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García-Rodríguez, Thalía; Gutiérrez-Ruiz, Margarita Eugenia; Ceniceros-Gómez, Águeda Elena

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# ARSENIC AND LEAD BIOACCESSIBILITIES MEASURED WITH A NOVEL REACTOR SYSTEM USING THE MEXICAN STANDARD AND PBET METHODS: COMPARISON WITH IN VIVO AND IN VITRO REPORTED DATA

Bioaccesibilidad de arsénico y plomo determinada con un novedoso sistema de reactores usando el método PBET y el de la Norma Oficial Mexicana: comparación con datos reportados in vivo e in vitro

Thalía GARCÍA-RODRÍGUEZ, Margarita Eugenia GUTIÉRREZ-RUIZ*
and Águeda Elena CENICEROS-GÓMEZ

Laboratorio de Biogeoquímica Ambiental, Facultad de Química, Universidad Nacional Autónoma de México, Ciudad Universitaria, Av. Universidad No. 3000, Coyoacán, CDMX, C.P. 04510, México.

*Author for correspondence: ginny@unam.mx

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Key words: As bioaccessibility, Pb bioaccessibility, novel bioaccessibility equipment, NOM-147 method, in vivo-in vitro comparison, in vitro bioavailability.

**ABSTRACT**

Contaminated soils can become exposure routes of elements toxic to human beings. The health risk of a toxic element by ingestion depends on its bioavailability in the gastrointestinal system, measured in vivo or in vitro. This study aimed to use a novel, versatile reactor (gastrointestinal simulation reactor system to determine bioaccessibility -GSRSB-) to measure lead and arsenic bioaccessibility in the gastric and intestinal phases by applying a modified physiologically based extraction test (PBET). Three composite samples of polluted soils with As (0.50 - 3.25%) and Pb (0.02 - 0.10%) and the certified reference material NIST 2710 were analyzed with this GSRSB-PBET method and the NOM-147 Mexican standard method, which uses an end-over-end shaker. All results were compared to one another. The NIST 2710 results were contrasted with those reported in vivo and in vitro by 14 laboratories. The (GSRSB-PBET) gastric phase ranges were 35.9-55.1 % (As) and 59.6-96.1 % (Pb), while (NOM-147) gastric phases were 35.8-60.4 % (As) and 61.0-70.7 % (Pb). The (GSRSB-PBET) intestinal phase ranges were 39.5-46.9 % (As) and 19.9-31.5 % (Pb). The As and Pb compounds and the stirring technique seem to influence bioaccessibility. On the other hand, the comparison of NIST 2710 results with those reported in vitro and in vivo indicated that As and Pb gastric bioaccessibility obtained with GSRSB-PBET falls into the in vivo results range, while NOM-147 results are higher and fall outside the in vivo range, possibly overestimating the risk. Thus, the proposed method is adequate for modifying the current Mexican Standard Method (NOM-147), which only allows the calculation of Pb gastric bioavailability in vitro.

Palabras clave: bioaccesibilidad de As, bioaccesibilidad de Pb, equipo novedoso de bioaccesibilidad, método NOM-147, comparación in vivo-in vitro, biodisponibilidad in vitro.

