MERCURY DETERMINATIONS IN BIOHAZARD SAMPLES BY GFAAS USING NOVIPLEX CARD SAMPLING

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ABSTRACT: This paper presents a new method for sampling biohazard samples (mercury-contaminated human and fish blood/plasma and muscle and liver tissue homogenates) using percolation on Noviplex cards for total mercury determination by graphite furnace atomic absorption spectrometry (GFAAS). For the sampling process, 50 μL of the biological samples was percolated onto the sampling disk of the Noviplex card. Three minutes after percolation of the sample aliquot, the sample adsorbed on the card-sampling disk was already dry and showed stability at

room temperature for six months. After the sampling process, the card sampling discs with the percolated samples were mineralized in an acidic medium containing 18 mol L^{-1} H_2SO_4 and 0.02 mol L^{-1} $KMnO_4$ in a 1.0:0.50 (v/v) ratio. Mercury determinations of the acid extracts were performed via GFAAS by injecting 15 μL of sample + 5 μL of zirconium nitrate (chemical modifier) into the graphite tube of the spectrometer, in which the inner wall was coated with tungsten carbide (permanent chemical modifier). The reaction conditions provided thermal stabilization of mercury at atomization temperatures up to 1700 °C. The method was validated for total mercury determinations with extracts from DORM-4 and DOLT-4 reference materials. The calculated LOD and LOQ ranged from 12 to 43 μg kg^{-1}, respectively. The sampling method proved to be quite robust to mercury determinations in biohazard samples.
KEYWORDS: Noviplex cards, mercury in biological samples, electrothermal atomization, chemical modifier.

INTRODUCTION

Mercury is a highly toxic element that is dangerous, responsible for environmental contamination and human intoxication, and is considered a global pollutant [1]. It is known that during the colonial period in Spanish America and later in North America, mercury was widely used in amalgamation processes for gold and silver mining [2]. Intoxication symptoms related to mercury exposure in humans vary depending on the mercury chemical form to which they are exposed, which can cause serious to irreversible damage to the urinary, respiratory, immune, reproductive and central nervous systems [3-8].

Sample collection and preparation is one of the most important steps in the process for monitoring mercury toxicity in biological samples. In recent decades, several strategies for sample preparation and subsequent mercury determinations have been published [9-16]. However, the difficulties related to sampling of biological materials *in situ* and subsequent determination by manipulating the sample as little as possible have not yet been overcome.

In this context, the *Noviplex™* card system developed by Kim and collaborators [17] is an innovative biological fluid collection device that can be used to collect blood aliquots and/or biological extracts. Its viability lies in the sophisticated collection method, which does not require special bottles, equipment, or refrigeration, and in the ability to transport samples without losing the physical and chemical properties of the material. An absorbent disc is saturated with the sampled biological fluid and percolation occurs within three minutes through capillary action, making it possible to transport the cards economically and with minimal biohazard risk to a central laboratory for further analysis, e.g., for mercury.

To overcome the difficulties of mercury sampling, the present study is designed to optimize and validate sampling processes for blood/plasma and homogenates of muscle and liver tissue from mercury-contaminated fish by using the Noviplex™ card system. The card sampling disks are subjected to mineralization by using an acid mixture with chemical modifiers and then used for mercury quantification by GFAAS.

MATERIAL AND METHODS

Sampling procedures with Noviplex cards

The Chico Mendes Institute for Biodiversity Conservation and Authorization and Information System on Biodiversity (ICMBio; Ref. SISBIO 43890-1), Committee on Ethics in the Use of Animals (CEUA), protocol number 0186/2017 and National Research Ethics Commission - NREC, under CAAE No. 43167420.7.0000.5411, authorized this research.

