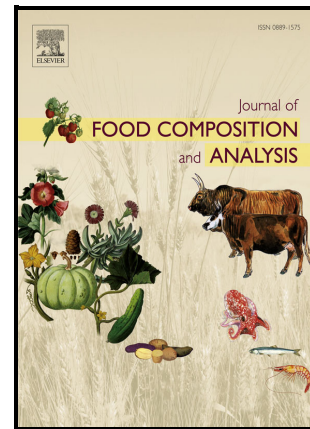# Journal Pre-proof

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Isis S. Permigiani, Nicolas K. Vallejo, Paul E. Hasuoka, Raúl A. Gil, M. Carolina Romero

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Arsenic speciation analysis in cow's milk and plant-based imitation milks by HPLC-ICP-MS

Isis S. Permigiani[a,b,*], Nicolas K. Vallejo[a], Paul E. Hasuoka[a], Raúl A. Gil [a], M. Carolina Romero[b],

[a]Laboratorio de Espectrometría de Masas – Instituto de Química de San Luis (CCT-San Luis) – Universidad Nacional de San Luis. Ejército de los Andes 950, Ciudad de San Luis. Argentina. (5700)

[b]Centro Austral de Investigaciones Científicas (CADIC) - CONICET, Houssay 200, V9410CAB. Ushuaia, Tierra del Fuego, Argentina

*Corresponding author: sabrinapermigiani@gmail.com

Full postal address: 40 Viviendas, N°31. CP: 9410. Ushuaia, Tierra del Fuego. Argentina

Telephone number: +5402901422310 Ext. 142

Fax: +5402901430644

Email address of the other authors

Nicolas K. Vallejos: nkvallejo@unsl.edu.ar

Paul E. Hasuoka: phasuoka@gmail.com

Raúl A. Gil: raulandresgil@gmail.com

M. Carolina Romero: carofrau@gmail.com

Abstract

Imitation milks based on vegetables have become relevant in recent years as a substitute for cow´s milk. Total and inorganic content of arsenic (As) determination in food is of utmost importance due to toxicological concerns. This study aimed to (1) optimize and validate a methodology for speciation analysis using anion exchange high performance liquid chromatography coupled to plasma mass spectrometry and (2) analyze the arsenic content in milk and imitation milks.

Determination of As (III) and As (V) was done following a simple and innovative treatment, which consisted on the dilution of a small volume of sample in mobile phase and further filtration. Furthermore, acid microdigestion for total As determination with small amounts of acid and sample was optimized. The detection limits of the method ranged from 0.2 to 0.7 µg L^{-1}. Regarding to arsenic content we found that imitation milks based on peanuts and nuts presented the lowest inorganic As content (between 1.7 to 1 µg L^{-1}) whereas the highest values were found in imitations based on soy, rice and vetch (27.3, 13 and 8 µg L^{-1}, respectively). The cow's milk presented intermediate levels of inorganic As (4 µg L^{-1}). Also, almond- and quinoa-based imitation milks showed inorganic As values below detection limits, so these imitation milks may be the safest to consume with respect to arsenic content.

Keywords: Milk and imitation milks; trace elements; Inorganic arsenic; speciation; HPLC-ICP-MS analysis; *Microdigestion procedure.*

1. Introduction

The consumption of beverages based on vegetables, cereals, seed and even roots has increased in recent years, trying to replace animal milk consumption, especially cow's milk (Lacerda Sanchez et al., 2020). Although cow's milk is recognized for its nutritious high quality, especially regarding protein content, an increasing number of consumers have been reported to develop several kinds of allergies to certain components (Scholz-Ahrens et al., 2020). In this context, plant-based imitation milks become relevant as alternative products, becoming a crucial factor to ensure their nutritional quality.

The paradigm shift in food gave rise to imitation milks that use mainly rice, soybeans, almonds, coconut, nuts, and some seeds as their base (Arbach et al., 2021). In general terms, the production of a plant-based imitation milk consists of disintegrating plant material in water, in some cases being pre-sewn, presoaked or peeled to improve the treatment. Then, steps of grinding, homogenization, pressing and filtering take place to obtain the final food product (Silva et al., 2020). Soy and rice-based imitations appear as the best candidates to replace cow's milk; however, the supplementation of some nutrients is necessary to ensure nutritional quality, especially protein in rice-based beverages (Walther et al., 2022). Imitation milk based on soy contains between 3% and 4% protein and is therefore close to the nutritional composition of milk in this respect, while its carbohydrate and fat content is lower. The protein quality of legumes is higher than that of cereals and almost matches some animal proteins (for example, from beef), but is lower than cow's milk (Fructuoso et al., 2021).