**RESUMEN**

Los suelos contaminados pueden ser fuentes de exposición de elementos tóxicos para los seres humanos. El riesgo a la salud de un elemento tóxico por ingestión depende de su biodisponibilidad gastrointestinal, medida in vivo o in vitro. El objetivo de este estudio fue evaluar un reactor novedoso y versátil (GSRSB) para medir la bioaccesibilidad de plomo y arsénico en las fases gástrica e intestinal aplicando un método de extracción de base fisiológica (PBET, por sus siglas en inglés) modificado. Se analizaron tres muestras compuestas de suelos contaminados con As (0.50 - 3.25%) y Pb (0.02 - 0.10%) y el material de referencia certificado NIST 2710 usando el método GSRSB-PBET y el método estándar mexicano NOM-147, utilizando un agitador axial. Los resultados del NIST 2710 se contrastaron con los informados in vivo e in vitro por 14 laboratorios. Los resultados en la fase gástrica (GSRSB-PBET) fueron 35.9-55.1% (As) y 59.6-96.1% (Pb), en fase intestinal (GSRSB-PBET) 39.5-46.9% (As) y 19.9-31.5% (Pb); mientras que la fase gástrica (NOM-147) fueron 35.8-60.4% (As) y 61.0-70.7% (Pb). Los compuestos de As y Pb y la técnica de agitación parecen influir en la bioaccesibilidad. La comparación de los resultados del NIST 2710 con los reportados in vitro e in vivo indicó que la bioaccesibilidad gástrica de As y Pb obtenida con GSRSB-PBET está en el intervalo de los resultados in vivo, mientras que los resultados obtenidos con la NOM-147 son mayores y fuera del intervalo in vivo, posiblemente sobrestimando el riesgo. El método propuesto es adecuado para modificar el actual método estándar mexicano (NOM-147) que sólo determina la biodisponibilidad gástrica del Pb in vitro.

## INTRODUCTION

The primary anthropogenic activities responsible for higher levels of potentially toxic elements (PTE) in the environment are energy production and mining-metallurgy (Panayotova 2016, Sposito 2008, Masindi and Muedi 2018). The biogeochemical cycles of several elements, including arsenic (As) and lead (Pb), have been disturbed (Masindi and Muedi 2018), and populations can be exposed to polluted soil and water. Depending on the dose and chemical species, As and Pb ingested may harm human health (Kumpiene et al. 2017). Other factors influence toxicity, mainly the route of exposure, solubility, particle size, environmental matrix type, and the presence of certain substances that reduce or improve their absorption. The Pb acetate is one of the most bioavailable compounds because it is very soluble (Freeman et al. 1994) but most of the Pb in soils is forming compounds with low solubility (Rooney 2002). In the gastrointestinal tract, iron, zinc, and calcium decrease Pb absorption, possibly competing for absorption receptors in the intestine. Biogeochemical transformations control As bioavailability, toxicity, and its environmental fate. As can be oxidated by diverse compounds or reduced by organic compounds, forming more toxic inorganic species. Direct reduction of Fe (III) by microorganisms can lead to As sequestration by sorption (Borch et al. 2010). These processes have a big influence on the As bioavailability in vivo and, consequently, on in vitro determinations.

Several toxic arsenic species may form in the intestine because of the reaction between arsenic and food compounds. (Conrad and Barton 1978, Mushak 1991, Diamond et al. 1998, Calatayud and Llopis 2015, Ollson et al. 2017)

The damages caused by arsenic are classified as carcinogenic, mutagenic, or genotoxic, producing immunological, reproductive, developmental, neurological, renal, hepatic, hematological, gastrointestinal, cardiovascular, pulmonary, respiratory, and dermal harm (Mandal and Suzuki 2002, Sattar et al. 2016). Pb is classified as "probably carcinogenic to humans" (Group 2A), according to IARC (2006). It has mainly neurocognitive and behavioral effects (Chiodo et al. 2004, Mason et al. 2014) and decreases children's intellectual quotient (Schnaas et al. 2006, Mitra et al. 2017). However, because of various factors, most above mentioned, only part of the ingested element is absorbed through the gastrointestinal tract. This absorbed fraction is named bioavailable (Zhu et al. 2015). It can be measured as an absolute amount or a relative amount (Ruby et al. 1996, 1999, Juhasz et al. 2009, USEPA 2012, Koch et al. 2013).

Bioavailability is determined using animal models (in vivo), which is expensive, time-consuming (days or months), and raises ethical considerations. As an alternative to bioavailability tests, bioaccessibility

tests mimic the human gastrointestinal processes (in vitro) being less costly and faster; these are also named in vitro bioavailability methods (Ruby et al. 1999, Juhasz et al. 2014, Liu et al. 2017).

