



Analytical Methods

Direct determination of Pb in raw milk by graphite furnace atomic absorption spectrometry (GF AAS) with electrothermal atomization sampling from slurries



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ABSTRACT

Milk is an important food in the human diet due to its physico-chemical composition; therefore, it is necessary to monitor contamination by toxic metals such as Pb. Milk sample slurries were prepared using Triton X-100 and nitric acid for direct analysis of Pb using graphite furnace atomic absorption spectrometry – GF AAS. After dilution of the slurries, 10.00 μl were directly introduced into the pyrolytic graphite tube without use of a chemical modifier, which acts as an advantage considering this type of matrix. The limits of detection and quantification were 0.64 and 2.14 $\mu\text{g l}^{-1}$, respectively. The figures of merit studied showed that the proposed methodology without pretreatment of the raw milk sample and using external standard calibration is suitable. The methodology was applied in milk samples from the Guarapuava region, in Paraná State (Brazil) and Pb concentrations ranged from 2.12 to 37.36 $\mu\text{g l}^{-1}$.

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1. Introduction

Milk and its derivatives are important components of the human diet, especially for the elderly and children, because of the presence of compounds such as essential proteins and minerals that are responsible for the growth and sustenance of human life (Maas, Lucot, Gimbert, Crini, Badot, 2011; Soares et al., 2010). The composition of bovine milk is about 85.8% water, 4.9% lactose, 3.9% fat, 3.5% proteins and 0.9% salts (Soares et al., 2010).

As milk is widely used in the human diet, it needs to be collected from healthy animals that are contamination-free (Soares et al., 2010). However, there may be some unwanted contaminants, such as toxic metals, present on pastures or low quality feeds and supplements. Many implements used in the feeding of these animals may contain residues of toxic metals that, when excreted in the milk, end up entering the food chain with humans often being the final receptor (Gonçalves, Mesquita, & Gonçalves, 2008).

Toxic metals can be introduced into the environment by human activities or geological origins, such as from the use of fertilizers and pesticides, atmospheric deposition and/or weathering of rock

matrices (Maas et al., 2011). Toxic metals are one of the largest environmental sources of contamination, causing great environmental, economic and public health impacts and, unlike many other toxic pollutants, they are not biodegradable (Bakkali et al., 2009; Baytak, 2007; Rodríguez et al., 2005). Among the toxic metals, lead (Pb) causes various biochemical changes, all of which are harmful, and there is no evidence of any essential function of Pb in the human body (Larine, 1997 d). Since lead has no biological function their presence even in low levels can cause multiple irreversible damage in human health. Several studies correlated the metal toxicity with neurological, hematologic, renal and cardiovascular effects, therefore lead exposure can promote enzymatic inhibition, severe pathologies and death (Geraldés et al., 2016).

In Brazil, the dairy sector is very important for agribusiness, occurring on approximately one million rural properties. According to data from the Brazilian Institute of Geography and Statistics (IBGE (Instituto de Geografia e Estatística), 2015), milk production in Brazil in 2014 was 35.17 billion litres, and the southern region accounted for 34.7% of the national production. The state of Paraná ranked the second largest producer of milk in the southern region; thus, the determination of trace metals such as lead is important for quality control of milk produced in the region.

The atomic absorption spectrometry technique is widely used for the quantification of metal analytes because of its selectivity and sensitivity. Due to the advantageous technical features (Rodríguez et al., 2005), atomic absorption, it was used for the

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quantification of lead in the milk samples investigated in this work. To ensure that the analytical methodology used provided reliable data on the sample under study, validation of the data was performed (Ribani, Bottoli, Collins, Jardim, & Melo, 2004). The parameters evaluated in the validation of analytical methods were selectivity/specificity, linearity and application range, precision, accuracy, detection limit, quantification limit and robustness (Ribani et al., 2004; Inmetro, 2013).

