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Nitric acid and hydrogen peroxide in milk and cheese digestion for the detection of metals

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ABSTRACT

Objective: To determine the concentration of arsenic (As), cadmium (Cd), copper (Cu), chromium (Cr), lead (Pb), and zinc (Zn) in milk and cheese by comparing the use of nitric acid (HNO_3) and a mixture of nitric acid and hydrogen peroxide ($HNO_3+H_2O_2$) in acid digestion.

Design/methodology/approach: The milk for the study was collected from storage tanks in various localities of Huejotzingo, and the different types of cheese came from Santa Ana Xalmimilulco, Huejotzingo, Puebla, Mexico. Digestion was carried out in a CEM MarsX microwave. Elemental concentrations were determined by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES).

Results: The combination of $HNO_3+H_2O_2$ in milk digestion resulted in higher concentrations of Cd, Pb, and Zn. Conversely, the concentration of As was higher when using only HNO_3 for digestion. No significant differences were found for Cr and Cu between the two digestion methods. In the case of Oaxaca cheese, digestion with HNO_3 resulted in higher concentrations of As. Acid digestions did not affect the concentrations of the remaining elements. The same behavior was observed for Ranchero cheese, since the digestion combining $HNO_3+H_2O_2$ resulted in lower As concentrations, as compared to the digestion with only HNO_3. Pb and Zn were not significantly affected by the treatments, while the concentrations of Cd, Cr, and Cu could not be determined when samples were treated with the $HNO_3+H_2O_2$ combination.

Limitations: In this work we were able to determine the concentration of a number of metals present in milk and cheese using nitric acid and hydrogen peroxide in acid digestion. However, we recommend more tests are carried out to establish which acid or combination of acids allows for a broader detection of metals.

Findings/Conclusions: According to the results obtained in this work, we can conclude that there is specificity for metal detection, with HNO_3 being the most efficient.

Keywords: Acid digestion, Metal contamination in milk, Cheese.

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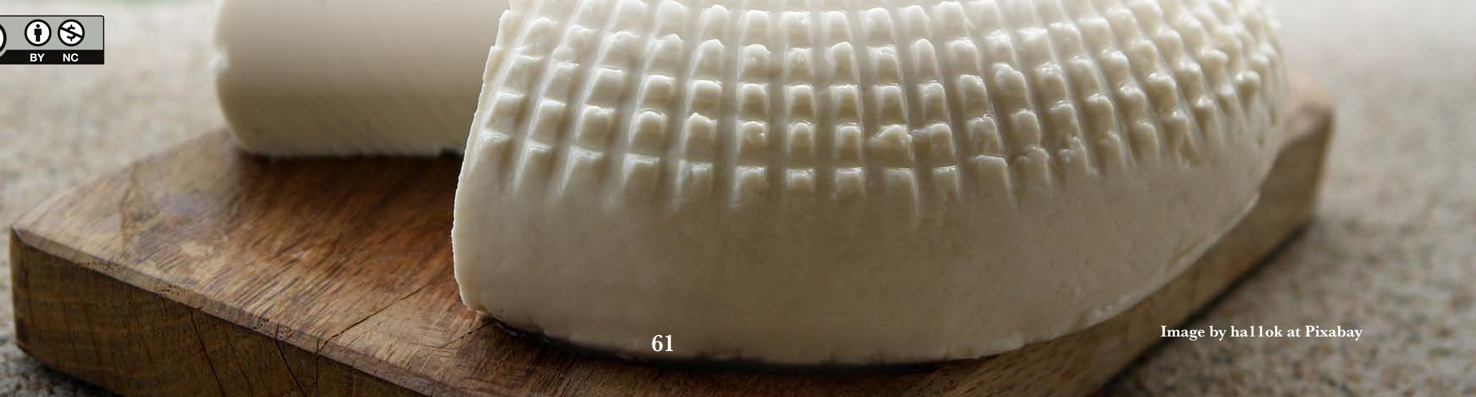
INTRODUCTION

