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Validation of a method for direct cadmium determination in breast milk by graphite furnace atomic absorption spectrometry using tantalum treated graphite tube and co-injection of iridium as chemical modifier

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ABSTRACT

A method for direct cadmium determination in breast milk by graphite furnace atomic absorption spectrometry (GF AAS) was developed in this work. Twenty six experiments were conducted for optimization of the pyrolysis temperature and time, type of modifier and sample treatment. The optimum results were 550ºC and 30 seconds for pyrolysis temperature and time, respectively, permanent Ta + co-injection of Ir solution as modifiers and samples prepared at room temperature with nitric acid. This method allowed cadmium determination under the optimized conditions with a calibration curve ranging from 0 to 3.0 µg L^{-1}*. Limit of detection, quantification and characteristic mass obtained were 0.11 µg* L^{-1}*, 0.36 µg* L^{-1} *and 0.37 pg, respectively. Recovery studies at three concentration levels presented results ranging from 96 to 99%. Intra and inter-assay studies revealed coefficients of variation ranging from 1.7 to 11.4%, respectively. The method was applied to determine cadmium in six samples and concentrations ranged from 0.23 to 1.56 µg* L^{-1}*.*

Key words*:* Cadmium, Breast Milk, Method Validation, Graphite Furnace Atomic Absorption Spectrometry

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INTRODUCTION

Acute ingestion of cadmium salts causes nausea, vomiting and diarrhea, whereas inhalation of cadmium fumes gives rise to acute pulmonary edema and to pneumonitis. Cadmium entering the body is bound to a low molecular weight protein metallothionein, which contains a high proportion of cysteine residues. Initial concentrations are in the liver, followed by translocation to the kidney. Long term absorption of cadmium causes renal tubular damage with a marked increase of cadmium excretion in the urine and a low molecular weight proteinuria. It has significant effects on calcium metabolism, which may result from renal damage, although the mechanism remains unclear [1].

Milk and dairy products are important components of the human diet from childhood to adulthood. Breast milk is the principal means of nutrition in an individual’s first years of growth and development. It may also be a source of contamination by cadmium and other metals if the mother is exposed to such substances.

One of the limitations of trace analysis in complex matrices is that sample digestion requires several steps, long preparation times and may result in losses or contamination. Graphite Furnace Atomic Absorption Spectrometry (GF AAS) has been employed to analyze complex samples with little or no sample preparation [2-5] with the advantage of reducing analysis time, reagent costs and avoiding contamination. Because of this limitation, a microwave oven is used for digestion of the milk samples in most of the works published. Likewise, Leotsinidis *et al.* [6] determined Zn and Fe by flame atomic absorption spectrometry (FAAS) and Cd, Pb, Cu and Mn by GF AAS in colostrum and transitory breast milk from Greek women after acid digestion under pressure in an autoclave at 150^{o}C for 3h. The digested sample was then quantitatively transferred to a clean polyethylene tube and the volume corrected to 10 mL with high purity water. Rahimi *et al.* [7] also determined Cd in human milk digesting it in a 25 mL sample with 7 mL HNO_3 and 7 mL of H_2O_2 30% in a partially opened digestion flask. After cooling, the volume was adjusted to 50 mL with double-distilled water. Cd concentrations were determined by GF AAS. Moussa [8] determined Pb and Cd in human milk by eliminating the organic part of the sample (0.50 g). The author added 6 mL of HNO_3, 1 mL 30% H_2O_2 and 0.1 EDTA. The mixture was then heated in a heating plate at 50°C to concentrate, and then diluted to 25 mL with distilled water. The concentrations of Pb and Cd were determined by GF AAS using $Mg(NO_3)_2$ as a chemical modifier in solution. Turan *et al.* [9] determined heavy metals in human colostrum using a tungsten-palladium-citric acid chemical modifier mixture and a wet-ashing procedure was applied to dissolve the samples and to remove the fat. Additionally, two works in the literature involve direct determination of metals in human milk. Falomir *et al.* [10] determined lead in breast milk. The optimum conditions found by the authors for lead determination in breast milk was a sample dilution in Triton X-100 (0.25%), solution chemical modifier (75 µg of palladium) and nitric acid contents (2%). The authors employed univariate optimization and evaluated precision exclusively by intra-assay studies. Furthermore, no tests with permanent modifiers were performed. These substances generally increase graphite-tube lifetime and provide a better *in-situ* digestion of the sample inside the graphite furnace, requiring minimum preparation of the samples. Lara *et al.* [11] developed a method for direct manganese analysis in human milk using multivariate tools for optimization and hydroximethylaminomethane 80% v/v, pH = 8 (TRIS) for sample dilution (1:1). One of the variables studied by the authors in the multivariate analysis was the use of permanent chemical modifiers in solution or non-use of chemical modifiers. The other variables were temperature and time of pyrolysis and atomization temperature. The analytical conditions obtained with multivariate optimization were: pyrolysis temperature of 1347^{o}C, 10s pyrolysis time, atomization temperature of 2691^{o}C and non-use of chemical modifiers. However the pyrolysis and atomization temperatures used in GF AAS temperature program were 1350^{o}C and 2690^{o}C, respectively.