Regulatory frameworks related to the remediation of polluted soils follow a risk assessment approach, and bioaccessibility is an adjustment factor in the calculations (USAEC, 2003). Mexican environmental regulation NOM-147-SEMARNAT/SSA1-2004 follows the same approach. Nevertheless, this regulation only allows the calculation of soil cleanup target levels based on gastric bioaccessibility of Pb measured through the simple bioaccessibility extraction test (SBET) (Dabin et al. 2012, Koch et al. 2013) using an end-over-end shaker.

In the Environmental Biogeochemistry Laboratory of the School of Chemistry of the National Autonomous University of Mexico (UNAM), PTE bioaccessibility is measured frequently. A novel agitation system called gastrointestinal simulation reactor system to determine bioaccessibility (GSRSB) was designed and built to facilitate this determination. It allows continuous control of the pH and improves gas flow stability, simplifying the reagents' addition and aliquots sampling during phase change. Characteristics of the vessels of the agitation equipment avoids sedimentation and soil erosion (Quiroz-Vivanco 2018). This study aimed to use the GSRSB with the physiologically based extraction test (PBET) to measure Pb and As gastric and intestinal bioaccessibility in polluted soils, to show the performance of this novel equipment, and to have data to propose this method (GSRSB-PBET) as an alternative to the current NOM-147 standard method. As and Pb bioaccessibilities of three composite soil samples and the certified reference material from the National Institute of Standards and Technology (NIST) Montana High number 2710 (NIST 2710) were measured by GSRSB-PBET and NOM-147 methods to reach this goal, both methods results were compared. Additionally, only NIST 2710 results were compared with those reported in vivo and in vitro by 14 laboratories, applying 17 methods (Koch et al. 2013).

## METHODS AND MATERIALS

### Sampling

The soil samples were collected at a site in Central Mexico, where Pb and Cu smelters and an $As_2O_3$ production facility were operating for more than 90 years (Gutiérrez-Ruiz et al. 2003, Villalobos 2010, Martínez-Jardines 2018). The area has a BSk climate according to the Köppen-Geiger classification, with an annual temperature of 16.8 ºC and an annual rainfall of 341 mm (CLIMATE-DATA, 2021). The soils are acidic (Martínez-Jardines 2018). According to Gutiérrez-Ruiz (2003) and Martínez-Jardines (2018), the soils were polluted with slag containing Si, Fe, Ca, K, Pb, Cu and much lower quantities of Mn, As, Ni, Ba and Cd, mainly as oxides but also as sulfides. Other important wastes that polluted the site and were characterized are converter flue dust, smelting furnace flue dust, calcine, and black arsenic.

### Sample preparation

The polluted site was in a remediation process, and from the complete set of soil samples collected (N = 800), nine were selected based on their arsenic content, using a non-probability judgment sampling method (Frey 2018). The coordinates of the sites chosen are presented in **table I**. The nine samples (S1-S9) and wastes (converter flue dust, smelting furnaces flue dust, calcine, and black arsenic) were dried (40 ºC), ground, sieved (mesh #10 < 2 mm), and homogenized by quartering (Hesse 1971). Portions of 100 g were re-milled (ball grinder Fritsch), sieved (mesh #200 < 74 μm) (same particle size of NIST 2710 certified material) and preserved at room temperature (15-20 ºC) in hermetically sealed plastic containers. The three composite samples were prepared (C1, C2, and C3), mixing 100 g of three individual soils (**Table I**) selected by geographic proximity and As concentration: C1 (S1, S2, S3); C2 (S4, S5, S6) and C3 (S7, S8, S9).

### Analytical determinations

All chemicals used were analytical reagent grade (AR grade). Deionized water was used to prepare all solutions for the leaching tests and all analytical procedures. All determinations were done in triplicate, and their relative standard deviations (RSD) calculated (values in tables). All the extracts were preserved in the dark at 4 ºC. Elements were quantified using ICP-OES (Agilent Technologies model 5100), applying method 6010C (USEPA 2000). Digestions were done using an Ethos Easy microwave digestion system (Millestone Inc.) using Teflon PFA beakers applying US-EPA method 3051A (USEPA 2007b).