Milk and dairy products are consumed by more than 6 million people in the world, most of whom live in developing countries. However, per capita consumption is higher in developed countries (FAO, 2020). This is because dairy products have a great nutritional value and are considered a complete and balanced food. Therefore, it should be a basic



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component in the diet of human beings, primarily for children due to its relation to linear growth and bone health (Fernández-Fernández *et al.*, 2015; Miedico *et al.*, 2016; Wallace *et al.*, 2020). Nevertheless, when milk is produced in contaminated environments, it can contain toxic substances and metallic elements that can affect its quality and consequently food safety and public health (Harlia *et al.*, 2018; Khaneghah *et al.*, 2020). The presence of elements such as lead (Pb), cadmium (Cd), zinc (Zn), chromium (Cr), and arsenic (As) has been reported in the milk of cattle that had ingested contaminated forage, water, and silage. Consequently, other dairy products may also contain toxic elements that come either from milk or from contamination during the production process. Evidently, this represents a health risk (Moreno-Rojas *et al.*, 2010; Çetinkaya *et al.*, 2016; Castro-González *et al.*, 2017; Castro-González *et al.*, 2018a; Zhou *et al.*, 2019).

Any toxic material contained in milk must be considered serious. Hence, metals contained in food, particularly in milk and its derivatives, constitute a highly relevant issue because they involve a broader contamination of the food chain, and because of the damages this can cause to public health (Mubbasher *et al.*, 2003; Ismail *et al.*, 2017b).

The consumption of metal-contaminated foods has caused various pathologies, such as neurotoxic damage, poor cognitive development, and inadequate organ development, which can lead to death from cardiovascular disorders, kidney failure, and different types of cancer (Dorea and Donangelo, 2006; Arora *et al.*, 2008; ATSDR, 2013; Ismail *et al.*, 2017a; Sujka *et al.*, 2019).

Metals enter milk primarily via the animal's ingestion of contaminated water and food (Castro-González *et al.*, 2018a). To quantify metal concentration, inductively coupled plasma optical emission spectroscopy (ICP-OES) has been used by various authors (Ríos-Arana *et al.*, 2004; Wang *et al.*, 2004; Bakircioglu *et al.*, 2011; Di Giuseppe *et al.*, 2014; Antunović *et al.*, 2018). To prepare the sample, it must be digested in order to destroy the organic matter and obtain a complete solution of analytes. This will allow to conduct the analysis with the greatest possible accuracy (Ayala-Armijos and Romero-Bonilla, 2013). The digestion procedure may have variations that influence the quantification of metals for specific components. It is therefore necessary to assess different methods and find those that will effectively identify the presence of these toxic elements. This in turn will lead to results that allow for control strategizing and risk mitigation.

The town of Santa Ana Xalmimilulco, Huejotzingo, Puebla, Mexico, concentrates a daily production of approximately 45 tons of raw milk —part of which is used to make cheese— from different neighboring communities. Here, animals used for production are fed with forages that are grown in soils irrigated with wastewater containing discharges of different industries located in the area, among them a textile industrial park, the Quetzalcóatl industrial park, and a petrochemical plant.

The objective of this research was to determine the concentration of metals —arsenic (As), cadmium (Cd), copper (Cu), chromium (Cr), lead (Pb), and zinc (Zn)— in raw milk and in two types of cheese, Oaxaca and Ranchero, derived from it, comparing the use of nitric acid and of a combination of nitric acid and hydrogen peroxide in the acid digestion process.

MATERIALS AND METHODS

Location of milk and cheese collection points

The work was carried out in the summer of 2019, in the municipality of Huejotzingo, in the state of Puebla, Mexico (19.12° 30.81' N and 98.24° 36.78' W), at an altitude of 2,228 m (Figure 1).