Plant and cereal crops used as raw material for these imitations could be susceptible to containing high levels of toxic elements such as arsenic (As) (Oskarsson & Alexander, 2021; TatahMentan et al., 2023). Arsenic is a metalloid that may occur naturally in rocks and soils and is transferred to groundwater through solubilization or leaching processes. Given the growing damage that our environment is currently undergoing, toxic elements

such as As have reached the food chain of animals and humans (Kandhro et al., 2023). As origin is not necessarily associated with anthropic activities. In many cases, As is a natural "contaminant". Particulary in Argentina, it is found in groundwater in the western region of Buenos Aires, South of Cordoba, North of La Pampa, Southeast of San Luis, and in the provinces of Chaco and Formosa. Knowledge of the natural contamination of water for human consumption with As in Argentina dates back more than 100 years and has not yet been resolved. There is ample scientific evidence indicating that a significant partition of the Argentine territory has water with As levels that are detrimental to health (more than 3000 µg L^{-1}) and are comparable to levels reported in regions such as Bangladesh and India (Astolfi, et al., 198; Mandal & Suzuki, 2002; McClintock et al., 2012). The presence of arsenic is significantly influenced by the geology of the country-Many of the regions affected by Chronic Regional Endemic Hydroarsenicism (HACRE) are located in areas with geological formations rich in arsenic minerals. Natural decomposition and erosion processes release arsenic into groundwater and surface water. Historical and current mining activities in Argentina have contributed to the release of arsenic into the environment. (Nicolli et al., 2012).

This element enters the food chain mainly through: (1) consumption of water with high As content, (2) absorption of crops irrigated with contaminated water and (3) ingestion of water-filtering organisms or their direct predators (Upadhyay et al., 2019). Among the main arsenic compounds, organic arsenic (oAs) (combined with carbon and hydrogen) and inorganic arsenic (iAs) (combined with oxygen, chlorine, and sulfur) can be distinguished. The iAs content is usually considered as the sum of the As (III) (arsenite) and As (V) (arsenate) compounds, whereas within the oAs concentration, the main species are monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) in terrestrial systems. In seafoods and marine organisms, arsenobetaine and arsenosugars

are the predominant oAs forms. The total content of arsenic (AsT) represents the sum of the organic and inorganic species (AsT=oAs+iAs). Toxicity depends on the chemical form of As. Inorganic compounds are considered more toxic and harmful to health than organic ones (Guillod-Magnin et al., 2018; Redan et al., 2019), for this reason, quantifying the amount of iAs with an appropriate analytical technique is essential.

Arsenic has been designated as a carcinogen by the International Agency for Research on Cancer since 1980 (Cancer, 2012). Such risk depends on the degree of exposure to the element and the type of As species: iAs compounds are considered Group 1 carcinogens, while organic compounds, MMA and DMA, are considered possibly carcinogenic, included in Group 2. Among the main consequences of a long exposure to high doses of As are different types of cancer, skin lesions, respiratory diseases and difficulties in the urinary tract. On the other hand, chronic exposure to low-doses of As can cause, among other conditions, digestive problems, and diabetes (Chávez-Capilla et al., 2016; Kollander et al., 2019). Approximately 100 years after the discovery of environmental exposure to As, there is still no accurate information on the magnitude of the exposed population (Navoni, 2013). Available literature indicates that a portion of the Argentine territory has water with levels of As harmful to health (Benitez et.al., 2000; Hopenhayn-Rich et al., 1998). In some studies, As toxicity has been related to negative effects on young children intelligence (perception, reasoning, memory, among others) (Wasserman et al., 2014). Particularly, in Argentina, at the beginning of the 20th century, "Bell Ville disease" was defined in the province of Córdoba, later renamed chronic regional endemic hydroarsenicism.

The speciation analyses carried out to identify the species of certain elements in foods have been developed over time. In this context, anionic exchange high performance liquid chromatography (HPLC) coupled to inductively coupled plasma mass spectrometry (ICP-

MS), is a technique that allows the perfect identification of the As species to study: As (III) and As (V). HPLC allows excellent separation and reproducibility followed by detection of As using ICP MS, which is a very sensitive detector.

The difference in the anionic nature of the As (III) and As (V) species make anion exchange chromatography suitable for their separation. As (V) is the slowest eluting component due to its low pKa value whereas As (III) compounds elute rapidly resulting in short retention times due to their high pKa values (Reid et al., 2020). Among the most used mobile phases we can find solutions of $NH_4H_2PO_4$, $(NH_4)_2HPO_4$, $(NH_4)_2CO_3$ or NH_4HCO_3. Previous works performed an arsenic extraction before injection into the column. This method involves the use of organic solvents such as hexane and HNO_3 (Meharg et al., 2008), or trifluoroacetic acid (TFA) followed by 6 hours of contact between the sample and the acid at 100 °C (Munera-Picazo et al., 2014).

In this context, the aim of the present study was to optimize and validate methodologies for speciation of inorganic arsenic in milk and imitation milks using HPLC-IPCM-MS. For this purpose, it was first necessary to determine total arsenic concentration presented in each sample, which also involved optimizing the sample pretreatment process with the goal of eliminating the use of substantial solvent quantities and reducing the time required to process each sample analysis.

