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Total Mercury Determination in Muscle and Liver Tissue Samples from Brazilian Amazon Fish Using Slurry Sampling

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Abstract
This paper presents a slurry sampling method for total mercury determination by graphite furnace atomic absorption spectrometry (GFAAS) in tissue of fish from the Amazon. The tissue samples were lyophilized and macerated, and then the slurry samples were prepared by putting 20 mg of tissue, added to a solution containing Triton X-100, Suprapur HNO_3, and zirconium nitrate directly in sampling vials of a spectrometer. Mercury standard solutions were prepared under the same conditions as the slurry samples. The slurry samples and the mercury standard solutions were sonicated for 20 s. Twenty microliters of slurry samples were injected into the graphite tube, which contained an internal wall lined with tungsten carbide. Under these conditions, it was possible to thermally stabilize the mercury up to an atomization temperature of 1700 °C. The method was validated by mercury determination in reference materials DORM-4 and DOLT-4. The LOD and LOQ were 0.014 and 0.045 mg kg^{-1}, respectively, and recovery percentages in relation to the concentration values were certified in the order of 98%.

Keywords: Amazon fish, Mercury in fish tissues, Slurry samples, GFAAS

Introduction

Several studies developed in the last two decades have highlighted high mercury concentrations in Brazilian Amazon fish species. In general, all of these papers point to a bioaccumulation along the trophic chain as being responsible for the high mercury content in species from this region. Since the algae and aquatic plants of the Amazon rivers have higher mercury concentrations than the waters of these rivers, the fish that feed exclusively on these algae and plants have higher mercury concentrations than the algae. This phenomenon is heightened in predatory fish, which have mercury concentrations one million times higher than the water of the rivers [1–3].

The main source of food for the human population of the Brazilian Amazon is fish. The species of fish that are most consumed by the population of this region include: dourada (*Brachyplatystoma rousseauxii*), filhote (*Brachyplathystoma filamentosum*), tucunaré (*Cichla* spp.), tambaqui (*Colossoma macropomum*), pirarucu (*Arapaima gigas*), piranha preta (*Serrasalmus rhombeus*), and barba chata (*Pinirampus pirinampu*) [4]. Among these species, dourada, filhote, tucunaré, pirarucu, piranha preta, and barba chata feed mainly on smaller fish, while the tambaqui is omnivorous, feeding principally on aquatic plants but also on small fish [2]. Thus, mercury may be contaminating the riverine population from the Brazilian Amazon region, through their consumption of large amounts of fish. Therefore, the mercury concentrations in fishes consumed in the Brazilian Amazon region should be constantly monitored. In this context, new methodologies for mercury determination in fish tissue samples will contribute to the control of mercury concentration in fish [2–6].

The problems that living beings experience due to mercury exposure are discussed in several papers [7–11]. "This toxic element, mainly in organic form, may accumulate in tissues and organs of aquatic organisms in concentrations higher than those found in water. In humans, mercury can pass through biological membranes from the mother to the fetus, and thus cause anatomical abnormalities and severe damage to the central nervous system. High concentrations of mercury in rodents and humans can cause hepatotoxicity, nephrotoxicity and neurological damage" [5].

Papers published in the last decades have highlighted slurry sampling for metal determination by graphite furnace atomic absorption spectrometry (GFAAS) as a robust technique [12–14]. Besides presenting such advantages as high sensitivity and low detection limits, the graphite tube allows the injection of solid sampling to be used, thus enabling the determination of volatile elements without requiring a mineralization sample step. In this case, the volatile element can be thermally stabilized by the use of an appropriate chemical modifier [12–14]. The elimination of the mineralization sample step is a significant advantage in determining trace elements such as mercury, because it reduces the sample manipulation and consequently the possibility of contamination.

From the viewpoint of the discussion above, the objective of this present work was to describe a new slurry sampling method for mercury

determination by GFAAS, using muscle and liver tissue samples of fishes from the Brazilian Amazon.