Sampling and sample transport

Milk samples were collected randomly from milk storage tanks at the different localities studied (Figure 1). This milk comes from small production units, where cattle is fed with forages that are grown in soils irrigated with wastewater. Meanwhile, both types of cheese were acquired from the factory with the largest cheese production in the region, located in Santa Ana Xalmimilulco, Puebla.

Ten 100 g cheese samples were collected —five of Oaxaca cheese and five of Ranchero cheese— every week for five weeks. Milk samples were collected using 50 mL Falcon tubes (Fisher Scientific, Waltham, MN, USA) previously treated according to the methodology described by Castro-González *et al*. (2018a). All samples were transferred in a refrigerator at a temperature of 4 °C to the laboratory, where they were frozen at −80 °C. Subsequently, all samples were lyophilized using a LABCONCO lyophilizer (FreeZone 4.5 Liter Benchtop, Kansas City, MO, USA).

Sample digestion

The milk and cheese samples were digested in a microwave oven (CEM MarsX, CEM Corporation, Mathews, NC, USA). We took 0.5 g samples of the lyophilized matrices for subsequent digestion. We carried out two variants of the digestion process per sample and per matrix. For the first one we used 10 mL of HNO_3 and for the second one, 5 mL of HNO_3 and 5 mL of H_2O_2. Nitric acid was of high purity (65%; Merck, Darmstadt, Germany), while H_2O_2 was 30% v/v (Merck, Darmstadt, Germany). In both cases, the

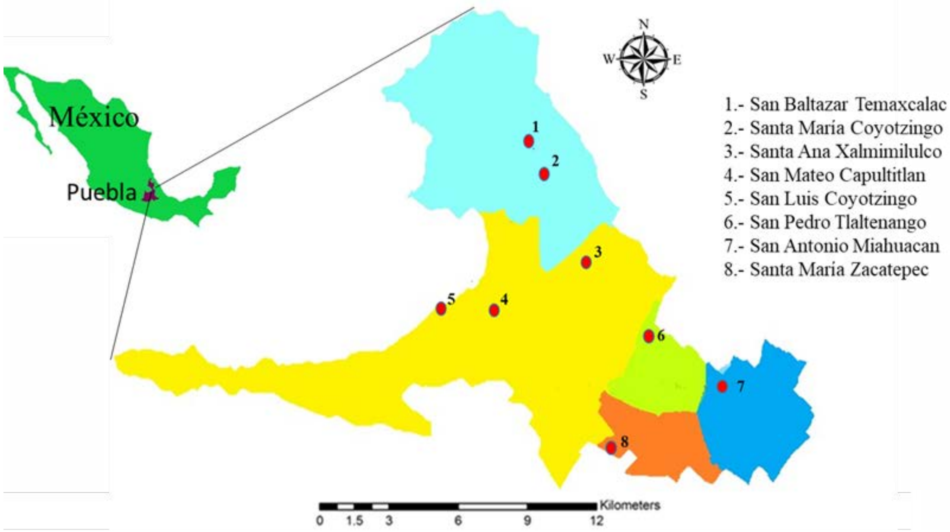

Figure 1. Location of milk collection centers in the municipality of Huejotzingo, Puebla, Mexico.

samples were placed in the microwave at 1600 W power, with a ramp time of 15 min to reach a temperature of 200 °C at a pressure of 800 psi and a holding time of 15 min. Once digested, the samples were filtered using Whatman grade 42 paper (GE Healthcare, Little Chalfont, UK), diluted to 50 mL with deionized water, and left cooling at 3 °C until analysis.

Analyte quantification

The concentrations of As, Cd, Cu, Cr, Pb, and Zn were determined using inductively coupled plasma optical emission spectroscopy (ICP-OES, Varian 730, Mulgrave, Victoria, Australia). The solutions were prepared in type I deionized water (18.2 MΩ cm^{-1}). All chemicals used were analytical reagent grade. Calibration standards were prepared using an ICP multi-element standard solution XVI (Merck, Darmstadt, Germany).