Blood

The sampling procedure was utilized with fish species from the Madeira River, Rondônia/Brazil, which is contaminated by mercury, and with volunteers from the riverside population of the Jirau region - Madeira River, Rondônia/Brazil. After collecting the fish, aliquots of blood were collected from the tails of the animals at the collection site (still on the boat) and percolated onto the sampling disks of the Noviplex cards. The collected aliquots of blood (50 μL) were applied directly to the "test area" on the top layer of the Noviplex card extraction plate. After approximately one minute, a "checkpoint" on the side of the application site changed from white to red, indicating that lateral spreading had occurred and that there was a sufficient volume for the application. After three minutes, the top layer of the extraction plate was peeled off to expose the collection disk, which was adhered to a base plate and contained the stored sample. The sample on the collection disk was dried for approximately 15 minutes and stored at room temperature for further analysis [17]. In total, 24 blood samples were collected from each fish species and humans (volunteers from the riverside population Madeira River).

Plasma eluates and muscle and liver tissue homogenates

Samplings of muscle and liver tissue homogenates were performed by using the following procedure: Approximately 40 mg samples were collected from muscle and/or liver tissue pools of each fish species and homogenized in 500 μL of buffer NP-40 (50 mmol L^{-1} NaCl – *Merck*, 50 mmol L^{-1} Tris-HCl - *GE Healthcare*, pH 8) and centrifuged for 10 minutes at 10,000 rpm and 4 °C, from which the protein extracts were obtained. Subsequently, 50 μL aliquots of protein extract were transferred to the Noviplex card collection disk and allowed to dry for 15 minutes. This procedure was also adopted for plasma elution from the disks of the Noviplex cards used in the collection of blood samples. In total, 12 cards were prepared per pool of protein extracts (muscle and liver) from each fish species. The cards were stored at room temperature until further analysis. For the plasma samples, of the 24 Noviplex cards used in sampling the blood of fish and humans, 12 were used for plasma elution from the Noviplex card sampling discs. Validation of the sampling methodology using Noviplex cards

for determination of total mercury in biological fluid was carried out with extracts from trace metal reference materials DORM 4 - fish protein and DOLT 4 - fish liver, which were certified by the National Research Council Measurement Science and Standards, Ottawa/Canada to contain 0.410±0.055 mg kg^{-1} and 2.58±0.22 mg kg^{-1} of total mercury, respectively.

Mineralization process with Noviplex card sampling discs and plasma eluates

For determinations of total mercury in the blood samples, plasma eluates and muscle and liver tissue homogenates of the fish were collected on Noviplex cards, acid mineralization of the sampling discs and plasma eluates was carried out with an oxidizing mixture containing 18 mol L^{-1} H_2SO_4 (Merck) + 0.02 mol L^{-1} $KMnO_4$ (Merck) in a 1.0:0.5 (v/v) ratio while heating in the range 60 °C to 120 °C in a digester block until the extracts were transparent and showed a light purple color. The acid extracts obtained were transferred to 5 mL volumetric flasks, diluted with ultrapure water (18.2 MW cm^{-1}) obtained from a PURELAB Ultra ELGA purification system. Subsequently, the total mercury concentrations in the extracts were determined by GFAAS according to the procedure described in Section 2.3.

Total mercury determination

Total mercury determinations for acid extracts obtained via mineralization of the sampling disks from the Noviplex cards were carried out by GFAAS using a SHIMADZU model AA-6800 atomic absorption spectrometer equipped with a background absorption corrector with a deuterium lamp, a pyrolytic graphite integrated platform and an ASC-6100 autosampler. A SHIMADZU mercury hollow cathode lamp was used with a current of 12 mA. The wavelength was 253.7 nm, and the spectral resolution was 0.5 nm. Argon was used as the inert gas, and a constant flow of 1 L min^{-1} was maintained throughout the heating program, except during the atomization step, when the gas flow was interrupted. Absorbance signal peak areas were measured. The pyrolytic graphite tubes with the integrated platforms used for mercury determinations had their internal walls coated with tungsten to increase their useful life according to the procedure described by Silva *et al.* [18].

Analytical curve preparation

An analytical curve was prepared by using a Merck standard mercury stock solution and subsequent dilutions to obtain total mercury concentrations ranging from 0.25 to 2.00 μg L^{-1}. Zirconium nitrate was added to the standard solutions to give final concentrations of 20 mg L^{-1} zirconium, which acted as a chemical modifier [19]. Then, 20 μL of the standard solution was injected into the graphite tube of the spectrometer coated with tungsten carbide [18] by using a micropipette of the autosampler. Absorbance measurements were performed in triplicate. The graphite tube heating program optimized for mercury determination is described in Table 1.