This study aimed to evaluate the Pb contamination in raw bovine milk samples from the Guarapuava region of Paraná with graphite furnace atomic absorption spectrometry – GF AAS. Existing studies have analysed Pb using GF AAS in different types of milk (breast, raw, processed), but there is a lack of studies using direct sampling. Milk is a complex matrix and because of the presence of fat, direct analysis is difficult because there are problems with clogging of the capillary, deposition of organic material into the graphite tube and spectral and non-spectral interferences (Ataro, McCrindle, Botha, McCrindle, & Ndibewu, 2008). Thus, there is a significant need for the development of direct analysis methods which minimize and/or eliminate contamination and loss of analyte, minimize the use of reactants, and increase the speed of analysis (Falomir, Alegría, Barberá, Farré, & Lagarda, 1999).

2. Experimental

2.1. Materials and methods

All measurements were performed in a graphite furnace atomic absorption spectrometer (Varian GTA120 AA240Z) equipped with an autosampler (PSD 120) and background corrector (Zeeman). A hollow multi-element cathode lamp (Varian) was used, operating at a current of 10 mA, a slit width of 0.5 nm, and 283.3 nm wavelength. Graphite tubes coated pyrolytically were used. All reagents used were of analytical grade. The solutions were prepared using ultrapure water (TKA-GenPure). Nitric Acid (Sigma-Aldrich, 65%) and Triton™ X-100 (Sigma-Aldrich) were also used for sample preparation. The standard solutions were prepared by dilution of stock standard solution of 1000 mg l⁻¹ Pb (Biotec, Brazil). Glassware and plastic containers used were cleaned by soaking in a nitric acid bath of 10% (v/v) for 24 h, then rinsed 3 to 5 times with ultrapure water and allowed to dry at room temperature. The glassware that was greasy (due to the fat in dairy) was left in a potassium hydroxide/ethanol bath 10% (m/v) for 24 h, and rinsed before soaking in nitric acid as described above.

2.2. Samples

For the optimization study and validation of the analytical methodology, raw milk samples were obtained from cattle belonging to the Animal Nutrition Laboratory of the Department of Veterinary Medicine, UNICENTRO, Guarapuava, Paraná, Brazil. All milk samples were collected from producers in Guarapuava-PR region, Brazil. The samples (50 ml) were collected in polyethylene bottles and stored at –2 °C until analysis.

2.3. Analytical procedure

For direct determination of Pb in raw milk samples, slurries were prepared using 2.00 mL of raw milk, nitric acid (0.70 mol l⁻¹) and Triton X-100 (0.5% v/v). The slurries were diluted in 25.0 mL volumetric flask and then 10.00 µl were injected into the graphite furnace.

For optimization of the graphite furnace temperature program, the samples were fortified with 20.00 µg l⁻¹ standard solution of Pb. First, the pyrolysis temperature was optimized in the range

of 300–800 °C and subsequently, the atomization temperature was optimized in the range of 1500–2300 °C. The pyrolysis and atomization temperatures that produced the highest signal/background ratio were considered optimal and applied in the validation study.

2.4. Study validation and application of analytical methodology

2.4.1. Calibration and linearity

Analytical curves were made for calibration (in duplicate) with and without the presence of sample matrix, in order to investigate if the matrix affects the quantification of Pb in raw milk by the proposed method. The curves were made by the external standard method using aqueous solutions (no matrix) and by the method of standard addition (presence of the matrix) in a working range 0–20 µg l⁻¹. The linearity was evaluated by linear regression analysis, where a lack of fit test was used at a 95% confidence level. Statistical analysis was performed using statistical software (Minitab for Windows v. 16.2.2).

2.4.2. Limits of detection and quantitation

The limits of detection (LD) and quantitation (LQ) were calculated by the method of calibration curve construction according to the National Association of Testing Authorities – Guidelines for the Validation and Verification of Quantitative and Qualitative Test Methods (NATA (National Association of Testing Authorities), 2012), where the following equations were used:

$$LD = \frac{3SD}{m}$$

$$LQ = \frac{10SD}{m}$$

where SD is the standard deviation of 10 consecutive measurements of blank solution and *m* is the slope of the analytical curve.