Hydroxymethylaminomethane diluent (TRIS) in the studied concentration (80% v/v), together with the optimized conditions by factorial design and central planning was efficient for sample digestion. This fact can be confirmed by the absence of matrix effect and, therefore, use of aqueous calibration. Quináia and Nóbrega [12] determined chromium concentration in cow milk using a mixture of tertiary amines (CFAC) as a diluent. This reagent can also be applied to analyze metals in breast milk. However, due to its high costs, analysis using CFAC is less viable. An alternative is using other amine reagents as TRIS.

This work was developed to determine Cd in breast milk in order to use tools that allow for the smallest sample preparation to avoid losses or contamination. For this we tested the use of permanent chemical modifiers and/or solution, as well as pre-digestion of the sample with HNO_3 suprapur and TRIS. Similarly to CFAC, TRIS is also an amine reagent.

EXPERIMENTAL SECTION

Reagents and chemicals

All solutions were prepared using water previously deionized in a Milli-Q system from Millipore (Bedford, MA, USA). Nitric acid 65% Suprapur® from Merck (Darmstadt, Germany) was used. Standard solutions were prepared from stock solutions of cadmium 1000 ± 2 mg L^{-1} (Titrisol – Merck) in HNO_3 5% v/v and . For the graphite tube treatment a 1000 mg L^{-1} standard solution of rhodium, ruthenium, iridium and tantalum (Titrisol - Fluka) was used. Tungsten standard solution of 1000 mg L^{-1}, prepared by dissolving 0.18 g of Na_2WO_4 (Merck) in 100 mL of water, iridium atomic spectroscopy standard solution – Fluka, Buchs, Switzerland (part n^o 58195), containing 1000 mg L^{-1} in 1 mol L^{-1} hydrochloric acid and tantalum 1000 mg L^{-1} Fluka, Buchs, Switzerland (part n^o 86275) in 1 mol L^{-1} hydrochloric acid, were used as permanent chemical modifiers. Ammonium dihydrogen phosphate (Reagen, São Paulo, Brazil) and Triton X-100 (Merck) were also used as solution chemical modifiers. Plastic bottles, autosampler cups and glassware materials were cleaned by immersion into 20% (v/v) HNO_3 for at least one day, rinsed several times with Milli-Q water and dried.

An autosampler washing solution containing 0.1% (v/v) Triton X-100 (Merck) plus 0.2% (v/v) nitric acid was used to avoid clogging the autosampler sampling capillary tip due to analyte adsorption and also to improve sample solution dispersion onto the platform.

Apparatus

The absorbances were obtained using an atomic absorption spectrometer SpectrAA Zeeman-220 from Varian

(Victoria, Australia) equipped with a graphite furnace, an autosampler (PSD – 31-972) and a polarized Zeeman background correction, all from Varian. A cadmium hollow cathode lamp from Varian (Part number: 5610124800) was used as light source and operated at 6.0 mA with a spectral band-pass of 0.5 nm at 228.8 nm. Argon 99.999% from Air Products® (Contagem, MG, Brazil) was used as purge gas. Pyrolytic graphite coated tubes (Varian, part no. 63-100023-00) with L'vov platform (Varian, part no. 63-100024-00) were used for Cd analysis.

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Sampling and Sample Treatment

Breast milk samples were obtained through a partnership with Maternity Hospital Odete Valadares (Belo Horizonte, MG, Brazil). The samples were collected and stored in hermetically closed glass flasks and kept in a freezer at -20 °C. They were removed from the freezer to reach room temperature before being employed. One sample was randomly selected for all subsequent studies (reference sample). Sample treatment for Cd determinations, consisted of predigesting of 1 mL of sample with 40 µL of suprapur $HNO_{3,}$ 1-minute agitation with vortex-type agitator and completing the volume to 2 mL. A 1:1 HNO_3 solution was used as blank. Alternatively, samples were diluted 1:1 with 80% v/v TRIS solution for both analytes.