Steps	Temperature (°C)	Stages Ramp (s) Heating (s)	Argon Flow (L min^{-1})
Drying	90	5 5	1
Drying	120	5 5	1
Pyrolysis	400	6 6	1
Pyrolysis	800	6 10	1
Atomization	1700	3 0	0
Cleaning	1900	5 5	1

Table 1. Graphite tube heating program optimized for determination of mercury in acid extracts obtained from mineralization of sampling disks from Noviplex cards.

Analytical procedures for sample analyses

The total mercury determinations used 20 μL aliquots obtained by mixing 10 μL of the acid extract from the Noviplex card-sampling disc with 4 μL of 100 mg L^{-1} zirconium nitrate and 6 μL of ultrapure water. The 20 μL aliquots were injected into the graphite tube coated with tungsten carbide using the spectrometer [15, 16]. Measurements were made with three repetitions and used the graphite tube heating program shown in Table 1.

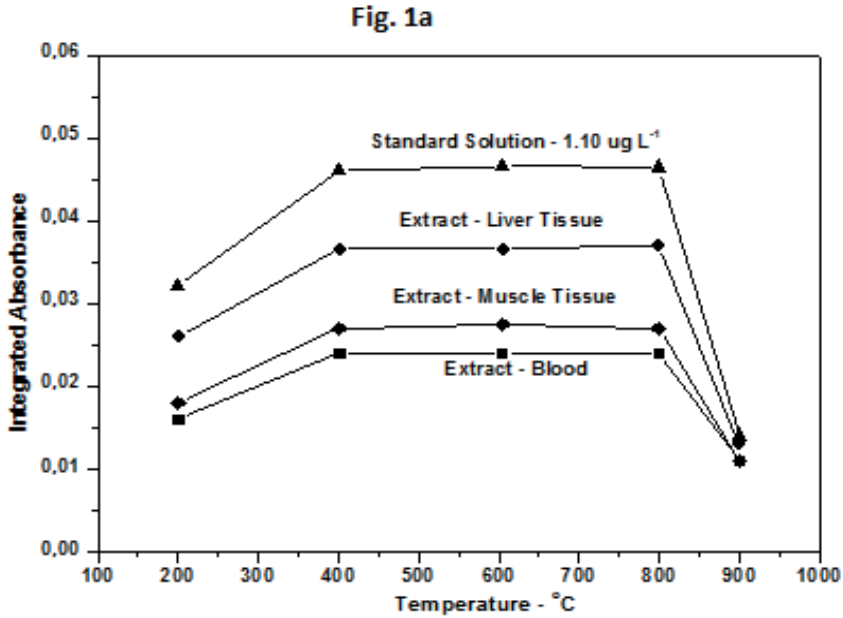
Statistical analyses

The results of the mercury determinations were expressed as the mean ± standard deviation and analyzed with Student's t test and the F test in SAS statistical software version 8 to verify significant differences. The level of significance used was 5% ($P < 0.05$) [15, 16, 20].

RESULTS AND DISCUSSION

Optimization of instrumental parameters

The mercury thermal stabilization temperatures used for pyrolysis of acid extracts obtained from mineralization of the Noviplex card sampling disks and in the mercury atomization process were investigated. For this, the absorbance signals obtained for mercury in a standard solution containing 1.10 μg L^{-1} and in the acid extracts of the samples were compared. The results are shown in Figure 1.