2.4.3. Precision

The precision of the method was evaluated by the reproducibility and the intermediate precision. For reproducibility, sample measurements were performed at three levels of concentration. The milk samples were spiked with 5.0, 10.0 and 15.0 µg l⁻¹ of Pb. Measurements of each concentration level were performed in triplicate. To assess the intermediate precision, the samples were spiked with 10.0 µg l⁻¹ of Pb. Tests were conducted in triplicate on four different days. The results of the precision study were evaluated by calculating the relative standard deviation (RSD,%) according to equation (AOAC (Association of Official Analytical Chemists), 2002):

$$RSD = \frac{s}{\bar{x}} \times 100$$

where *s* = standard deviation and \bar{x} = average Pb concentration.

2.4.4. Accuracy

The accuracy of the method was assessed by Pb recovery tests in the milk samples using two fortification levels, 5.0 and 15.0 µg l⁻¹, and the tests were performed in triplicate. The accuracy assessment was performed by the recovery rate (%), according to the guide of the Association of Official Analytical Chemists (AOAC, 2002).

3. Results and discussion

The objective was to validate a methodology for direct analysis of Pb in raw milk using GF AAS. Slurries were prepared using Triton X-100 in order to stabilize the samples. In Table 1 are the metal

Table 1
Recovery of Pb using different conditions of preparation of the milk slurries.

Volume of milk (ml)	Volume of Triton X-100 (ml)	Volume of Nitric Acid (ml)	Recovery of Pb ^a (%)
1.00	0.125	0	325.80 ± 11.74
1.00	0.250	0	345.47 ± 12.94
1.00	1.250	0	337.75 ± 24.54
1.00	0	1.14	305.72 ± 4.63
1.00	0	2.26	344.35 ± 1.77
1.00	0.125	1.14	175.15 ± 5.02
2.00	0.125	1.14	96.30 ± 1.77
2.00	0.250	1.14	131.13 ± 0.50

^a Fortified samples with 20.00 µg l⁻¹ of Pb.

recovery results for Pb in milk samples using Triton X-100, nitric acid and the combination of two reagents.

As can be seen in Table 1, recoveries higher than 300% were obtained when either Triton X-100 or nitric acid alone was added to 1.00 ml of milk sample, showing that the proportions used in the preparation of slurries were not suitable for analytical validation according to the association of analytical communities AOAC (2002), which allows 70–125% recovery. As the recovery is directly related to the accuracy, recoveries higher than suggested by the AOAC are related to systematic errors such as the interference of the reagents and a sample matrix effect, which was not completely eliminated during pyrolysis step. Triton X-100 was added to the milk sample to ensure homogeneity of the solution, while nitric acid was added to aid in removing the matrix in the pyrolysis step. There was an improvement in the recovery of analyte (175.15%) when the combination of the two reagents were added to 1.00 ml of milk, suggesting a greater efficiency in the pyrolysis step. To evaluate a possible interference, the volume of the milk was increased relative to the reagents used (2.00 ml of milk and 0.125 ml of Triton X-100 and 1.13 ml of nitric acid), and obtained one adequate recovery (96.3%), i.e. within the limits proposed by the analytical validation guide of the AOAC (2002). Therefore, this condition was chosen for the preparation of milk slurries for the direct determination of Pb by GF AAS.

Pyrolysis and atomization temperatures for determination of Pb using the technique of graphite furnace atomic absorption spectrometry indicated by the equipment manufacturer are 400 °C and 2100 °C, respectively. In this work, optimizations were carried

out for the pyrolysis and atomization temperatures of Pb and the results are shown in Figs. 1 and 2, respectively. First, the pyrolysis temperature was evaluated by varying the temperature between 300–800 °C using the milk slurry sample while keeping the atomization temperature fixed at 2100 °C. Fig. 1 shows the ratio between the analytical signal (absorbance) versus peak height for the pyrolysis temperature.

As can be seen in Fig. 1, at a temperature of 500 °C, a higher absorbance intensity for Pb was obtained and at ≥600 °C, there was a decrease in the analytical signal indicating loss by evaporation. In Fig. 1 are also shown background (BG) signals, which are derived from the molecular absorption and sample particles scattering by radiation, which were not completely removed in the pyrolysis step. At 300 °C, a high BG value is observed; between 400 and 500 °C, the background signals decreased and remained fairly constant; at 600 °C, the lowest BG signal was observed, but there was also a decrease in the analytical signal for Pb. Therefore, 500 °C was chosen as the optimum temperature for pyrolysis of in raw milk sample, since it displayed the best ratio between the analytical signal of the sample and background signal.