2. Material and methods

2.1 *Chemical and reagents*

Distilled and de-ionized water, with a resistivity of 18.2 MΩ cm (Easy pure RF system, Barnstead, USA) was employed. Concentrated nitric acid (HNO_3, 65% v/v) from Sigma-Aldrich (Germany) and hydrogen peroxide (H_2O_2, 30% v/v) from J. T. Baker (Mexico) were used as samples in mineralization. Standard As (III) solution (1000 µg mL^{-1}) was

prepared by dissolving 0.3300 g of As_2O_3 (Sigma, St. Louis, USA) in 10 mL of 1 M NaOH and diluting to a final volume of 250 mL with 2 M HCl. Standard As (V) solution (1000 µg mL^{-1}) was prepared by dissolving 0.6933 g Na_3AsO_4 (Merck, Darmstadt, Germany) in 10 mL of 1 M NaOH diluted to a final volume of 250 mL with 2 M HCl. As (III) and As (V) standards were used in speciation studies. Multi-element calibration standard 3 from Perkin Elmer Pure Plus containing 10 mg L^{-1} of As in 5% HNO_3 was used for total arsenic determination. For the external calibration against aqueous standards, the standard solutions were prepared in 1.0% v/v HNO_3 The As concentrations were 0.5; 1; 5; 10; 20; 40; 80 and 120 µg L^{-1}.

2.2 Samples and standard reference materials

Liquid samples of cow's milk and imitation milks were obtained from different markets in Argentina. All selected samples were commercially produced. Three different brands were chosen for each type of sample, and three samples per batch of each one of the brands were selected. Analyses were carried out in triplicate. The samples were named as: cow's milk (CM), imitation milks based on soy (SIM), peanut (PIM), almond (AIM), quinoa (QMI), vetch (VIM), nut (NIM) and rice (RIM). A certificated reference material (CRM) of rice flour (NIST, 1568b) was analyzed to validate the results of the total and inorganic arsenic concentration.

2.3 Sample preparation and procedures

Once the samples arrived at our laboratory, a lyophilization process was conducted. Aliquots of each sample were lyophilized for a period of 24 hours, at a pressure of at least

0.25 mBar and a temperature of -53°C (L-T8, Rificor, Buenos Aires, Argentina). The lyophilized sample was collected in As-free falcon tubes and stored at -80 °C until analysis.

2.3.1 Total arsenic content: acid microdigestion

Traditional techniques for determining total arsenic content often employ substantial quantities of acid, which subsequently result in a significant sample dilution during ICP-MS analysis. In order to reduce the quantities of both sample and acid, the microdigestion acid for the sample was optimized. Samples of 180 mg of lyophilized samples and 800 µL of fluid samples were weighed, placed in a 15 mL As-free falcon tube, 600 µL of HNO_3 (65%) were added and placed in an ultrasonic bath for 15 minutes. Then, 500 µL of H_2O_2 (30%) was added and placed in a thermostatic bath for 90 minutes at 60 °C. After this time, the samples were brought to final volume with ultrapure water and immediately analyzed by ICP-MS. This method was developed and optimized during this study. As it was well accepted, $^{40}Ar^{35}Cl^+$ ion contribution at 75 m/z is negligible due to Chlorine elimination under concentrated HNO_3 conditions. For the validation of the methodology, 180 mg of CRM was analyzed, following the same methodology described for the lyophilized samples.

2.3.2 As (III) and As (V) determination: speciation analysis

Aliquots of lyophilized samples of 0.1 g were suspended in 2 mL of mobile phase $((NH_4)_2HPO_4)$ and homogenized in an ultrasonic bath for 10 minutes. Before injection in the chromatographic column, the samples were centrifuged at 1000 x g for 10 minutes and 3-fold diluted and filtered through a 0.45 µm pore size filter (Fig. 1).

2.4 Instrumentation

2.4.1 Instrumental for Inductively coupled plasma mass spectrometry

An inductively coupled plasma mass spectrometer, Perkin-Elmer SCIEX, ELAN DRC-e (Thornhill, Canada) was used. The argon gas with minimum purity of 99.996% was supplied by Air Liquid (Río Cuarto, Córdoba, Argentina). An HF-resistant and high performance perfluoroacetate (PFA) nebulizer model PFA-ST, coupled to a quartz cyclonic spray chamber with internal baffle and drain line, cooled with the PC^3 system from ESI (Omaha – NE, USA) was used. Tygon black/black 0.76 mm i.d. and 40 cm length peristaltic pump tubing was used. The instrument settings are shown in Table 1.

2.4.2 HPLC

Sample extracts for As speciation were injected into an anion exchange chromatography (HPLC) Hamilton column (PRP-X100) coupled to the ICP-MS instrument by tubing connections from the column attached to the ICP-MS nebulizer. Chromatographic separations were performed using an isocratic HPLC pump (200; Perkin-Elmer, Thornill, Canada). To obtain the best possible separation, two different mobile phases were evaluated: ammonium carbonate, $(NH_4)_2CO_3$, and ammonium biphosphate, $(NH_4)_2HPO_4$. The flow rate and pH conditions of the mobile phase were optimized. The selected operational conditions of HPLC are summarized in Table 1. Under these conditions, the obtained retention timed of about two minutes comes from the As (III) and the retention timed of 6 min comes from the compounds containing As (V) (Fig. 2a).