#### *General composition and geoavailability*

The pH in soils and wastes was measured following ISO-10390:2005 with a model Orion Star A211 Thermo Scientific potentiometer. Total concentrations of As, Pb, Fe, Ca, Cd, Cu, Zn, and Mn were measured with a portable model DP-6000-CC

**TABLE I.** SAMPLING SITE UTM COORDINATES, pH, AND TOTAL AND GEOAVAILABLE ELEMENT CONCENTRATIONS OF COMPOSITE SOIL SAMPLES, WASTES AND SUBPRODUCTS.

| **UTM coordinates of the sampling sites** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Composite soil | C1 | | | C2 | | | C3 | | |
| Individual soil | S1 | S2 | S3 | S4 | S5 | S6 | S7 | S8 | S9 |
| UTM W [a] | 291265 | 291265 | 291215 | 291115 | 291165 | 291115 | 291265 | 291365 | 291315 |
| UTM N | 2452135 | 2452035 | 2452185 | 2452185 | 2452285 | 2452235 | 2452085 | 2452085 | 2452085 |

| **Total and geoavailable concentrations of the composite samples** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Element Total (mg/kg) [b] | C1 | | | C2 | | | C3 | | |
| | Total | Geoavailability [b] | | Total | Geoavailability [b] | | Total | Geoavailability [b] | |
| | (mg/kg) [b] | (mg/kg) [b] | (%) | (mg/kg) [b] | (mg/kg) [b] | (%) | (mg/kg) [b] | (mg/kg) [b] | (%) |
| As | 32490 ± 418 | 2855 ± 66 | 8.8 | 10648 ± 137 | 480 ± 61 | 4.5 | 4964 ± 31 | 801 ± 12 | 16.1 |
| Pb | 760 ± 17 | <D.L. [c] | - | 1027 ± 19 | <D.L. [c] | - | 236 ± 5 | <D.L. [c] | - |
| Fe | 20474 ± 330 | 28 ± 2 | 0.1 | 24520 ± 217 | 22 ± 3 | 0.1 | 23034 ± 244 | <D.L. [c] | - |
| Ca | 12364 ± 233 | 1119 ±128 | 9.3 | 15170 ± 438 | 5083 ±430 | 33.9 | 26776 ± 452 | 1452 ± 96 | 5.4 |
| Cd | 470 ± 14 | 183 ± 8 | 38.9 | 178 ± 6 | 56 ± 1 | 31.4 | 100 ± 5 | 54 ± 1 | 54.1 |
| Cu | 254 ± 21 | 67 ± 4 | 26.4 | 666 ± 24 | 44 ± 2 | 6.5 | 199 ± 19 | 17 ± 1 | 8.5 |
| Zn | 1004 ± 23 | 61 ± 3 | 6.0 | 1411 ± 29 | 269 ± 32 | 19.1 | 330 ± 8 | 19 ± 1 | 5.8 |
| Mn | 594 ± 38 | 24 ± 3 | 4.1 | 420 ± 36 | 79 ± 4 | 18.7 | 473 ± 19 | 4 ± 1 | 0.9 |
| S | 1800 ± 17 | ND [d] | - | 4000 ± 37 | ND [d] | - | 900 ± 8 | ND [d] | - |
| pH [b] | 5.32 ± 0.03 | | | 5.17 ± 0.25 | | | 7.49 ± 0.29 | | |