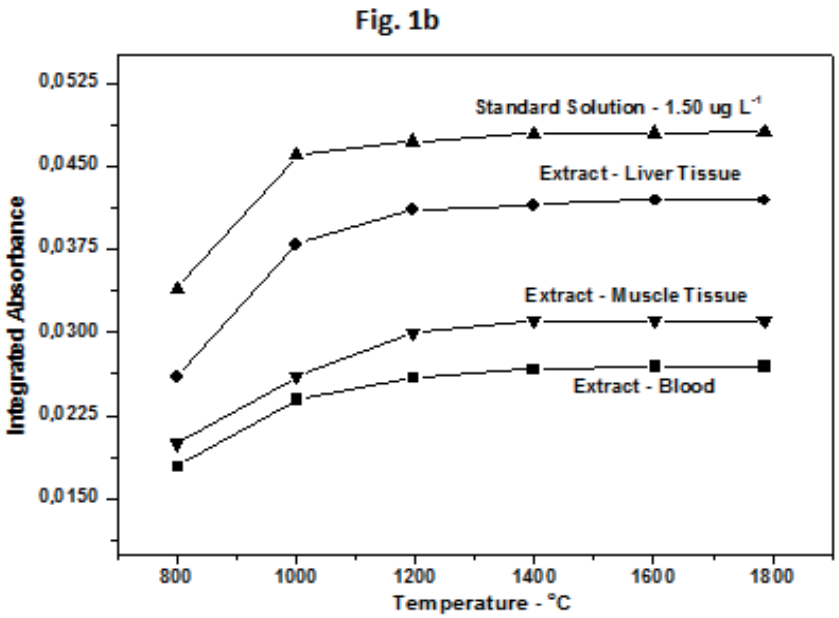


Figure 1. Pyrolysis and atomization curves obtained for acid samples extracted from Noviplex card sampling discs and from a standard solution containing 1.10 μg L-1 mercury.

A pyrolysis temperature of 700 **°C** was chosen because, as shown in Figure 1a, the absorbance signals obtained for mercury remained constant above 400 **°C** and exhibited rapid decreases after treatment above 800 **°C**, which indicated that other components of the sample solution and/or standard solution were pyrolyzed (Moraes et al. 2013). Regarding the atomization temperature (Figure 2.b), it was observed that the absorbance peaks obtained for mercury were constant after heating within the range 1300 **°C** to 1800 **°C**. Considering this, the atomization temperature chosen was 1700 **°C**. The good thermal stability of mercury obtained in these experiments demonstrated the efficiency of zirconium nitrate, which acted as a chemical modifier.

It is possible that the gradual increase in temperature (1000-1800 °C) caused formation of intermetallic bonds between mercury and zirconium during reduction of Hg to the elemental state, which would require a significant increase in the temperature of mercury volatilization and, consequently, of the atomization temperature [18, 19, 20]. In addition, it should be noted that in mineralization of the Noviplex card sampling disks, the extracts showed a slightly purple color, indicating the presence of permanganate ions in the solution. Thus, in the graphite tube heating process, the permanganate ions were reduced to MnO_2, which, together with the tungsten carbide film deposited on the graphite tube wall (permanent chemical modifier), may have contributed to the thermal stabilization of mercury.

Optimization of the analytical curve

After optimizing the pyrolysis and atomization temperatures, an analytical curve was obtained for concentrations ranging between 0.25 and 2.00 μg L^{-1}. The analytical curve for mercury is shown as Equation 1 (with a linear correlation coefficient of 0.9990):

$$\textbf{Conc. (Hg - } \mu\textbf{g L}^{-1}\textbf{)} = \frac{\textbf{Abs. – 0.00086}}{\textbf{0.05106}} \qquad \textbf{(1)}$$

The absorbance values obtained for the mercury standards (n=3) showed good repeatability and reproducibility (relative standard deviations less than 2%), which reinforced the efficiency of adding zirconium nitrate/manganese dioxide/tungsten carbide as chemical modifiers for thermal stabilization of mercury during pyrolysis and atomization with the heating program used. The characteristic mass calculated for the 1.50 μg L^{-1} of mercury standard was 1.93 pg, and the limits of detection (LOD) and quantification (LOQ) for mercury were 0.016 and 0.052 mg L^{-1}, respectively; these were calculated by considering the standard deviation from 10 measurements of the blank and standard solutions and the slope of the analytical curve (LOD = 3s/slope and LOQ = 10s/slope) [21]. The lifetime of the graphite tube was equivalent to 467 burns, which confirmed that the mineralization method used with the disks of the Noviplex cards and the eluates of the plasma samples was efficient for digestion of organic matter and preserved the analyte contained in the aqueous extract.