Material and Methods

Reagents, Sample Collection, and Preparation

The fish species were captured from the Madeira River in the area covered by the Jirau Hydroelectric Power Plant, in Porto Velho, Rondônia, Brazil. Fish catch points are as follows: (a) S 09° 16′ 12.8″ and W 064° 41′ 14.1″ and (b) S 09° 11′ 16.98″ and W 064° 36′ 44.53″. Seven adult carnivorous filhote fish (*Brachyplathystoma filamentosum*) have an average length of 105 cm and a mean weight of 20.00 kg, eight adult carnivorous tucunaré (*Cichla spp*.) averaging 40 cm and a mean weight of 3.50 kg, eight adult carnivorous pirarucu (*Arapaima gigas*) averaging 60 cm and a mean weight of 15.00 kg, seven adult carnivorous piranha preta (*Serrasalmus rhombeus*) averaging 35 cm and a mean weight of 2.50 kg, ten barba chata (*Pinirampus pirinampu*) averaging 40 cm and a mean weight of 3.00 kg, and seven adult omnivorous tambaqui fish (*Colossoma macropomum*) averaging 50 cm and a mean weight of 12.00 kg. Fish were collected using the capture method through nets, then sedated in 1 g/15 L benzocaine anesthetic solution of water until complete desensitization and death [15]. A portion of the muscle tissue as well as a portion of the hepatic tissue were removed, identified, placed in sterile tubes, and stored in liquid nitrogen. From these, muscle and liver tissue samples were ground in *mixer* to obtain a *pool* of each sample by species and stored in a freezer at − 80 °C [5].

Approximately 10 g of each *pool* of muscle and liver tissue samples were lyophilized by 72 h, and then approximately 2 g of each sample pool (muscle or liver) were macerated in mortar and pestle in the presence of liquid nitrogen, to obtain particles with a size of approximately 60 mm. Then, one part of the lyophilized and ground tissue was used for the slurry samples preparation, and the other part was mineralized in an ultrasonic cold-water bath, according to the procedure described by Moraes et al. [16].

All the reagents used in this work were analytical grade. The solutions were all prepared with high-purity deionized water (18.2 MΩ cm^{-1}), obtained with an Elga Ionic system (PURELAB Option, USA).The bottles for storing solutions— along with glassware and other containers used—were immersed in 10% *v/v* nitric acid for 24 h, rinsed with ultrapure water, and dried before use.

Slurry Samples Preparation

The slurry samples of muscle and liver tissues were prepared in triplicate directly in the autosampler vials of the spectrometer, according to the procedure described by Silva et al. [14], although with the following modifications: 20 mg of samples pool were mixed with aliquots of 1 mL of

a solution containing 0.05% *v/v* Triton X-100, 0.50% *v/v* Suprapur HNO_3, and 100 mg L^{-1} zirconium (chemical modifier), obtained after shaking slurry samples. Then the slurry samples were homogenized by sonication for 60 s before being injected on the graphite tube of the spectrometer.

Apparatus

The mercury determinations were ascertained using a SHIMADZU AA-6800 atomic absorption spectrometer equipped with an ASC-6100 autosampler. Argon was used as the furnace sheath gas. Pyrolytic graphite tubes with integrated platforms were used in the mercury determinations. These tubes had their internal walls covered with tungsten carbide, which acted as a permanent chemical modifier. The absorbance values were measured in peaks area [14].

The slurry samples of muscle and liver tissue, the slurries of fish protein, the certified reference material (DORM-4), and the liver certified reference material (DOLT-4) were sonicated in the Unique ultrasonic cell disruptor. All samples of biological tissues (muscle and liver) and biologically certified materials (DORMA-4 and DOLT-4) were lyophilized using the CHRIST–ALPHA, model 2-4 LD *plus* equipment. The Unique Ultrasonic Cleaner, model USC1800A, was used in an ultrasonic water bath, in the acid mineralization of the samples of biological tissues and biological-certified materials.

Analytical Procedures

The analytical curve was prepared by diluting a 20 μg L^{-1} mercury standard solution with a concentration range of between 0.10 and 2.00 μg L^{-1}. The mercury standard solutions contained the same concentrations of Suprapur HNO_3, Triton X-100, and zirconium as described in the item for slurry samples. Then, 20 μL of these mercury standard solutions were injected into the graphite tube of the spectrometer, using the autosampler. With regards to slurry samples of tissues, volumes of 20 μL were used in the injection step as well. The absorbance values were measured in triplicate, and the graphite tubes heating program, optimized for mercury analysis, is described in Table 1.

Results and Discussion

Adjustment of the Graphite Furnace Heating Program

The correct adjustment of pyrolysis and atomization temperature is fundamental for obtaining exact and reproducible analytical results in determinations of metals and/or metalloids by GFAAS using slurry samples. The pyrolysis temperature deserves more attention, because at this stage, all matrix components of the sample should be eliminated, which favors the atomization process of the analyte. Thus, pyrolysis and atomization temperatures were adjusted to achieve the best thermal

stability of the mercury in the determinations of this analyte in the slurries of fish protein, certified reference material DORM-4, and liver-certified reference material DOLT-4, under the conditions described in the section "Slurry Samples Preparation." Figure 1 (curves a, b, c, and d) illustrates the influence of pyrolysis and atomization temperatures on the thermal stability of mercury in the analysis of reference materials slurry (DORM-4 and DOLT-4).