| **pH, total and geoavailable concentrations of As, Pb y Cd in subproducts and wastes** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| "Wastes" (include subproducts stored in the open) | pH | As | | | Pb | | | Cd | | |
| | | Total | Geoavailability | | Total | Geoavailability | | Total | Geoavailability | |
| | | (mg/kg) | (mg/kg) | (%) | (mg/kg) | (mg/kg) | (%) | (mg/kg) | (mg/kg) | (%) |
| Converter flue dust | 4.7 | 18800 | 878 | 4.7 | 106200 | 87 | 0.08 | 4800 | 1126 | 23.5 |
| Smelting furnaces flue dust | 4.3 | 92500 | 17560 | 19.0 | 315600 | 85 | 0.03 | 15200 | 2040 | 13.4 |
| Calcine | 7.2 | 15400 | 1344 | 8.7 | 176900 | 5 | 0.00 | 11200 | 50 | 0.4 |
| Black arsenic | 3.9 | 612100 | 28430 | 4.6 | 24600 | 133 | 0.54 | 1700 | 437 | 25.8 |

[a] Zone 14; [b] Mean ± SD (n=3); [c] Detection Limit (D.L.) Pb =0.6 mg/kg, and Fe = 1 mg/kg; [d] ND: Not determined.

Thermo Scientific X-ray fluorescence Olympus analyzer using the 6200 method (USEPA 2007, Zamora-Martínez et al. 2008). Total sulfur was determined in a model S-832 Leco analyzer (Bremner and Tabatabai 1971). All composite soils were analyzed by SEM-EDS in a Hitachi TM1000 tabletop scanning electron microscope with an energy dispersive spectroscopy module, and by X-ray diffraction (XRD) with a Shimadzu XRD-6000 equipped with a Cu tube and a graphite monochromator. Blanks and certified reference materials were used for quality control for individual and composite soil analysis: pH (NIST-traceable buffer solutions), XRF (NIST 2711a), sulfur content (Oreas 45b) and ICP-OES determinations (HPS, QCS-26). Geoavailable concentrations in soils and wastes were determined with the D3987-06 method (ASTM 2006).

***Soils bioaccessibility***

Two methods have been used to measure the bioaccessibility of soils: NOM-147, which only includes the gastric phase and was applied as indicated by Mexican regulation (SEMARNAT 2007) using glycine at pH = 1.50 ± 0.05 (HCl) and an end-over-end shaker (37 ± 2 ºC). This method is analogous to the SBET, RBALP and SBRC (Dabin et al. 2012). The other method used was the PBET (Ruby et al. 1996), which determines gastric and intestinal phases. The reagents used include pepsin, citrate, malate, lactic acid, and acetic acid in the gastric phase and bile salts and pancreatin in the intestinal phase. Reagent quantities, ratios (solid/liquid), and residence times were applied as indicated by Ruby et al. (1996). A modification was made in the gastric phase, adjusting the pH to 1.50 ± 0.05 with HCl (11 M) as was reported to increased in vitro and in vivo correlations (Drexler and Brattin 2007, Juhasz et al. 2014). After the gastric phase concluded, pH was gradually increased to 7.00±0.10 with a $NaHCO_3$ saturated solution and subsequently, pancreatin and bile salts were added.

Intestinal extracts were digested ($HNO_3$-microwave-assisted, method 3051A) (USEPA 2007b) using an Ethos Easy (Milestone) to destroy organic compounds that interfere with PTE quantification by ICP-OES (USEPA 2000). Argon was used as a stirring gas in the novel reactor (GSRSB). As mentioned above, it was exclusively designed in our laboratory. It is used to improve the control pH and gas flow stability, simplifying the reagents' addition and taking of aliquots during the phase change. It has conical bottom glass reactors placed in a heating bath (37±1 ºC). The vessels contain a thin tube through which the argon enters and stirs the suspension (1 L/min) (**Fig. 1**). The conical design is used to prevent the sedimentation and erosion of soil particles. The pH electrodes are placed inside each reactor, allowing measurements without stopping the agitation process. A small orifice is used to take aliquots or add reagents during the gastric-to-intestinal phase change and to introduce pH electrodes (García-Rodríguez 2017, Quiroz-Vivanco 2018). The NOM-147 end-over-end shaker (**Fig. 1**) controls the temperature with an immersion recirculation heater. Nevertheless, it does not allow one to measure pH, take aliquots, or add reagents when the shaker is in motion.