Precision and accuracy levels (Table 1) were established using five targets and ten repetitions. Quality control was carried out with a standard and a control sample, which was applied after every 20 samples analyzed. The method described by Khan *et al.* (2014) was used to determine the limit of detection (LOD) and the limit of quantification (LOQ).

The experimental design was completely randomized. First, data were subjected to the Lilliefors (Kolmogorov-Smirnov) normality test, establishing a value of $\alpha=0.05$. Subsequently, a non-parametric analysis of variance was carried out: the Kruskal-Wallis test. To determine the significant differences between matrices, the Kruskal-Wallis post-hoc test was applied for each metal, using software R version 4.0.4 R Commander.

RESULTS AND DISCUSSION

Table 2 presents the concentration of metals found in milk samples, while Table 3 shows the concentrations found in Oaxaca and Ranchero cheeses.

Milk

The combination of nitric acid and hydrogen peroxide ($HNO_3+H_2O_2$) for milk digestion resulted in higher concentrations of Cd, Pb, and Zn. Conversely, the concentration of As was higher when using only nitric acid (HNO_3). In the case of Cr and Cu, no significant differences were found between the two types of digestions.

Table 1. Precision and accuracy levels and quality control of the milk and cheese samples analyzed.

Standards	Elements					
	As	Cd	Cr	Cu	Pb	Zn
RDS (%)	16.84	12.59	3.19	4.58	14.54	1.66
LOD (mg L^{-1})	0.01	0.001	0.001	0.002	0.008	0.003
LOQ (mg L^{-1})	0.03	0.004	0.002	0.007	0.03	0.009
r^2	0.999	0.999	0.999	0.999	0.999	0.999
Vr (%)	104	106	106	99	101	103

RDS=relative standard deviation; LOD=minimum detectable quantity; LOQ=minimum quantifiable quantity of equipment; r^2=correlation coefficient; Vr=recovery value.

Table 2. Metal concentrations (mg kg^{-1}) in milk, after digestion with nitric acid (HNO_3) and a mixture of nitric acid and hydrogen peroxide ($HNO_3+H_2O_2$).

Metals	**Milk digestion**	
	HNO_3 (mg kg^{-1})	**$HNO_3+H_2O_2$ (mg kg^{-1})**
Cd	0.001±0.001 b	0.010±0.006 a
Pb	0.026±0.013 b	0.035±0.016 a
As	0.130±0.070a	0.080±0.080 b
Cr	0.028±0.003 a	0.024±0.020 a
Cu	0.014±0.004 a	0.016±0.007 a
Zn	0.540±0.080 b	0.740±0.090 a

Means±SD with different letters within each row indicate significant differences ($p \leq 0.05$).

Table 3. Metal concentrations (mg kg^{-1}) in Oaxaca and Ranchero cheeses, after digestion with nitric acid (HNO_3) and a mixture of nitric acid and hydrogen peroxide ($HNO_3+H_2O_2$).

Metals	**Oaxaca cheese**		**Ranchero cheese**	
	HNO_3 (mg kg^{-1})	**$HNO_3+H_2O_2$ (mg kg^{-1})**	**HNO_3 (mg kg^{-1})**	**$HNO_3+H_2O_2$ (mg kg^{-1})**
Cd	0.001±0.001a	ND	0.003±0.001 a	ND
Pb	0.060±0.05 a	0.08±0.03 a	0.110±0.040 a	0.11±0.04 a
As	0.180±0. 110a	0.05±0.07 b	0.160±0.070 a	0.05±0.07 b
Cr	0.010±0.003 a	ND	0.020±0.020 a	ND
Cu	0.010±0.006 a	ND	0.018±0.010a	ND
Zn	0.710±0.180 a	0.84±0.19 a	0.240±0.100 a	0.36±0.2 a

Means±SD with different letters within each row indicate significant differences ($p \leq 0.05$).

