R}{R}\right)^2+\left(\frac{\mu_{\Pr ecisão}}{\Pr ecisão}\right)^2+\left(\frac{\mu_{C_{aliq}}}{C_{aliq}}\right)^2+\left(\frac{\mu_D}{D}\right)^2} \qquad (14)$$

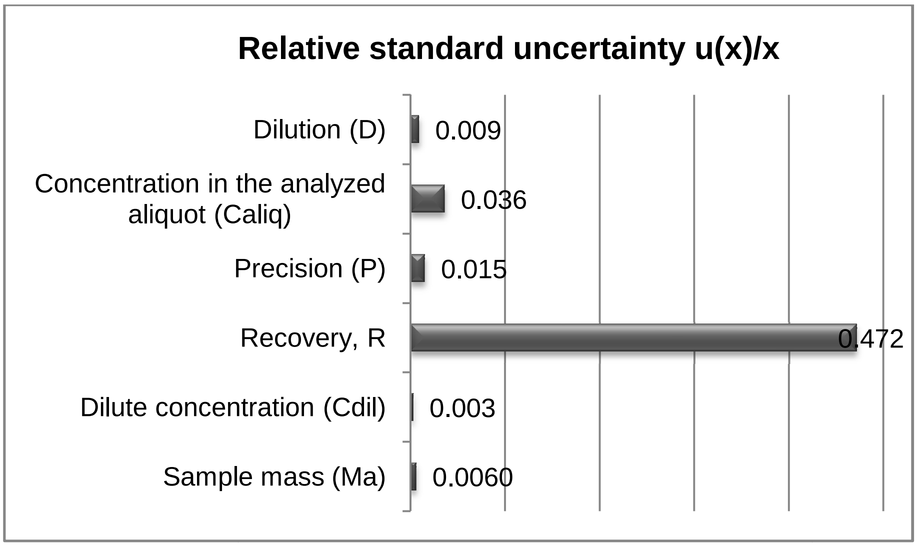
Where: C_{elemt} is concentration of Pb in the sample; μ_{Ma} is uncertainty of certified of balance; μ_1, μ_2, μ_3 is standard deviation obtained in weighing; M_a is initial mass of the sample; μ_{Cdil} is uncertainty of the diluted stock standard; C_{dil} is Concentration of the diluted stock standard; µ(R) is uncertainty of recovery of reference material certified; R is the recovery of reference material certified; $\mu_{precision}$ is uncertainty of the precision calculated; μ_{Caliq} is uncertainty calculated for the analyzed aliquot; C_{aliq} is concentration of element in the aliquot analyzed; μ_D is uncertainty calculated for dilution; D is dilution for analysis.

$$\mu_{C_{elemento}} = 8.679x\sqrt{\left(\frac{0.0013}{0{,}200}\right)^2+\left(\frac{0.0042}{1.4}\right)^2+\left(\frac{0.4724}{1.004}\right)^2+(0.015)^2+\left(\frac{31.0367}{0.37}\right)^2+\left(\frac{0.947374}{100}\right)^2}$$

$\mu_{Celemento} =$ 83.88 x 2 = U = 167.76 µg.Kg^{-1} = 0.17 µg.g^{-1}

Based on this value, the expanded uncertainty was calculated using a coverage factor of two, which gives a level of confidence of approximate 95%. The obtained result is: u (C_{Pb}) = 0.17 $\mu g.g^{-1}$.

The contributions of the parameter to the measurements uncertainty are illustrated in grafic 2.



Graphic 2. Illustration of contributions to the uncertainty budget in $\mu g.g^{-1}$.

The concentration value obtained and the corresponding global uncertainties estimates are presented in Table 3.

Table 3 - Levels of Hg, Zn, Cu in the gray catfish, region of Cosipa and Pb of region of Araçarã and the respective uncertainty associated U in $\mu g.g^{-1}$.

Common name/gender or species	$\mu g.g^{-1}$ ± U			
	Hg	Zn	Cu	Pb
Gray Catfish/ Ariidae	7.70 ± 0.88	2.58 ± 0.30	3.13 ± 0.37	2.32 ± 0.17

***expanded uncertainty for k = 2 with a confidence level of 95%**

In analytical reports is common to express the expanded uncertainty as U ± y, where y is the value of the measurand [3].

2. CONCLUSION

The estimate of uncertainty is an important analytical tool for quality assurance of analytical measurement, providing traceability and reliability of the results presented by the laboratory. In this work we observe the distinction between techniques. The dominant parameter for the uncertainty in the analysis by AAS and by HR ICP-MS was the recovery of reference material, obtaining 11.4% of uncertainty for Hg and 7.2% for Pb, this result could be improved by opting for a certified reference material with lower uncertainty.

Finally, it would be interesting to use a simulation of the components of uncertainty to see what plan of action is best suited to reduce the final uncertainty for each analytical method.

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