By analyzing Fig. 1 (curves a and b), it can be observed, as a function of the measured absorbance signals in the pyrolysis stage, that the mercury exhibits thermal stability up to 900 °C, when the absorbance signals undergo a rapid decline. In relation to the atomization temperature, curves c and d (Fig. 1) show values constant in the absorbance signals up to 1800 °C. Thus, the pyrolysis and atomization temperature were selected for all other experiments, given that the thermal stability of the mercury in the pyrolysis stage and the higher absorbance signals in the atomization stage were 300–900 and 1700 °C, respectively (Table 1).

The analyte absorbance signals (AA) and background absorbance signals (BG) are also important parameters in the analysis of slurry sampling. Thus, Fig. 2 (curves a, b, and c) shows the comparison of the best measured absorbance signal (AA) for the mercury with the background absorbance signal (BG), using the optimized pyrolysis and atomization temperatures to the standard of reference materials slurries.

The curves illustrated in Fig. 2 show a relatively small background signal, indicating that the combination of zirconium (coinjected chemical modifier) and tungsten carbide (permanent chemical modifier) was efficient in the thermal stabilization of mercury during the stages of pyrolysis and atomization. In these stages, Hg^{2+} ions should form intermetallic bonds, first with zirconium during the reduction process for Hg^0, which then must be absorbed with the tungsten carbide film deposited on the graphite tube platform. Thus, the combinations of these processes contribute to the thermal stabilization of mercury at an atomization temperature of 1700 °C.

Attainment of the Analytical Curve

After the optimization procedures of the graphite furnace heating program for mercury determination (described in Table 1), an analytical curve was prepared with mercury aqueous standard solutions in the concentration range of 0.10 to 2.00 μg L^{-1}, as described in the section "Analytical Procedures." The line equation obtained for the mercury analytical curve was as follows: $C_{(Hg)} = A_{int} - 0.00324/0.0832$, which gave r (linear correlation coefficient) = 0.9986. The characteristic mass (m_0), detection limits (LOD), and quantification limits (LOQ) were calculated as described by [17]. Thus, the m_0 was calculated in relation to the aqueous standard solution containing 0.50 μg L^{-1} of mercury, producing a value of 1.10 pg; the LOD (LOD = 3r/slope) and LOQ (LOQ = 10r/ slope) values were 18 and 61 ng L^{-1}, respectively.

Application of the Proposed Method

After the procedures of optimization and the LOD and LOQ determinations, the applicability of the developed method was evaluated in total mercury determination of slurry tissue samples (muscle and liver) from the fish species specified in the section "Reagents, Sample Collection, and Preparation." Table 2 shows the results obtained in these determinations. The mercury concentrations determined in muscle tissue samples are below 0.500 mg kg^{-1}, the maximum value permitted by the World Health Organization (WHO) [18]. However, the mercury concentrations in liver tissue samples are above the value recommended by the WHO and are approximately ten times higher than the concentrations determined for muscle tissue samples. These results are in agreement with other works in the literature, which highlight a greater accumulation of toxic metals (such as mercury) in fish liver than in fish muscle [2, 3, 16]. The method's accuracy was evaluated by mercury determination in certified reference materials DORM-4 (fish muscle protein) and DOLT-4 (liver of fish). The mercury concentrations determined for DORM- 4 and DOLT-4 (n = 6), as shown in Table 2, were approximately 1.50% lower than the certified values (DORM-4 determined values, 0.404 ± 0.006 and 2.54 ± 0.04 mg kg^{-1}; DOLT-4 certified values, 0.410 ± 0.055 and 2.58 ± 0.22), showing excellent accuracy of the proposed method (Table 3). The method reproducibility was evaluated using the HORRAT value (Eq. 1), which was calculated by dividing RSD^R (relative standard deviation obtained collaboratively) by $PRSD^R$ (relative standard deviation calculated from the Horwitz equation, Eq. 2) [19]. These calculations were performed on the basis of the mercury concentrations determined for DORM-4 and DOLT-4.

$$HORRAT\ value = RSD_R / PRSDR \quad (1)$$

$$PRSD_R = 2^{(1-0.5\ logC)} \quad (2)$$

where C represents the mercury concentration obtained for DORM-4 and DOLT-4.