Application of the proposed method in total mercury determinations

The sampling method using Noviplex cards was applied to total mercury determinations for a pool of blood samples and extracts from muscle and liver tissue from corvina, jaraqui, pirarucu, piranha preta and tambaqui fish species from the Amazon region and for blood samples from the riverside population of the Jirau/Rondônia region located on the margins of the Madeira River in the Brazilian Amazon, a region highly impacted by mercury due to gold mining) [20]. Validation of the method was performed with mercury determinations for extracts from the trace metal reference materials DORM 4 – fish protein and DOLT 4 – fish liver. The results are summarized in Tables 2 and 3.

Samples	Jaraqui fish[a] (μg kg^{-1})	Piranha Preta fish[a] (μg kg^{-1})	Pirarucu fish[a] (μg kg^{-1})	Corvina fish[a] (μg kg^{-1})	Tambaqui fish[a] (μg kg^{-1})	Certified materials[a] (mg kg^{-1})
Muscle	66±1.12	272±5.20	189±3,30	72±1.30	62±1.11	
Liver	113±2.03	470±8,35	341±6.05	283±5.10	202±3.60	
Plasma	4.40±0.08*	6.60±0.11*	5.10±0.08*	5.70±0.09*	4.60±0.08*	
Blood	5.46±0.09*	8.20±0.14*	6.30±0.11*	7.10±0.12*	5.60±0.10*	
DORM-4	-	-	-	-	-	0.402±0.007
DOLT-4	-	-	-	-	-	2.54±0.04

Average value ± standard deviation (n = 6); Concentration – (μg L^{-1})

Table 2. Results obtained for mercury determinations with pooled extracts from blood and plasma samples and muscle (MT) and liver (LT) tissues of Amazon fish species and from the trace metal reference materials *DORM 4* and *DOLT 4*.

Riverside population groups	Blood pool samples[a] (μg L^{-1})	Plasma pool samples[a] (μg L^{-1})
Group 1	0.84±0,07	0.64±0.05
Group 2	1.17±0,05	0.89±0.04
Group 3	3.43±0,20	2.60±0.14
Group 4	10.0±0,41	7.60±0.27

Average value ± standard deviation (n = 6); Group 1 - feed on fish three times a month, Group 2 - feed on fish three times a week, Group 3 - feed on fish until six times a week, Group 4 - feed on fish every day.

Table 3. Results obtained for mercury determinations with pooled extracts from blood and plasma samples from the riverside population of the Jirau region – Madeira River/Brazilian Amazon – using sampling with Noviplex cards.

The muscle and liver tissues and the blood and plasma samples of the fish species studied showed mercury concentrations ranging from 63 to 470 μg kg^{-1} and 4.40 to 8.20 μg L^{-1}, respectively. These results are in agreement with literature values determined using GFAAS and direct acid mineralization of tissue samples [16, 19, 20, 22 – 24]. The results for mercury determinations of blood samples from the riverside population, the Madeira River/ Brazilian Amazon (Table 3), were in the range 0.84 a 10 μg L^{-1}. In the plasma samples eluted from the sampling disks of the Noviplex cards, the concentrations were in the range 0.64 a 7.60 μg L^{-1}; these concentrations are approximately 24% lower compared to the results obtained for blood samples. It is noteworthy that the total mercury levels determined for the pooled blood and plasma samples showed a gradual increase with increasing frequency of fish ingestion by the volunteers of the riverside population, who were divided into the following groups: *pool* 1 - group of volunteers who eat fish three times a month; *pool 2* - group of volunteers who eat fish three times a week; *pool 3* - group of volunteers who eat fish up to six times a week; *pool 4* - group of volunteers who eat fish daily. Thus, a positive correlation was observed between bioaccumulation of mercury and consumption of fish by the volunteers from the riverside population of the Jirau region – the Madeira River/Brazilian Amazon. The values obtained for total mercury concentration in the DORM-4 and DOLT-4 certified materials (Table 3) showed differences of less than 2% when compared to the certified values. Method precision was determined from the relative standard deviations in mercury concentrations determined for the DORM-4 and DOLT 4 NRCs using the HORRAT parameter [25], these values were 1.40% and 1.20%, respectively. The LOD and LOQ calculated by using 20 mg of DROM-4 and/or DOLT-4 NRCs were 12 and 39 mg kg^{-1} and 13 and 43 μg kg^{-1}, respectively. The mercury values determined for blood/plasma and muscle and liver tissue samples from fish (Table 2) and in human blood/plasma samples (Table 3) were higher than the LOQs of the Noviplex card sampling method, attesting to the viability of the method for sampling biohazardous materials.