2.4 Figures of merit

The As speciation analysis was validated according to, ISO/IEC 17025:2005 protocol. To evaluate the linearity and working range, a blank and six samples with analyte addition at increasing concentrations were measured (1.0, 8.0, 16.0, 32.0, 40.0, 48.0 μg L^{-1}) using

a correlation coefficient value (r^2). The precision was evaluated through the percent relative standard deviation (%RSD) value of 10 replicated samples prepared independently and added with six increasing concentrations of the analyte as follows: 1.0, 8.0, 16.0, 32.0, 40.0, 48.0 µg L^{-1}. The analytical accuracy of the method was analyzed through spike-recovery tests, where 5 replicates of each sample with selected standard at two different As concentrations addition (i.e. 6.0 and 12.0 µg L^{-1}) were analyzed. Then, the As recovery percentage was calculated as:

$$Recovery\ \% = \frac{(C_f - C)}{C_{sp}} \times 100 \qquad (1)$$

where C_f is the analyte concentration found after the analysis of the spiked sample; C is the sample concentration without spike and C_{sp} is the spike concentration added. The LOD and LOQ were calculated as 3 and 10 times the standard deviation of the analytical signals from the blank method (n = 11), respectively.

A Certified Reference Material (NIST, 1568b) with certified iAs and AsT content, was analyzed by HPLC-ICP-MS and ICP-MS to assess the iAs and AsT content.

2.5 Statistical Analyses

Descriptive statistics were done with MINITAB software. Data is presented as mean ± standard deviation. Analysis of variance (one-way ANOVAs) were performed to determine differences among samples on arsenic content. Data was checked for normality and homogeneity of variance by Kolmogorov–Smirnov and Levene tests, respectively. Significant differences ($p < 0.05$) were compared using Tukey, post hoc test (Sokal and Rohlf, 1995).

3. Results and discussion

3.1 Validation of the methodologies

The parameters of the calibration curves for As (III), As (V) and AsT, where y= signal intensity (counts per second, cps) and x= concentration (µg L^{-1}), are shown in Table 2. The linearity test was performed to ensure the analytical system's ability to provide a linear response in the working range. The analyte concentration-account ratio was linear, ranging from 0.5 to 120 µg L^{-1}. The linear correlation coefficient for all As species was similar and satisfactory (r^2≈0.999). The method precision was evaluated by the %RSD, with %RSD valued from 0.47 to 4.1%. Accuracy was determined through analysis of certified reference material and recovery tests for the determination of AsT and iAs. A reference material of rice flour was used since it is the only rice-based product where iAs and AsT concentrations are certified.

Table 3 displays the findings of the CRM analysis, indicating that the determined concentrations align with the certified values. The determination of the total content of As recovery tests were carried out spiking each type of sample, in two different presentations (fluid and lyophilized) at 3 increasing and equally spaced concentration levels (10, 40 and 70 µg L^{-1}).

The results obtained are summarized in Table 4 and show that the use of previously lyophilized samples allows a decrease in the matrix effect and higher recovery percentages were obtained (above 85%).

To verify the accuracy of the applied method for As speciation, column recovery studies were performed and the results are shown in Table 5. Two types of samples were selected for this study, based on the composition of its matrices: rice-based imitation (RMI) and soy-based imitation (SIM). In these cases, the recoveries were between 89-107%, which can be considered a good result for highly complex matrices, such as those analyzed in

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this study (Table 5). Contribution of $^{40}Ar^{35}Cl^+$ ion at 75 m/z is not expected here due to column separation of Arsenic species prior ICPMS determination.

The Limit of Detection (LOD) and Limit of Quantification (LOQ) for As (III), As (V), and AsT were determined by assessing the standard deviation (SD) based on 11 blank measurements. We applied a criterion of 3 times the SD value for LOD and 10 times the SD value for LOQ, as outlined in Table 2. The LOD values ranged from 0.2 to 0.7 µg L^{-1}, while the LOQ values spanned from 0.8 to 2.5 µg L^{-1}. These values were considered appropriate for this type of analysis, as they were lower than those reported by Ruzik et al. (2022) and comparable to values reported by Guillod-Magnin et al. (2018).

3.2 Total arsenic content (AsT)

The results obtained demonstrate a significant difference in total arsenic content among the samples (ANOVA, F= 1303.16, p< 0.001; see Table 6). Specifically, SIM exhibited the highest AsT content, with significant differences compared to the other samples (Tukey, p< 0.05). Additionally, SIM, RIM, and VIM exhibited significantly higher AsT content than the CM samples (Tukey, p< 0.05), while NIM, PIM, AIM, and QIM samples displayed significantly lower AsT content than the CM samples (Tukey, p< 0.05; Table 6).

The potential occurrence of arsenic in CM might be linked to environmental contamination from soil and water in the animal's habitat. This could result in the uptake of arsenic into the animal's biological system, followed by metabolism and subsequent transfer to the milk (Alonso et al., 2002; Malhat et al., 2012). The values of AsT for CM found in our work are lower than those reported in India (0.44 mg kg^{-1}; Ahmed et. al., 2016) and higher than those reported in Brazil (0.003 mg kg^{-1}; Ciminelli et. al., 2017). This suggests that the geographical factor might be a potential cause for the variation in arsenic content observed in the milk samples. Other authors recorded that the percentage

that could be transported from the soil to cow's milk is approximately 1% (Upadhyay et al., 2019), which implies that higher concentrations in the soil where the animal grazes or in cattle water increase the total arsenic concentration in cow's milk. Our choice of samples for analysis was influenced by consumer preferences in the region and the higher availability of these brands in local markets. However, it was not feasible to determine the exact geographical origins of each milk within the Argentine territory. Argentinian food code (Argentino, 2001) establishes 0.01 mg L^{-1} as the maximum limit of As in drinking water, while it admits a maximum content of this element in fluid milk of 0.05 mg L^{-1} and 0.3 mg L^{-1} in cereals and products derived from cereals. Thus, all the samples examined in this study have AsT content below the established maximum limit.