After the pyrolysis temperature was chosen, it was fixed and then the atomizing temperature was varied between 1500–2300 °C and the results are shown in Fig. 2.

In Fig. 2, one can observe that the highest analytical signal Pb in the sample temperature was observed with an atomization temperature of 1700 °C. At the same temperature, it can be seen that the background signal is reduced compared to the other temperatures studied, indicating that the temperature of 1700 °C is the best temperature for atomizing as it displays the largest analytical signal and smallest background signal. The pyrolysis and atomization temperatures encountered in this study are consistent with those found in the literature for raw and processed milk, 700 °C and 1800 °C for pyrolysis and atomization, respectively (Kazi et al., 2009).

3.1. Analytical validation study

3.1.1. Calibration and linearity curve

The instrument calibration was evaluated in aqueous medium (external standard) in the presence of matrix (standard addition) to determine if the matrix contributed to interference in the quantification of the Pb metal. The curves generated for lead in an aqueous medium (external standard) in the presence of matrix (standard addition) are shown in Fig. 3. The linearity of the curves

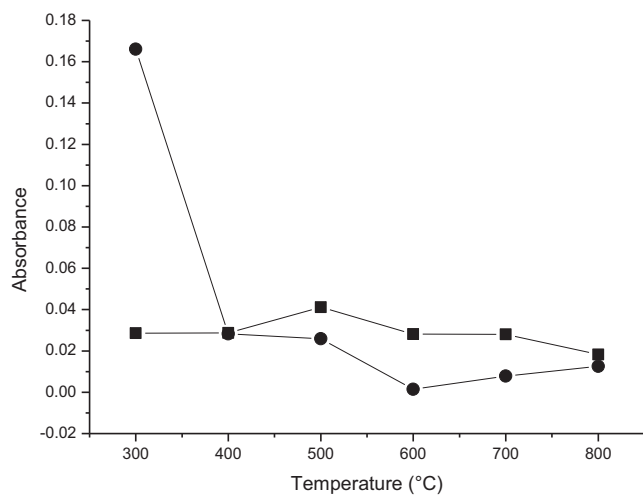


Fig. 1. Optimization of pyrolysis temperature used for direct determination of Pb in raw milk samples employing the GF AAS. Absorbance signals relate to peak height of Pb signals (square) and background signals (circle).

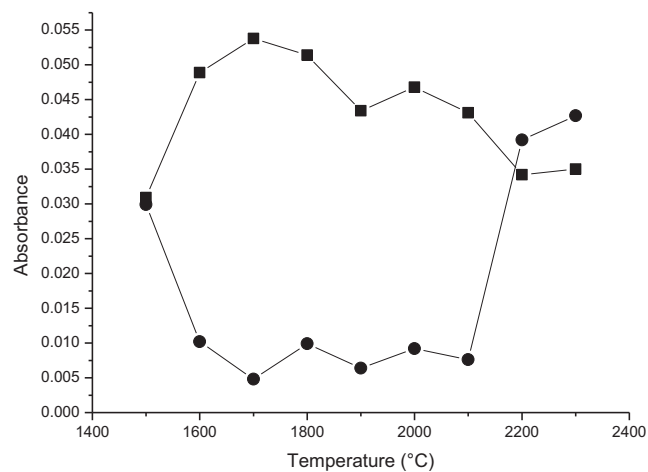


Fig. 2. Atomization optimization used for direct determination of Pb in raw milk samples using GF AAS. Signals relate to absorbance peak height Pb (square) and background signals (circle).

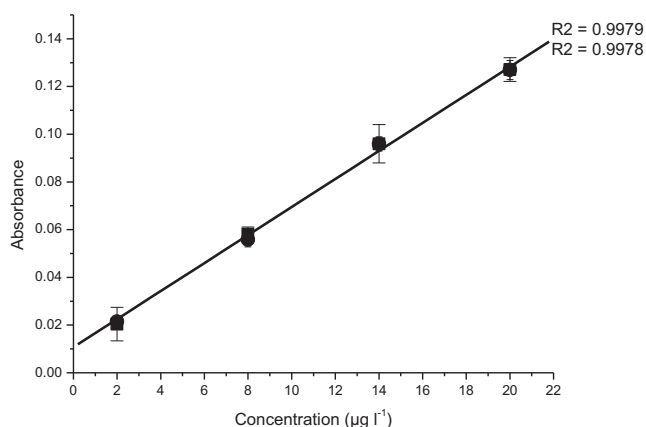


Fig. 3. Analytical curves obtained in the direct determination of Pb in raw milk samples by GF AAS using calibration by external standard (square) and with standard addition (circle).