In this study, the absolute bioaccessibility was calculated as the ratio between the element concentration (mg/kg) in the solution for each method or extraction phase, respect to the element concentration in the soil (mg/kg) in percentage. To calculate the relative bioaccessibility, the bioaccessibility results were corrected with the mean of three spike results for each method ($mean_{\%bioaccess}/mean_{\%spike\ recovery}$) according to Koch et al. (2013).

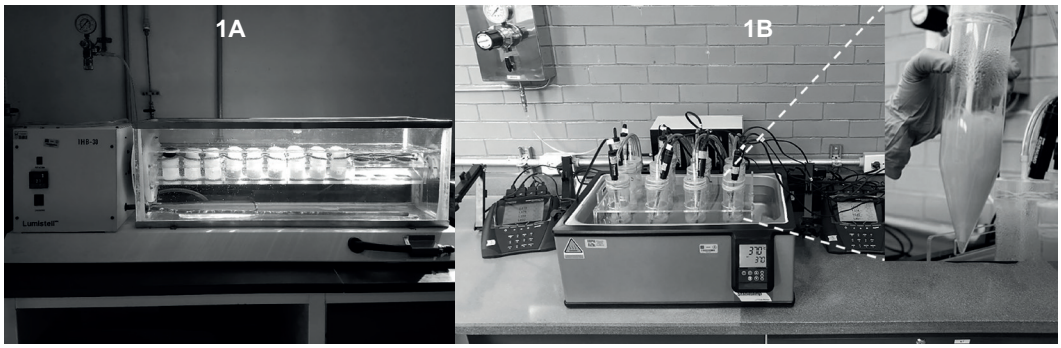



**Fig. 1.** Equipment used to measure oral bioaccessibility: (1A) End-over-end shaker NOM-147 method (gastric phase) and (1B) GSRSB-PBET method (gastric and intestinal phases).

## RESULTS

### General composition and geoavailability

The pH, total element concentration and geoavailability (soluble fraction in meteoric water at pH = 5.5 ± 0.3) of As, Pb, Fe, Ca, Cd, Cu, Zn and Mn were quantified in individual samples (data not shown) and in composite samples (**Table I**). In the nine individual soils, the range of pH was 3.7-8.0, while in groups S1, S2, S3 = 4.73-6.10, S4, S5, S6 =3.7-5.16 and S7, S8, S9 = 7.8-8.0. The range of total As for S1-S9 = 0.43-4.15 %, for S1, S2, S3 = 2.05-4.15 %, for S4, S5, S6 = 0.62-1.59 % and for S7, S8, S9 = 0.43-0.49 %. The range of total Pb for S1-S9 = 0.02-0.16 %, for S1, S2, S3 = 0.04-0.16, for S4, S5, S6 = 0.02-0.14 and for S7, S8, S9 = 0.02-0.03 %.

The formation of hydrolysable sulfates from the oxidation of sulfide could explain the acidity of most of the samples (Ward et al. 2004, Romero et al. 2008). Samples S7 to S9 had a weak positive reaction to acid due carbonates, explaining their basic pH. The As geoavailability of S1-S9 = 418-2048 (mg/kg), S1, S2, S3 =1381-2048 (mg/kg), S4, S5, S6 = 418-920 (mg/kg), and S7, S8, S9 = 682-1017 (mg/kg) (García-Rodríguez 2017). Pb geoavailability was lower than the detection limit (DL = 0.6 mg/kg). Total and geoavailable As are correlated (r = 0 .95), but not with the pH (As total vs pH (r = –0.62) and As geoavailable vs pH ( r = –0.33)). The total As range in wastes is 1.5-61.2 %, and geoavailable As varied from 878 to 28 430 (mg/kg). The full Pb range in wastes is 2.5-31.5 %, and geoavailable Pb varied from 5 to 133 (mg/kg) (**Table I**). Total Fe concentrations in all composite samples are similar and high (**Table I**). Total concentrations varied for the other elements present. Sample C1 has the highest total As, Cd and Mn. C2 has the highest concentrations of Pb, Fe, Cu, Zn and S, while C3 has the lowest of those metals except for Ca, which has the highest value (**Table I**).