Previous works have reported the presence of arsenic in rice-based beverages (da Rosa et al., 2019; Gu et al., 2020; Ruzik & Jakubowska, 2022). However, there is no available information related to arsenic content in vetch-based imitation milk. The amount of arsenic in vegetable drinks depends strongly on the type of plant used for the production of beverages and the agriculture techniques (agrochemicals used, soil, climate, irrigating water, and others) where these plants grew. Rice is one of the main pathways for arsenic to enter the human food chain. The problem of As' bioaccumulation in rice has been extensively studied over years (Cano-Lamadrid et al., 2016; Choi et al., 2016; Hojsak et al., 2015; Islam et al., 2017; Kara et. al., 2021; Šlejkovec et. al., 2021; Signes-Pastor et al., 2016). As bioaccumulation is transferred to all foods based on this cereal, including rice-based imitation milks. Several factors affect the concentration levels of arsenic and its species in rice grains. The geographical location, the irrigation regime, the environmental contamination, the quality of the irrigation water, the genotype of the rice as well as the processing of the grains, have shown how the As concentration can vary in the same crop field, when modifying any of these variables (Upadhyay et al., 2019). In

this investigation, the AsT concentration in rice-based beverages was similar to that determined in Brazil (29.48 µg L^{-1}, da Rosa et al. 2019) and higher than those reported in Poland (2.34 µg L^{-1}, Ruzik & Jakubowska, 2022), which could demonstrate how the geographical location of the cultivation area directly affects the concentration of AsT in rice-based beverages.

Soy is renowned for its exceptional nutritional attributes and its ability to thrive in even adverse soil conditions (Bustingorri et al., 2017; Finnegan & Chen, 2012). It stands as the predominant oilseed crop in Argentina, contributing to approximately 93% of the country's total production. Over the past five years, soybeans have consistently ranked as the leading grain crop in Argentina, yielding more than 53 million tons annually. The provinces of Buenos Aires (32.9%), Córdoba (29.2%), Santa Fe (17.5%), and Santiago del Estero (4.8%) play significant roles in this production, as reported by the SPE (2019). While soybeans were initially cultivated in the fertile soils of the Argentine Pampas, their adaptability has led to cultivation in less favorable conditions with higher arsenic content across the country (Bustingorri et al., 2015). Primary products derived from soybeans include oils, flours, and an increasing quantity of soy-based beverages aiming to replicate the nutritional characteristics of cow's milk. In our study, the AsT content determined in SIM was notably higher than values reported by Ruzik & Jakubowska (2022; 0.42 µg L^{-1}) and similar to those documented by Elaridi et al. (2021; 38.8 µg L^{-1}), despite the diverse geographical locations of sample collection (Poland and Lebanon, respectively).

In our study, we determined the total arsenic concentration in vetch-based imitation milk samples, revealing a concentration of 19.32 µg L^{-1}. Notably, there is a lack of prior scientific literature that evaluates the AsT content in such beverages. Based on the findings from our study, it can be concluded that imitation milks made from almonds,

peanuts, and vetch are the safest options in terms of inorganic arsenic content and the associated consumption risks.

Furthermore, to assess the impact of matrix on the determination of total arsenic content, we subjected liquid milk samples to acid microdigestion. The objective was to understand how sample pretreatment influences arsenic determination. Table 4 provides the recovery percentages for these analyses, performed on both liquid (LIQS) and lyophilized (LYOS) samples spiked at low, medium, and high concentration levels. Our results indicate that liquid milk samples exhibited recovery values of 55 to 78%, while lyophilized samples showed higher recovery rates, ranging from 85 to 107%. This discrepancy suggests that the presence of water in liquid samples hinders complete digestion, likely due to dilution effects in the digestion solutions. Consequently, future studies should consider the use of lyophilized samples for more accurate assessments.

3.3 Arsenic speciation by HPLC-ICP-MS

Speciation analysis of iAs compounds in cow´s milk and several imitation milks were performed based on HPLC-ICP-MS chromatograms of the samples studied (Fig. 2b). The signal in the chromatogram corresponding to the retention timed of about two minutes, comes from compounds of As (III) while the second signal corresponding to the retention time of six minutes was assigned to compounds of As (V). Thus, the sum of the concentration of As (III) and As (V) constitute the content of inorganic arsenic (iAs) present in the sample.

It was noted that the content of As (III) and As (V) exhibited significant difference among the samples (ANOVA, F= 1257.57 and 1077.64, p< 0.001 and 0.002, for As (III) and As (V), respectively). SIM and RMI presented the highest values for both species (Table 6).

The analyzed CM, SIM, RMI and VIM samples exhibited a higher concentration of As (V) than As (III). Particularly, in the PIM samples only As (III) was determined since the concentration of As (V) was less than the detection limit of the methodology. No detectable amounts of iAs were found in the QIM samples. Because in the almond-based imitation the AsT concentration was below the detection limit, the speciation did not generate any detection of the inorganic species.