Graphite Tube Treatment

The L'vov platform was treated with tungsten or tantalum as permanent modifiers. The treatment with 520 µg of each modifier was made by applying 40 µL of metal solution (1.0 g L^{-1}) onto the tube platform and subjecting it thirteen times to a furnace temperature program followed by thirteen 40 µL injections of Milli-Q water for tube cleaning [13].

Optimization Strategy

The reference sample spiked with 1.0 µg L^{-1} of Cd was employed to optimize pyrolysis temperature and time, type of modifier and sample treatment. The total volume introduced into the graphite tube was 10 or 20 µL, plus 5 or 10 µL of chemical modifier in solution when necessary.

First, temperature and drying time were optimized, starting from the conditions recommended by the manufacturer and proceeding until no bubbling could be observed in the sample inside the furnace. Next, several experiments were done to optimize the variables mentioned previously. One of the main problems regarding direct determination of cadmium or chromium by GF AAS in complex matrices is the high background absorption of samples, which does not allow for increasing pyrolysis temperature for some as these cited trace elements in breast milk samples. The optimization experiments were performed to minimize background signal without large losses in sensitivity. These experiments consisted of varying by trial and error the pyrolysis temperature and time, modifier type (tungsten, iridium or tantalum as permanent modifiers; iridium or $NH_4H_2PO_4$ 0.2% + HNO_3 0.2% (v/v) + Triton X-100 0.2% (v/v) as modifier in solution) and diluent (HNO_3 or dilution 1:1 with 80% v/v TRIS solution). Each experiment was performed in triplicate readings. Table 1 presents the results of experiments.

RESULTS

Optimization of the analytical conditions

The results of the experiments for optimizing the analytical conditions (Table 1) were plotted on a graph of Cd background versus experiment and absorbance versus experiment (Figure 1). In figure 1A, we can see that the experiments with samples prepared with HNO_3 generate results mostly with lower backgrounds (experiments marked by triangles), therefore, a greater number of experiments used sample diluent such as $HNO_{3.}$ TRIS, an organic substance, is not degraded within the graphite furnace according to the experimental temperatures, thus the obtained background absorbances in the experiments using TRIS were higher. Background absorbance less of than 0.2 units are often required in order to avoid as much interference from the matrix and ensure proper digestion of the samples inside the graphite furnace in addition to ensuring a perfect correction for Zeeman background corrector. The experiments that showed background absorption values less than 0.2 were numbered 15, 16, 20, 22 and 26 for Cd. Among these in the experiment that showed better sensitivity were number 26, as shown in in Figure 1. Experiment 26 corresponded to the following conditions: 550°C for pyrolysis temperature, 30 seconds to time pyrolysis, tantalum as permanent modifier and co-injection of 5µL of Ir as a solution modifier. The atomization temperature was maintained as recommended by the manufacturer, 1800°C. Table 2 presents the temperature furnace program used in the final determination of Cd in breast milk by GF AAS.

Analytical Figures of Merit

The figures of merit were acquired using optimum conditions (table 1, condition 26 and figure 1, condition triangle 26). Three curves using matrix matching calibration and three aqueous curves were obtained within the range of 0 and 3.0 µg L^{-1} using the optimized conditions for cadmium. Matrix effect was evaluated by comparing the averages of the angular and linear coefficients of the curves using statistical tools (F- and Student's t-Tests). The curves presented statistically significant differences at a 95% confidence level, which indicates the presence of a matrix effect. Therefore, all of the subsequent studies were carried out with matrix-matching calibration curves. The limits of detection (LOD) and quantification (LOQ) of the methods were defined by the equations LOD = 3S/b and LOQ = 10S/b, where S is the standard deviation of ten independent samples of the lowest point on the matrix matching calibration curve and b is the slope of the curves. LOD and LOQ were 0.11 µg L^{-1} and 0.36 µg L^{-1} for cadmium and 0.17 µg L^{-1}, respectively. Precision was evaluated through intra- and inter-assay studies. The intra-assay coefficient of variation (CV) was calculated using the standard deviation of the reference sample spiked with 0.5, 1.5 and 2.5 µg L^{-1} of Cd divided by the average of the respective concentration and multiplied by 100. Analyses were performed separately in seven replicates on the same day and read in triplicate. In order to calculate the inter-assay coefficient of variation, the same procedure was repeated with seven replicates of the sample and readings in triplicates over three days. The CV for intra- and inter-assay precision for Cd, ranged between 1.7 and 5.7% and 2.3 and 11.4%, respectively. The characteristic mass (1% absorption) was 0.37 ± 0.04 pg, calculated using data from the calibration curve (the characteristic mass recommended by the author for aqueous solution was 0.2 pg). Accuracy was evaluated using recovery studies with breast milk samples spiked with 0.5, 1.5 and 2.5 µg L^{-1}(n=7). Recoveries showed results ranging from 96 to 99%. Table 3 contains the main parameters of merit of the proposed method.