of the two methods was assessed by regression linear analysis, where the lack of fit test was used at a 95% confidence level. Both curves showed significant linearity in the investigated level of confidence as $F_{\text{regression}} (1472.59)$ was greater than the $F_{\text{critical}} (0.05, 1.6)$ (5.99) and the p values were less than 0.05 for both curves. At the same level of confidence, the two models showed no lack of fit because the $F_{\text{lof}} (1.66)$ values were lower than $F_{\text{critical}} (0.05, 2.4)$ (6.94), and p values were greater than 0.05, so both linear models can be accepted.

As can be seen in Fig. 3, the standard addition curves and external standard were similar, as if they were superimposed, indicating that there is no matrix interference for the proposed method. According to the literature, the nearer to 1.00 the ratio of the angular coefficients of the standard curves and external standard addition lies, the less the interference in the measurement matrix; further, the variation coefficient of the slope coefficients should not exceed 15.00%. The ratio of the angular coefficients for the external standard addition and the internal standard additions was 1.04 and the coefficient of variation was 2.69%; thus, we can conclude that under the conditions studied, the sample matrix does not interfere with the measurements for the direct determination of Pb using GF AAS. For this reason, we chose the external standard curve for quantification of Pb in milk because it is a faster and simpler method and is also cost-effective since there is savings in the lifetime of the graphite tube, due to the reduction in the number of analyzes.

3.1.2. Limits of detection and quantification

The sensitivity of the method was evaluated by the limits of detection and quantification, which were calculated according to the guide of the NATA (2012). The limits of detection and quantification obtained were 0.64 and 2.14 $\mu\text{g l}^{-1}$, respectively. The results were consistent with results found in literature for GF AAS: Lara (2008) in his study of breast milk using the direct sampling method, obtained a LD of 0.92 $\mu\text{g l}^{-1}$ and LQ of 3.07 $\mu\text{g l}^{-1}$ and Kazi et al. (2009), in their work with raw and processed milk using the extraction method assisted by microwave, found a LD of 0.50 $\mu\text{g l}^{-1}$ and LQ of 1.6 $\mu\text{g l}^{-1}$. The direct sampling method was sensitive and suitable for quantification of Pb in raw milk samples using GF AAS.

3.1.3. Precision and accuracy

The results of the precision test were evaluated for repeatability and intermediate precision. Repeatability was evaluated at 3 levels of concentration, and the relative standard deviations were

Table 2
Pb metal recovery in raw milk by GF AAS.

Added concentration ($\mu\text{g l}^{-1}$)	Determined concentration ($\mu\text{g l}^{-1}$)	Recovery (%)
5.00	4.86 ± 0.23	97.20 ± 4.67
15.00	15.16 ± 0.50	101.07 ± 3.35

Table 3

Pb concentration in milk samples collected from producers of Guarapuava region (Paraná State, Brazil).

Property	Concentration ($\mu\text{g l}^{-1}$)
A	13.50 ± 1.24
B	<LD
C	4.31 ± 0.27
D	6.38 ± 0.53
E	<LD
F	37.36 ± 1.75
G	18.50 ± 0.54
H	2.24 ± 0.06
I	11.19 ± 0.27
J	<LD
K	<LD
L	<LD
M	<LD
N	<LQ
O	2.12 ± 0.01
P	<LD

LD, detection limit; LQ, quantification limit.

calculated (RSD) to assess whether they were within the limits established by the AOAC (2002). The RSDs values found were 6.58%, 4.48% and 3.30% for samples spiked with concentrations of 5.00, 10.00 and 15.00 $\mu\text{g l}^{-1}$ Pb, respectively. According to the guide of the AOAC (2002), RSD values for repeatability at trace levels should not exceed 15.00%. As the RSD values were within the recommended range, the method was shown to have a good repeatability for all concentrations.