Usually, the toxic risk implied by arsenic depends on whether it is As (III) or As (V), being this last less dangerous. Considering the bibliographical research and the knowledge to date on the mechanism of action of As, in the organism, all the content of As (V) is reduced to As (III). Most of the metabolism of arsenic takes place in the liver, where As (V) is reduced to As (III) under anaerobic conditions. Unlike As (V), trivalent arsenic has a high affinity for sulfhydryl groups such as that of some proteins, where it binds and begins methylation, giving rise to organic arsenic compounds (MMA and DMA) (Guillod-Magnin et al., 2018).

In the RIM and VIM samples the amount of iAs represents 44% and 42%, respectively of the AsT, while in the SIM iAs constitutes almost 70% of the AsT. The high content of AsT found in the soybean-based beverage samples translates to the high content of iAs found in the speciation analysis. This may be largely due to the high iAs content reported in the areas with the highest soybean cultivation and production in the country, with up to 0.1 mg L^{-1} in Buenos Aires, up to 4.5 mg L^{-1} in Córdoba, in Santa Fe 0.13 mg L^{-1} and up to 2.4 mg L^{-1} of iAs in Santiago del Estero (Marchetti et al., 2021).

The results obtained allow us to infer that the oAr content is the difference between ArT and iAs. Samples with low AsT content, such as CM, PIM, and NMI, contain 26 and 45% content of oAs respectively, while in the quinoa-based imitation oAs represents 100% of the total arsenic.

International authorities, such as the World Health Organization (WHO, 1997) establishes as a safe consumption limit of iAs 2 µg · kg^{-1} of body weight (bw). It also sets a Benchmark Dose Lower Limit (BMDL$_{0.5}$, suggesting a 0.5% increased incidence of cancer) for iAs, being 3, 5.2 and 5.4 µg · day^{-1} · kg^{-1} bw for lung cancer, bladder cancer and skin lesion, respectively. Moreover, the European Food Safety Authority (EFSA, 2009) defines a BMDL0.1 ranging from 0.3 to 8 µg · day^{-1} · kg^{-1} bw, associated with a 1% elevated risk of lung, skin, and bladder cancer. In the context of a 250 mL serving, a daily portion of cow's milk contributes 0.42 µg, while rice and vetch-based vegetable drinks provide 3.15 and 2.02 µg of iAs, respectively. Notably, SIM contains 6.83 µg of iAs in a daily portion. When considering the weight of a 5-year-old child, the consumption of SIM constitutes 14% of the maximum daily intake limit set by the WHO (2011).

Previously employed methods for arsenic speciation in complex samples like milk and vegetable drinks have involved using organic solvents in extraction processes. However, these methods are environmentally unfriendly and pose health risks to operators due to high temperatures and labor-intensive procedures, consuming several hours per sample set (Cano-Lamadrid et al., 2015; Munera-Picazo et al., 2014; Pedron et al., 2016). In contrast, our optimized sample preparation process entails contacting the sample with the mobile phase for separation, followed by centrifugation, filtration, and a final dilution of the extract before injection into the column. This approach offers advantages such as avoiding the use of organic solvents, expediting the process, and preventing high-temperature conditions that could lead to interconversion between As (III) and As (V) species.

It's worth noting that certain steps in the production of imitation milks and vegetable drinks, including milling, pressing, and filtration, have the potential to alter the iAs content in the final food product. Redan et al. (2019) observed that the filtration process can increase inorganic arsenic content (ranging from 1 to 5 g L^{-1}) in commercial apple-based beverages, contingent on the material used in the filtration process.

4. Conclusions

This work has primarily focused on creating and validating a rapid and innovative analytical method for speciation analysis of arsenic in both cow's milk and imitation milk products available in Argentine supermarkets. The optimized procedure outlined in this research streamlines sample preparation, minimizes handling steps, and ensures precise HPLC separation using anionic exchange columns, followed by sensitive arsenic detection via ICP-MS. The method's validation adheres to established protocols and can be applied for determining total arsenic (AsT), as well as iAs, which encompasses both arsenate and arsenite. These findings hold particular significance in the realm of food sciences, especially since plant-based beverages known as "imitation milks" are favored by individuals who cannot consume cow's milk due to health considerations such as allergies or adherence to vegan/vegetarian diets. Our method provides a straightforward means to assess the potential intake of harmful arsenic due to the high consumption of such beverages.