The composite samples were analyzed through X-Ray Diffraction (XRD). The crystalline compounds identified were quartz, plagioclase, and $As_2O_3$. Weak signals were observed for augite (Ca, Na) (Mg, Fe, Al, Ti) $(Si, Al)_2O_6$, pharmacolite $CaH(AsO_4)\cdot 2H_2O$, clinomimetite $Pb_5(AsO_4)_3$

Cl, shultenite $PbHAsO_4$, and anglesite $PbSO_4$. Other compounds were expected because of previous findings in the soils of this area. Cu and Zn sulfates (Martínez-Jardines 2018) were not detected, probably due to low crystallinity or low concentration (Whitfield and Mitchell, 2008).

The SEM-EDS analysis of six particles from each of the composites (**Fig. 2**) showed analogous concentrations of the major elements (Al, Si, and Fe), whereas minor elements were variable.

The total concentrations of Ca and Fe were high, but with low geoavailability (**Table I**).

### *Soils bioaccessibility*

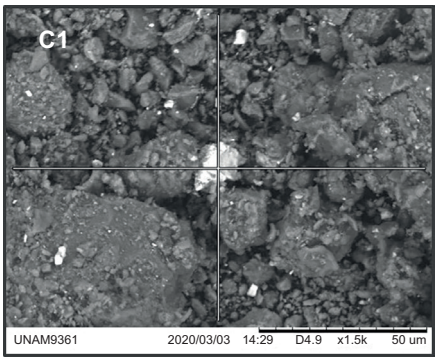
All bioaccessibility data is presented in **table II**. The range of As bioaccessibility (%) in the gastric phase (GSRSB-PBET) is 35.9-55.1 %, and for NIST 2710 is 46.3 %. The range of soil values using the NOM-147 method is higher = 35.8-60.4 %, and for NIST 2710 is 61.3 %. The percentage of As bioaccessibility in the intestinal phase by GSRSB-PBET is 39.5-46.9 %, and for NIST 2710 is 22.9 %. The Pb range in the gastric phase is 59.6-96.1 % from GSRSB-PBET, and the value for NIST 2710 is 72.0 %. The NOM-147 results range is 61.0-70.7 %, and for NIST 2710 is 90.8 %. In the intestinal phase, the range by GSRSB-PBET is 19.9-31.5 %, and for NIST is 36.6 %.

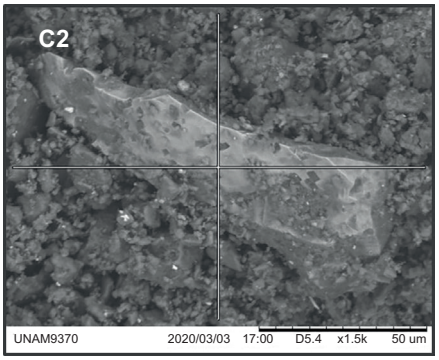
Pb, Cu and Zn exhibit higher bioaccessibility than geoavailability (**Tables I, II**), showing that the solubility of these soil pollutants is low in meteoric water but increases under gastric conditions (HCl, pH = 1.5). The bioaccessibility of Ca, Cu, Zn and Pb increases with higher total concentration. Geoavailability is not related to bioaccessibility, except for As and Zn (gastric phase, NOM-147, **Table II**). The bioaccessibility values of As, Pb Ca, Cu, and Zn in the gastric phase (**Table II**) are higher than their geoavailability.