Cheeses

Digestion with HNO_3 resulted in higher concentrations of As in the case of Oaxaca cheese. The remaining elements were not affected by either of the treatments. The same behavior was observed in the case of Ranchero cheese, since digestion with $HNO_3+H_2O_2$ resulted in lower As concentrations, as compared to digestion with only HNO_3. Pb and Zn were not significantly affected by either of the treatments, while the concentrations of Cd, Cr and Cu could not be determined when samples were treated with the $HNO_3+H_2O_2$ mixture.

Previous studies have reported As levels in milk below the values found in this work when HNO_3 was used for digestion. For milk produced in La Laguna region of Mexico, for instance, Rosas *et al.* (1999) found As values of 27.4 ng g^{-1} handling a combination of HNO_3, H_2O_2, H_2SO_4, and HCl. In Italy, Licata *et al.* (2004) reported 0.04 mg kg^{-1} of As, using HNO_3 and H_2O_2 (5:2) with atomic absorption spectroscopy. Castro-González *et al.* (2017) evaluated As in milk using HNO_3 for digestion in an area irrigated with wastewater in Mexico and reported 0.034 mg kg^{-1} using inductively coupled plasma optical emission spectroscopy.

The value obtained for As (0.13 mg kg^{-1}) in milk when using HNO_3 in digestion is close to the maximum of 0.15 mg kg^{-1} determined by the WHO-FAO (Codex Alimentarius Commission, 1995) and the corresponding European Union regulation (DOUE, 2006), and below the maximum of 0.2 mg kg^{-1} indicated by the Official Mexican Standard (SSA, 2010).

Taking the detected value of As into consideration is very important, since high concentrations of this element can cause carcinogenic effects by increasing oxidative stress, direct genotoxicity, altered DNA repair, and growth factor expression (Ghosh and Sil, 2015).

Milk digestion with H_2O_2 allows for a better detection of Cd, Pb, and Zn. In the case of Cd, this process evinced a value of 0.01 mg kg^{-1}, which is higher than the 0.001 mg kg^{-1} reported in Mexico by Castro-González *et al.* (2017), who used HNO_3 for digestion, and by Najarnezhad and Akbarabadi (2013) in Iran, who used a mixture of $HNO_3+H_2O_2$. The value of Cd found in this work is below the 0.10 mg kg^{-1} maximum threshold set by the MERCOSUR Technical Regulation (2011). In Mexico, the Official Mexican Standard (SSA, 2010) does not establish a maximum value for Cd in milk. The European Union regulation (DOUE, 2014) establishes maximum limits for Cd in various food products, including cereals, vegetables, meat, fish, shellfish, offal, and food supplements, but it does not do so in the case of milk and milk by-products. The presence of this metal in milk is important, since it brings no benefits to human metabolism, is considered extremely toxic, and has carcinogenic effects (IARC, 2012).

In the case of Pb, we observed that although values for both types of digestion were significantly different, in both cases they exceeded the maximum level of 0.020 mg kg^{-1} for milk according to CODEX (Codex Alimentarius, 1995) and the standards of the European Union (DOUE, 2015; UE, 2017). However, both values were below the maximum of 0.1 mg kg^{-1} set by the Official Mexican Standard (SSA, 2010), and of 0.046 mg kg^{-1} reported by Castro-González *et al.* (2017) using HNO_3 for digestion. It must be noted that these values are averaged over two seasons, unlike the values found in this work, which were limited to summer. The value found in this work with the mixture of $HNO_3+H_2O_2$ is higher when compared to that reported by Najarnezhad and Akbarabadi (2013), set at 0.008 mg kg^{-1} using 4 mL of HNO_3 and 4 mL of $HNO_3+H_2O_2$ per sample for digestion.

The results obtained for Zn in milk are below the values (4.96 mg kg^{-1}) reported by Semaghiul *et al.* (2008) using HNO_3, but they are higher for both digestions (Table 2) than those reported by Castro-González *et al.* (2017) (0.44 mg kg^{-1}) in Mexico using HNO_3.