Table 1: Optimization of the analytical conditions. Phosphate represents a solution of $NH_4H_2PO_4$ 0.2% + HNO_3 0.2 % + Triton X-100 0.2 %

Exper.	Sample treatment	Pyrolysis Temperature (ºC)	Pyrolysis Time (s)	Permanent Modifier	Co-injection	IA, s	Background
1	TRIS	600	30	Ta	Phosphate	0.1313	1.1255
2	TRIS	700	30	Ta	Phosphate	0.088	1.0561
3	TRIS	700	30	W	Phosphate	0.0444	0.28
4	TRIS	650	30	W	Phosphate	0.1177	2
5	TRIS	700	30	W	Phosphate	0.2463	2
6	TRIS	700	50	W	Phosphate	0.1073	1.7325
7	TRIS	700	30	W	Phosphate	0.0563	0.6108
8	TRIS	600	50	W	Phosphate	0.2095	2
9	TRIS	600	50	Ir	Phosphate	0.1825	2
10	TRIS	700	50	Ir	Phosphate	0.1128	2
11	TRIS	600	50	W	Phosphate	0.2341	2
12	HNO_3	600	30	W	Phosphate	0.1671	1.3819
13	HNO_3	700	30	W	Phosphate	0.018	0.2629
14	HNO_3	650	30	W	Phosphate	0.1692	1.5168
15	HNO_3	650	30	W	Ir	0.1137	0.0434
16	HNO_3	600	30	W	Ir	0.1114	0.0474
17	HNO_3	500	30	W	Ir	0.1472	1.5724
18	HNO_3	600	30	W	Ir + Phosphate	0.1715	2
19	HNO_3	700	30	W	Ir + Phosphate	0.1289	0.302
20	HNO_3	600	15	W	Ir	0.1161	0.059
21	HNO_3	600	15	W	Ir	0.1517	0.5711
22	HNO_3	600	15	Ta	Ir	0.1333	0.0609
23	HNO_3	500	30	Ta	Ir	0.1724	1.2908
24	HNO_3	500	30	Ta	Ir	0.1919	0.8572
25	HNO_3	500	50	Ta	Ir	0.2163	0.6891
26	HNO_3	550	30	Ta	Ir	0.1774	0.0915

IA, s = integrated absorbance, s

Table 2: Furnace temperature program optimized for analysis of Cd in breast milk using HNO_3 as diluent. The values of wavelength and slit width were 228.8 nm and 0.5 nm respectively.

Step	Temperature (°C)	Ramp time (s)	Hold (s)	Ar flow rate (L min^{-1})
1	85	5.0	-	3.0
2	95	10.0	-	3.0
3	150	40.0	-	3.0
4	550	20.0	30.0	3.0
5*	1800	0.6	2.0	0.0
6	2300	-	2.0	3.0

**reading*

Determination of cadmium in breast milk

Cadmium concentration was determined in six breast milk samples using the optimized experimental conditions and matrix matching calibration curves. Table 4 presents theses results. Cadmium levels in the analyzed samples ranged from 0.23 to 1.56 µg L^{-1}. The cadmium levels in the samples were less than those found in studies by Rahimi *et al.* [7], Turan *et al.* [9], and Moussa [8]. Rahimi *et al.* [7] determined cadmium and lead concentrations in 44 human milk samples, taken on the first to sixth postpartum week in Zarrinshahr, an industrial area of Iran. The mean ±