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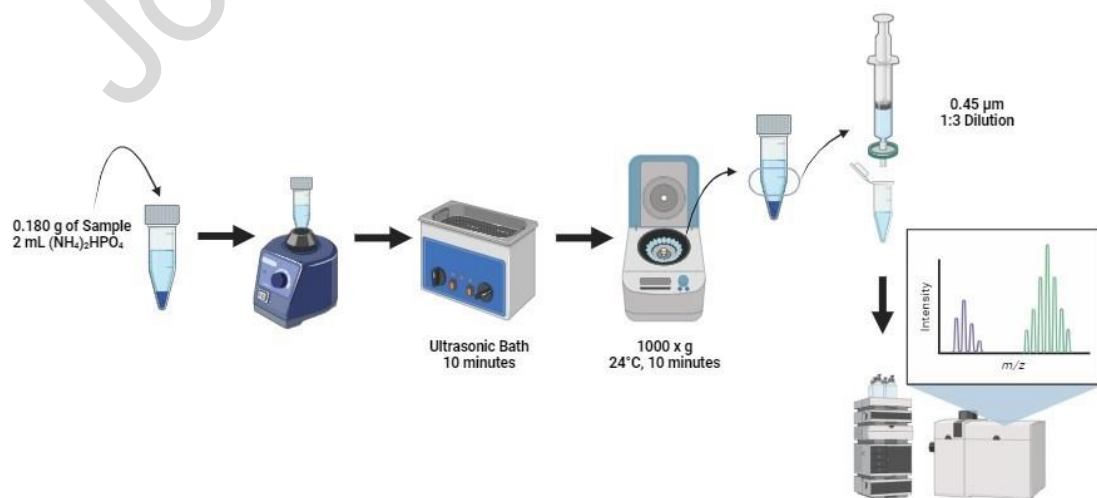
Figures captions

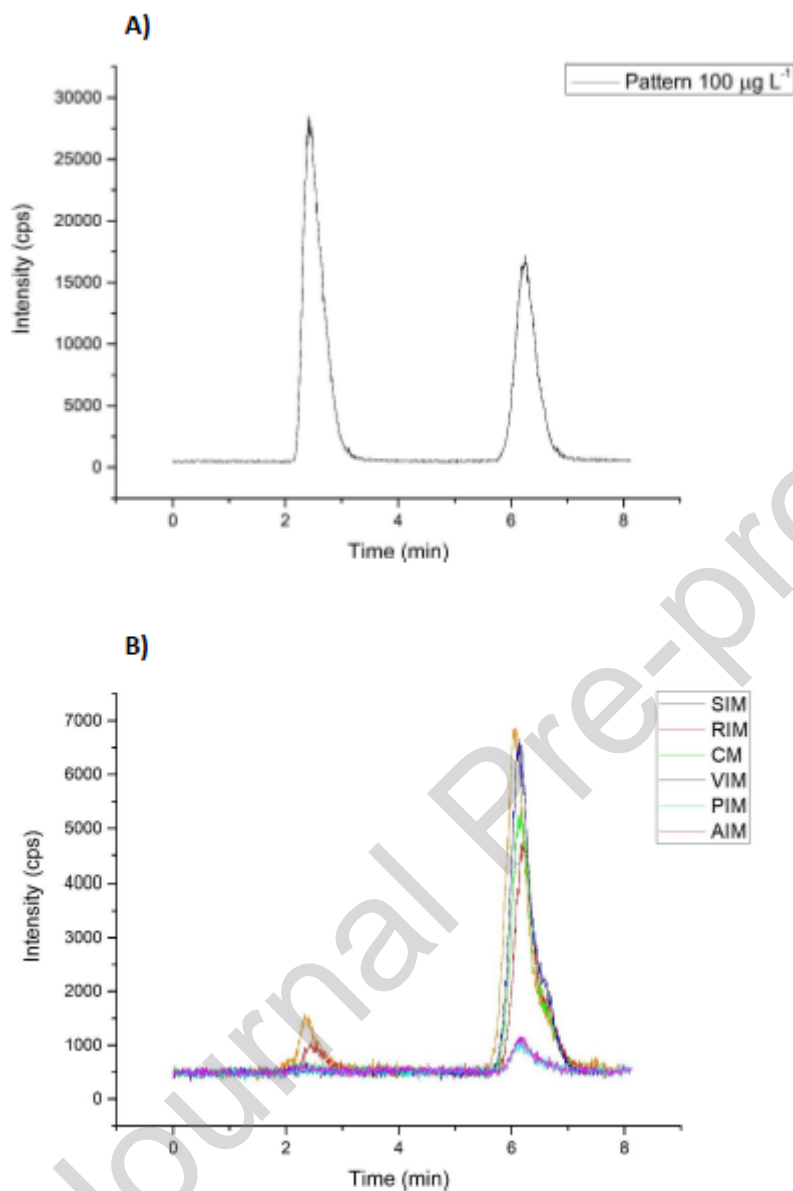**Fig. 1.** Sample preparation procedure for chromatographic separation.

Fig. 2. Chromatograms of As species: **A.** pattern solution with 100 µg L^{-1} of each species in water; **B.** samples of cow's milk and imitation milks diluted 1:3 in 20 mmol L^{-1} (NH$_4$)$_2$HPO$_4$.

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Table 1 – ICP-DRC-MS and HPLC Instrumental operational conditions for the determination of As total and As species in cow´s milk and milk imitations.