The average value we found for copper was of 0.016 mg Cu kg^{-1}, which was higher than that reported by Patra *et al.* (2008), set at 0.002 mg kg^{-1} using a combination of HNO_3:$HClO_3$ (4:1; v:v) for digestion, but lower than that reported by Castro-González *et al.* (2017), set at 0.029 mg kg^{-1} using only HNO_3. The concentrations of Cu found for both digestions in milk (0.014 and 0.016 mg kg^{-1}) (Table 2) are lower than those established by Bilandžić *et al.* (2011), of 0.937 mg kg^{-1}, and Malhat *et al.* (2012), of 2.8 mg kg^{-1}.

In both types of cheese, concentrations of As (0.16 mg kg^{-1}) and Pb (0.11 mg kg^{-1}) are below the maximum established by the Official Mexican Standard (SSA, 2010), set at 0.1 mg kg^{-1} and 0.2 mg kg^{-1}, respectively. However, they are higher than the concentrations reported by Castro-González *et al.* (2018b): 0.05 mg kg^{-1} for As and 0.17 mg kg^{-1} for Pb. Moreno-Rojas *et al.* (2010) reported Pb values of 0.013 mg kg^{-1} for Afuega'l pitu cheese and 0.026 mg kg^{-1} for Pasiego cheese, which are below the results obtained for Oaxaca and Ranchero cheeses regardless of the digestion method. Meshref *et al.* (2014) found 0.47 mg P kg^{-1} for Kareish cheese, which is above the value determined in this work for Oaxaca and Ranchero cheeses. However, these authors used a combination of HNO_3: $HClO_3$ (4:1; v:v) for digestion, and flame atomic absorption spectroscopy to perform the metal analysis. Christophoridis *et al.* (2019) reported values of 15.3 μg Pb kg^{-1} for Metsovone cheese and of 12.1 μg Pb kg^{-1} for Cream cheese, which are below the concentrations found in this work. The maximum value for cheese should be compared to the maximum established by milk standards, since cheese is the product of milk coagulation.

Values detected for cadmium using only HNO_3 for digestion were 0.001 mg Cd kg^{-1} for Oaxaca cheese, and 0.003 mg kg^{-1} for Ranchero cheese. Said values are below those found by Yüzbasi *et al.* (2009), set at 0.004 mg Cd kg^{-1} for fresh cheese using HNO_3 for digestion.

The detected values of 0.11 mg Pb kg^{-1} and 0.003 mg Cd kg^{-1} in our study for the case of Ranchero cheese are below the values reported in the case of Turkish Cami Bogazi cheese by Çetinkaya *et al.* (2016), established at 0.18 mg Pb kg^{-1} and 0.028 mg Cd kg^{-1}, respectively, using $HNO_3+H_2O_2$ for digestion in the same proportions as those used in this work. These authors also reported 2.75 Zn mg kg^{-1}, which is higher than the values detected in this research for both cheeses and both types of digestion.

The fact that element detection was better when using HNO_3 may be due to the amount used, since it reduces hydroscopic capacity and increases oxidation in the mixture to be analyzed. Also, it is known that nitrates allow for better readings. However, the detection of some metals requires the addition of other compounds, such as the H_2O_2 used in this work. Nevertheless, mixing compounds can interfere with the detection of some metals, probably because digestion might not be strong enough to provide an efficient detection.

CONCLUSIONS

According to the results obtained, we conclude that there is specificity for the detection of some metals, nitric acid being more effective in detecting As in digested milk. Meanwhile, the combination of nitric acid and hydrogen peroxide is more efficient for the detection of Cd, Pb, and Zn. In the case of cheese analysis, nitric acid is stronger for digestion and better for the detection of Cd, As, Cr, and Cu. It is important to continue testing different reagents to be even more accurate in detecting these elements in food, due to the danger they pose to health and food safety.

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