CONCLUSIONS

The sampling procedure used for biohazardous materials (blood plasma from humans, and blood, plasma and extracts from muscle and liver tissues of fish) proved to be effective for determining total mercury by GFAAS after optimizing the experimental conditions of the analysis. The advantage of the proposed method is that there is no need to refrigerate the samples because the volume percolated on the card disk is stable at room temperature; this eliminates the need for complicated logistics to preserve frozen samples. The use of zirconium nitrate/tungsten carbide/manganese dioxide as a chemical modifier provided thermal stabilization of mercury up to the atomization temperature of 1700 °C in determinations by GFAAS, which enabled determination of mercury at ppb levels (μg L^{-1} and/or μg kg^{-1}) in biohazardous materials contaminated with mercury.

AUTHORS' CONTRIBUTIONS

Conceptualization: Emerson C. Almeida and Nubya G. Cavallini; Methodology: Joyce A. Silva, Luane Benedita Gonçalves Andrade and Camila P. Braga, Jiri Adamec and Pedro M. Padilha; Formal analysis and investigation: Jiri Adamec and Pedro M. Padilha; Writing - original draft preparation: Emerson C. Almeida and Nubya Gonçalves Cavallini; Writing - review and editing: Pedro M. Padilha, Camila P. Braga and Jiri Adamec; Funding acquisition: Pedro M. Padilha; Resources: Pedro M. Padilha; Supervision: Pedro de Magalhães Padilha.

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DATA AVAILABILITY

All data are mentioned in the body of manuscript, tables, and figure.

AVAILABILITY OF DATA AND MATERIAL (DATA TRANSPARENCY)

All raw data will be made available on request.

DECLARATION

ETHICS APPROVAL

The protocols used in this study were approved by: Chico Mendes Institute for Biodiversity Conservation and Authorization and Information System on Biodiversity (ICMBio; Ref. SISBIO 43890-1); Committee on Ethics in the Use of Animals (CEUA), protocol number 0186/2017 and National Research Ethics Commission - NREC, under CAAE No. 43167420.7.0000.5411.

CONSENT TO PARTICIPATE

All volunteer patients (riverside population of the Jirau/Rondônia region - Brazil) agreed to participate of scientific study as stated in the "FREE AND CLEAR CONSENT TERM - FCCT" signed by all and approved by National Research Ethics Commission – NREC (CAAE No. 43167420.7.0000.5411).

CONSENT FOR PUBLICATION

All volunteer patients (riverside population of the Jirau/Rondônia region - Brazil) agreed to the disclosure of research data in scientific journals as stated in the “FREE AND CLEAR CONSENT TERM - FCCT” signed by all and approved by National Research Ethics Commission – NREC (CAAE No. 43167420.7.0000.5411).

COMPETING INTERESTS

The authors Emerson C. Almeida, Nubya G. Cavallini, Victor D. Faria, Felipe D. Cirinêu, Joyce A. Silva, José Cavalcante S. Vieira, Marcelo O. Lima, Luiz F. Zara, Camila P. Braga and Pedro M. Padilha declare no competing interests.

The author Jiri Adamec declare is a co-founder Novilytic, Telimmune and Tenax Bioanalytics.

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