Instrument	Elan DRC-e (Perkin-Elem SCIEX, Thornhill, Canada)
Parameter	Value / experimental setting
Sample uptake rate (μL min^{-1})	1100
Sample introduction	PFA micronebulizer model PFA-ST, with a quartz cyclonic spray chamber with an oxygen auxiliary gas kit
RF Power (W)	1050
Gas flow rates (L min^{-1})	0.81 (nebulizer), 4 (auxiliary) and 15 (cooling).
Interface	Ni cones (sampler and skimmer)
Standard ICP mode	^{75}As
Scanning mode	*PeakHopping*
Seeps	5 (1 for HPLC-ICP-MS)
Dwell time (ms)	50 (500 for HPLC-ICP-MS)
Number of replicates	3 (500 for HPLC-ICP-MS)
Instrument	**HPLC**
Pump	200; Perkin-Elmer, Thornill, Canadá
Column	PRP-X100, Anion exchange
Elution program	Isocratic
Mobile Phase solution	$(NH_4)_2HPO_4$
Mobile Phase concentration (mmol L^{-1})	20
Mobile Phase flow rate (mL L^{-1})	1
Mobile phase pH	5.6

Table 2 – Parameters of precision, accuracy and limits of detection (LOD) and quantification (LOQ) of the validation of the proposed method for the determination of inorganic and total As (As (III), As (V), AsT).

SPECIES	Calibration curves parameters			
	Equation	R^2	LOD (μg L^{-1})	LOQ (μg L^{-1})
As (III)	y=0.7117x + 0.0188	0.9992	0.6[a]	1.9[a]
As (V)	y=0.7166x + 0.021	0.9998	0.7[a]	2.5[a]
AsT	y=0.0602x + 0.0203	0.9990	0.2[b]	0.8[b]

[a] by HPLC-ICP-MS; [b] ICP-MS

Table 3- Analysis of certified rice flour.

	Analysis of certified rice flour NIST1568b	
	Certified value (μg kg^{-1})	Found (μg kg^{-1})
iAs [As (III)+ As (V)]	92±10	105±8
AsT	285±14	296±6

Mean± SD (n=3)

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Table 4- Recovery percentages of AsT at 3 different levels.

SAMPLE	AsT, Recovery studies					
	LIQS (Rec. %)			LYOS (Rec. %)		
	Spk 10 ug L^{-1}	Spk 40 ug L^{-1}	Spk 70 ug L^{-1}	Spk 10 ug L^{-1}	Spk 40 ug L^{-1}	Spk 70 ug L^{-1}
CM	73.9	69.9	77.5	100.98	107.7	99.4
SIM	65.5	70.1	71.2	91.7	96.9	95.8
PIM	54.6	60.8	62.3	98.3	104.8	97.4
AIM	68.5	76.3	72.2	90.9	96.4	91.7
NIM	59.7	66.9	67.5	90.9	98.0	89.6
QIM	75.5	60.2	66.1	88.3	90.1	91.2
RIM	62.9	64.6	73.4	87.1	102.3	101.9
VIM	65.4	64.9	56.2	85.4	90.9	86.2

LIQS: liquid sample, LYOS: lyophilized sample, Rec. %: Recovery percentage, Spk: spiked.

Table 5 – Column recoveries tests in RIM (rice-based imitation milk) and SIM (soybean-based imitation milk) samples at two different spike levels.

Sample and species	Column recoveries study				
	Found in the sample (μg L^{-1})	Found in the spiked sample (6 μg L^{-1})	Recovery (%)	Found in the spiked sample (12 μg L^{-1})	Recovery (%)
RIM					
As III	3.0	8.6	93.3	14.9	99.2
As V	9.9	15.4	91.7	22.7	106.7
SIM					
As III	6.9	12.5	93.3	18.6	97.5
As V	20.5	25.8	88.3	31.9	95.0

Table 6 – Arsenic III (As III), Arsenic V (As V), Inorganic arsenic (iAs: As III + As V) and total arsenic (AsT) concentration in cow's milk and imitation milks determined by ICP-MS and HPLC-ICP-MS.

Sample	(A) As (μg L^{-1})			
	As III	As V	iAs	AsT
SIM	6.9±0.2[a]	20.5±0.9[a]	27.3±1.0	39.6±0.9[a]
RIM	3.0±0.1[b]	9.8±0.3[b]	12.8±0.4	29.5±1.1[b]
VIM	< LOD	8.1±0.4[c]	8.1±0.4	19.3±0.8[c]
CM	< LOD	4.1±0.4[d]	4.1±0.4	5.5±0.7[c]
NIM	2.2±0.3[c]	< LOD	2.2±0.3	4.0±0.7[c, d]
PIM	1.7±0.1[d]	< LOD	1.7±0.1	2.3±0.2[d, e]
QIM	< LOD	< LOD	< LOD	1. 5±0.2[f]
AIM	< LOD	< LOD	< LOD	< LOD

Mean±SD (n=9), [a, b, c, d, e, f] indicates statistical difference among samples of each species ($p < 0.05$).

CRediT authorship contribution statement

Isis S. Permigiani: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing – original draft. **Nicolas K. Vallejo**: Investigation, Formal analysis, Writing – review & editing. **Paul E. Hasuoka**: Data curation, Investigation, Writing – review & editing. **Raúl A. Gil**: Supervision, Resource's. Funding acquisition, Project administration, Conceptualization, Writing – review & editing. **M. Carolina Romero**: Supervision, Funding acquisition, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal

relationships that could have appeared to influence the work reported in this paper

Highlights

- Speciation analysis of arsenic in milk and imitation milks.

- Acid microdigestion of samples using small volumes of sample and reagents.

- Comparison of inorganic As in imitation milks.

- Simple and fast sample pretreatment for chromatographic analysis.