The values of RSD^R were 3.71 and 1.72%, and the $PRSD_R$ values were 2.29 and 1.74, respectively, for DORM-4 and DOLT-4. Using this calculation strategy, the method reproducibility was 1.62 and 0.99 in relation to the DORM-4 and DOLT-4 certified standards, respectively (Table 3).

The LOD and LOQ values calculated using 20 mg of DORM-4 and/or DOLT-4 were 0.016 and 0.051 mg kg^{-1}, and 0.014 and 0.045 mg kg^{-1}, respectively (Table 3). It can be observed that the values of mercury concentrations, as presented in relation to the muscular and hepatic tissue samples of Amazon fish (Table 2), are all above the determined LOQ for the proposed method, thus indicating the viability of the slurry sampling method for mercury determination in fish tissue samples. The results obtained by the proposed method were checked with results that used samples mineralized in an ultrasonic water bath, according to the procedure described by Moraes et al. [16]. The results are also listed in

Table 2 and show no significant difference at a 95% confidence level (paired *t* test). In addition, the proposed method presented superior sample throughput when compared with other methods in the literature, but with comparable LOD and LOQ and life of the graphite tube (483 firings) [16, 20–24].

Conclusions

The proposed method for mercury determination by GFAAS using slurry sampling provided results that are equivalent to those obtained with the method in which the initial step involved sample mineralization in an ultrasonic water bath. The main advantage of the proposed method is that it does not require mineralization of the samples, which considerably reduces analysis time. In addition, the proposed method offers LOD and LOQ on the order of 0.014–0.016 mg kg^{-1} and 0.045–0.051 mg kg^{-1}, respectively, using only 20 μL of the slurry samples for each analytical determination. The proposed method can thus be used to monitor the levels of mercury in fish tissue samples, considering the low limits of detection, quantification, and its validation after mercury analysis using DORM-4 and DOLT-4 certified standards, which showed recovery percentages in relation to the concentration values certified in the order of 98%.

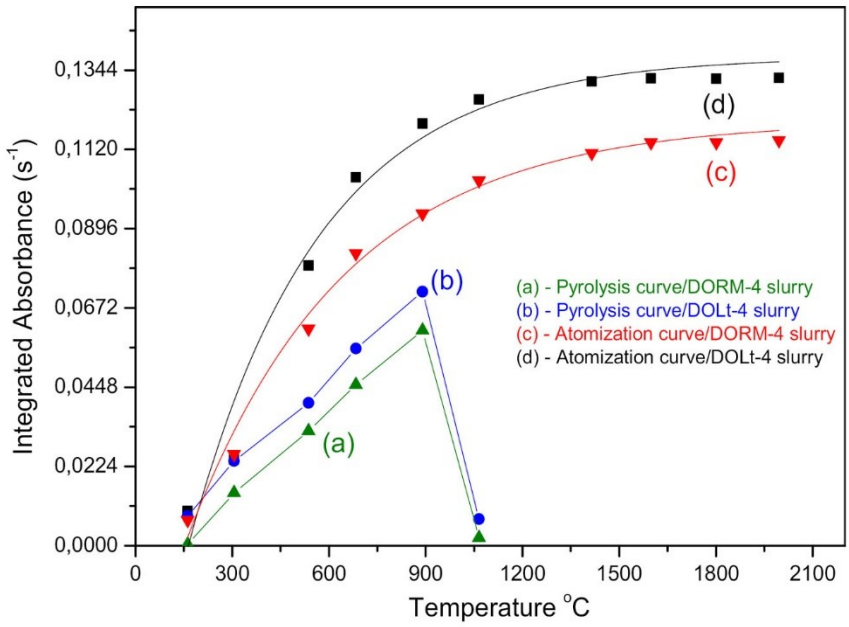

Fig. 1. Pyrolysis and atomization temperature optimized for thermal stabilization of mercury, in slurries of fish protein certified reference material DORM-4 and liver certified reference material DOLT-4