standard deviation (SD) of cadmium concentrations in human milk was 2.44 ± 1.47 μg L^{-1} (range 0.62 - 6.32 μg L^{-1}). According to Frkovic *et al.* (1997) [14], in a study using human milk, average background concentrations of cadmium was between 0.05-2 μg L^{-1}, while in the heavily polluted areas they may be up to 20 times higher. The results of cadmium concentration in milk in study, Rahimi *et al.* [7] are similar to those found in Croatia, (Frkovic *et al.* 1997) [14] and in Turkey by Turan *et al.* [9]. Studies by Turan *et al.* [9] had determined the concentration of cadmium and other metals in 30 samples of colostrum from middle class resident mothers of the city of Ankara (one of the most populated cities in Turkey). The average concentration of cadmium and the standard deviation found in these samples were 2.8 ± 1.7 μg L^{-1} with a range of 1.2 a 9.0 μg L^{-1}. Studies by Moussa [8] evaluated thirty lactating mothers, aged 25-35 (mean 30 years), living in three different cities of Egypt. The sampling centers were located in the following cities: (1) Nasr (2) Helwan (3) El Khanka. The results showed that Cd was (0.638 ± 0.032) μg L^{-1}, (1.84 ± 0.092) μg L^{-1} and (2.56 ± 0.12) μg L^{-1} in each city, respectively. In the above mentioned studies, the concentration range of cadmium that most closely matches the results in our study was by Moussa [8].

Table 3. Figures of merit

Regression equation (n= 7)	Abs = (0.11 ± 0.02)C_{Cd} + (0.014 ± 0.007)
R^2 (n = 7)	0.9929 ± 0.0004
Linear range (μg L^{-1})	0.36 – 3.0
Intra-assay precision (%, n = 7)	1.7 – 5.7
Inter-assay precision (%, n = 21)	2.3 – 11.4
Recovery (%, n = 63)	96 - 99
LOD (μg L^{-1})	0.11
LOQ (μg L^{-1})	0.36
Characteristic Mass (pg) (n = 7)	0.37 ± 0.04

Table 4: Cadmium concentration in six breast milk samples (value ± standard deviation, n=3)

Sample	Concentration (μg L^{-1})
1	0.58 ± 0.03
2	0.73 ± 0.08
3	0.38 ± 0.05
4	0.56 ± 0.05
5	1.56 ± 0.12
6	0.23 ± 0.01

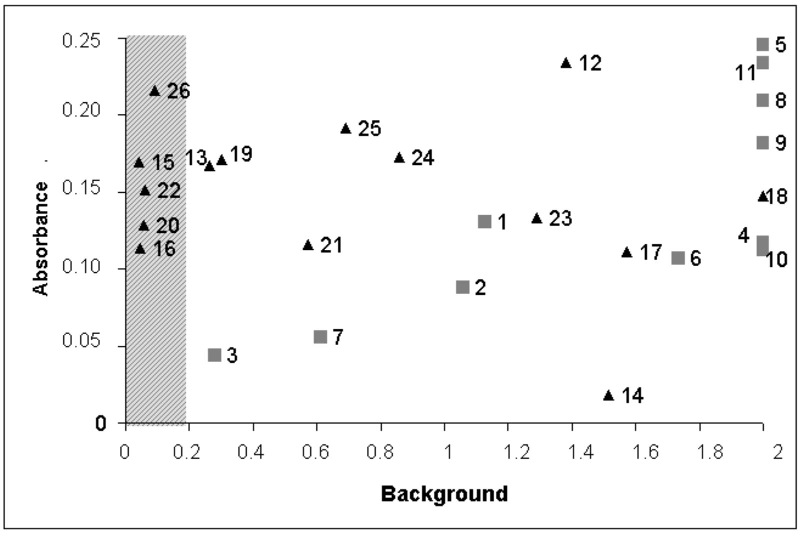

Figure 1: Cd absorbance *versus* background for experiments listed in table 1. The hatched area is the region of interest in which the background is smaller than 0.2 units

CONCLUSION

Nitric acid, compared with hydroximethylaminomethane promotes a predigestion of breast milk, whereas in most experiments using this acid the background signal was lower than when TRIS was used for cadmium determination. Added to this we have the use of permanent modifiers (Ta) and in solution (Ir). The use of these chemical modifiers allowed for a pyrolysis temperature much higher than that recommended by the manufacturer (250°C for 3 seconds), without jeopardizing the loss of sensitivity.

The optimal conditions obtained were 550ºC, 30 seconds, Ta + co-injection of Ir and HNO_3 for pyrolysis temperature and time, modifiers and sample treatment, respectively. The matrix-matching calibration presented sufficient linear correlation among the data. The figures of merit of the proposed method, such as limits of detection and quantification, linearity, intra- and inter-assay precision, recovery, accuracy and characteristic mass, were appropriate for the purpose.

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