The intermediate precision was obtained from analyses carried out on 4 different days, and the samples were analysed in triplicate at a concentration of 10.00 $\mu\text{g l}^{-1}$ Pb. In the evaluation of intermediate precision the RSD was used as a measure of the precision of replicas of samples for four different days (data not shown). The RSD calculated was 6.56% and therefore the intermediate precision was an appropriate result, within the limits proposed by the AOAC (2002).

The accuracy of the method was assessed by a Pb recovery test in a raw milk sample. To assess the accuracy of the method, measurements were made with the spiked sample in two concentration ranges: 5.00 $\mu\text{g l}^{-1}$ and 15.00 $\mu\text{g l}^{-1}$. Pb retrieval results in the milk sample are shown in Table 2.

The results obtained in the accuracy study were suitable because recoveries were within the range proposed by AOAC (2002) (70–125%) over the concentration range studied. Recoveries were found in the literature between 102–109% of Pb metal in milk samples using direct sampling (Lara, 2008; Rahimi, 2013). Bilandžić et al. (2011) studied the Pb levels in raw milk from the north and south of Croatia using microwave-assisted decomposition to treat the samples. The authors evaluated the accuracy of the method by metal recovery in the certified reference material, where a recovery of 98.9% was found. Based on the results obtained in this study and the results found in the literature, it can be affirmed that the direct analysis of lead in milk samples was efficient and showed adequate accuracy.

3.1.4. Application in milk samples

After the optimization study and validation of the analytical method, the method was applied to quantify lead concentrations

in 15 samples of raw milk producers of Guarapuava region of Brazil. The results are shown in Table 3. To ensure the integrity of producers in this study, the names of the properties were not disclosed and are represented by letters.

It can be seen from Table 3 that the Pb concentrations in certain milk samples were below the limits of detection and quantification; however Pb contamination was observed in one sample. The sample of the property F, with a content of $37.36 \pm 1.75 \mu\text{g l}^{-1}$, is above the maximum allowed by ANVISA, which is $20 \mu\text{g l}^{-1}$ (ANVISA (Agência Nacional de Vigilância Sanitária), 2013). This property is located away from the highway and industries. Possible contamination of the milk from this property may be related to animal feed. To increase milk production, farmers make use of dietary supplements that may be contaminated with toxic metals, including Pb, if they are of low quality (Marçal, Pardo, Nascimento do, Veras, & Moreno, 2003).

Pb levels obtained in this study were lower than the Pb values found in the literature. Soares et al. (2010) studied the presence of toxic metals in pasteurized cow's milk in the Paraíba Valley region (Brazil), and the average concentration of Pb was found to be $230 \mu\text{g l}^{-1}$, above the permitted by applicable Brazilian legislation it is $20 \mu\text{g l}^{-1}$ (ANVISA, 2013). Gonçalves, Mesquita and Gonçalves (2008) evaluated the incidence of metals in pasteurized cow's milk in the state of Goiás (Brazil). The researchers found that the average amount of lead in mesoregions of Goiás was $238 \mu\text{g l}^{-1}$, concentration that was above the permitted by Brazilian legislation and Codex Alimentarius Commission. Food Standards Programme. (1995), 0.05 mg l^{-1} . According to existing studies, contamination of milk may occur during processing (Kazi et al., 2009).

The low lead levels that were found in some milk samples may be related to the location of the properties where the samples were collected, as they were located far from the city, industries and metallurgy, which may release Pb metal to the middle environment.

4. Conclusion

Since the Pb is an accumulative metal, which causes harmful effects to human health, accurate and precise measurement methods are needed to quantify lead in food products. This study showed that the methodology proposed for direct analysis without pretreatment of bovine raw milk samples for Pb quantification was adequate. The matrix effects were found to be minimal, allowing the use of the external standard method and thereby minimizing the time for analysis and lowering costs. Validation studies of the analytical methodology demonstrated accuracy and precision in the data, which allowed the application of the method to generating reliable data from raw milk samples. The results indicated Pb contamination in milk from one studied property; for this reason, it is necessary to perform routine analyses to quantify Pb.

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