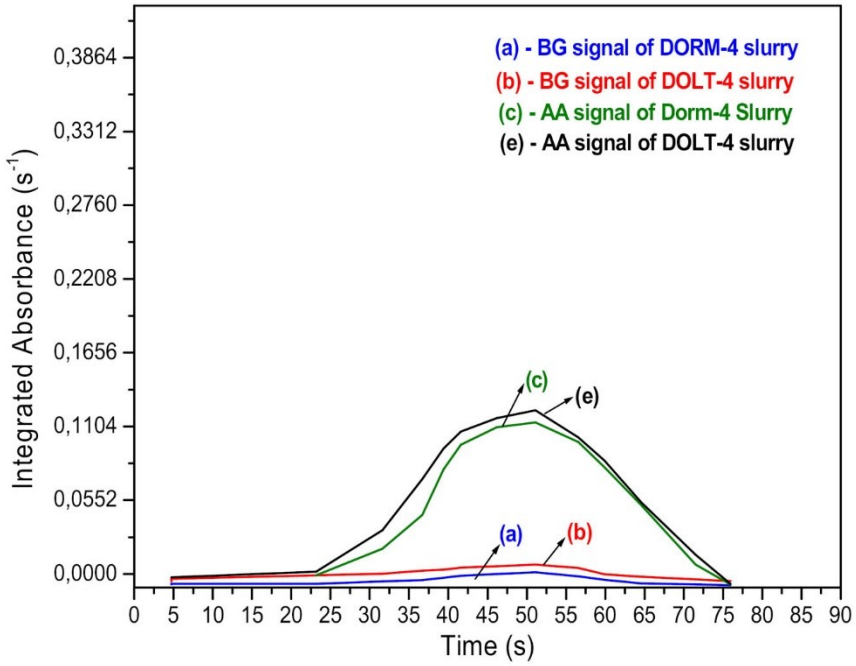

Fig. 2. Transient atomic absorption (AA) and background (BG) signals obtained for mercury atomization, in slurries of fish protein certified reference material DORM-4 and liver certified reference material DOLT-4

Table 1. The graphite furnace heating program optimized for mercury determination from slurries of tissue samples (muscle and liver), slurries of certified reference materials (DORM-4 and DOLT-4), and mercury aqueous standard solutions.

Steps	Temperature (°C)	Ramp (s)	Hold (s)	Argon flow L min^{-1}
Drying	80	5	0	1
Drying	160	5	10	1
Pyrolysis	300	5	10	1
Pyrolysis	900	5	1	0 1
Atomization	1700	2	5	0
Cleanup	2000	5	0	1

Table 2. Results obtained in the total mercury determination in muscle and liver tissue samples from Brazilian Amazon fishes.

Fish species and certified standards	Slurry sampling Muscle tissue (mg kg^{-1})	Acid mineralization Muscle tissue** (mg kg^{-1})	Slurry sampling Liver tissue (mg kg^{-1})	Acid mineralization Liver tissue** (mg kg^{-1})
Filhote	0.091 ± 0.0012	0.087 ± 0.0009	1.09 ± 0.014	1.04 ± 0.013
Tucunaré	0.105 ± 0.0016	0.101 ± 0.0014	1.26 ± 0.018	1.22 ± 0.015
Tambaqui	0.057 ± 0.0007	0.051 ± 0.0009	0.193 ± 0.0027	0.197 ± 0.0031
Pirarucu	0.195 ± 0.0021	0.190 ± 0.0020	0.349 ± 0.00	0.344 ± 0.00382
Piranha preta	0.268 ± 0.0028	0.263 ± 0.0029	0.459 ± 0.00523	0.456 ± 0.00533
Barba chata	0.065 ± 0.0008	0.060 ± 0.0007	0.556 ± 0.0068	0.550 ± 0.0072
DORM-4*	0.404 ± 0.006	0.403 ± 0.012	–	–
DOLT-4*	–	–	2.54 ± 0.04	2.53 ± 0.07

* Fish protein certified reference material DORM-4 containing 0.410 ± 0.055 mg kg^{-1} of total mercury. Liver certified reference material DOLT-4 containing 2.58 ± 0.22 mg kg^{-1} of total mercury.

** Mineralized samples in an ultrasonic water bath

Table 3. LOD and LOQ values, recovery percentage, and reproducibility percentage (HORRAT values) obtained in the mercury determination in the certified standards DORM-4 and DOLT-4

Method validation parameters	Certified standards DORM-4	Certified standards DOLT-4
Determine mercury concentration (mg kg^{-1})	0.404 ± 0.006	2.54±0.04
Certified mercury concentration (mg kg^{-1})	0.410 ± 0.055	2.58±0.22
LOD (mg kg^{-1})	0.016	0.051
LOQ (mg kg^{-1})	0.051	0.045
Recovery percentage (%)	98.54	98.44
HORRAT* values	1.62	0.99

* Reproducibility percentage—calculated using the HORRAT values

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Compliance with Ethical Standards — All the work in this paper that involved animal experimentation was approved by the Ethics Committee on the Use of Animals (CEUA) of the Faculty of Veterinary Medicine and Zootechnics (FMVZ) of the São Paulo State University (UNESP), School of Veterinary Medicine and Animal Science, Botucatu, Brazil, under the number of protocol 110/2015.

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