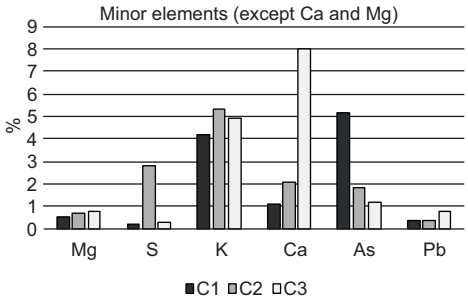
The bioaccessibility of Pb measured during the intestinal phase (PBET) is always lower than in the gastric phase using both methods. Nevertheless, As behavior is the opposite except in C3, the only basic sample.

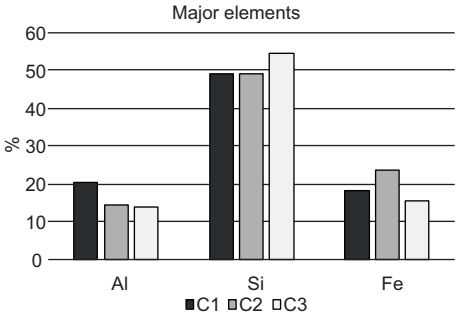
One-way ANOVA tests for As and Pb bioaccessibilities in the gastric phase among both methods (NOM-147 and GSRSB-PBET) were applied to compare whether the samples means were significantly different or not (using the F distribution).

For As we concluded that both gastric methods are statistically different, comparing bioaccessibilities for samples C1-C-3 ($F_{calculated}$ 4.71 > $F_{critical}$ 3.89, two-way ANOVA), and for reference material NIST 2710 ($F_{calculated}$ 45.08 > $F_{critical}$ 7.71, one way ANOVA). For Pb we also concluded that methods are statistically different, evaluating ANOVA for samples C1-C-3 ($F_{calculated}$ 26.36 > $F_{critical}$ 3.89, two-way ANOVA), and NIST 2710 ($F_{calculated}$ 34.88 > $F_{critical}$ 7.71, 1 factor ANOVA. All tests were done at 95 % of confidence.




| Composite samples | % w/w (average of 6 particles) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Al | Si | Fe | Mg | S | K | Ca | As | Pb |
| C1 | 20.6 | 49.3 | 18.4 | 0.5 | 0.2 | 4.2 | 1.1 | 5.2 | 0.4 |
| C2 | 14.3 | 49.3 | 23.5 | 0.7 | 2.8 | 5.3 | 2.1 | 1.8 | 0.4 |
| C3 | 13.8 | 54.5 | 15.7 | 0.8 | 0.3 | 4.9 | 8.0 | 1.2 | 0.8 |

**Fig. 2.** Average composition of particles of the composites. C1 and C2 micrographs are shown.

***Comparison between NIST 2710 bioaccessibility data and the in vitro and in vivo values reported by Koch et al. (2013)***

The magnitude of the As and Pb gastric bioaccessibility percentages of NIST 2710 calculated through the GSRSB-PBET method fall into the range for in vitro and in vivo results for the same standard (**Table III** and **Fig. 3**). It is important to mention that the result obtained is like those reported for in vitro by Koch et al. (2013) for the analogous method RMC-PBET. For the comparison of NIST 2710 results, we carried out statistical tests (one-way ANOVA) for As and Pb bioaccessibilities among the gastric phase (GSRSB-PBET method) and in vivo data reported (swine). We obtained that for As $F_{calculated}$ 2.07 < $F_{critical}$ 7.71, and for Pb $F_{calculated}$ 4.14 < $F_{critical}$ 7.71, concluding that there is no significant statistically difference between GSRSB-PBET method and in vivo swine results.

The values obtained with the NOM-147 method are higher than the others and fall out of the in vivo range (**Table III** and **Fig. 3**). The in vitro mean value reported by Koch et al. (2013) is 59 % for Pb. A higher Pb bioaccessibility percentage in the gastric phase was obtained using the NOM-147 method (90.8 %), Pb value measured with the RBALP method by Koch et al. (2013), it is the same as the NOM-147 method. We carried out statistical tests (one-way ANOVA) for both elements between the gastric phase (NOM-147 method) with swine As: $F_{calculated}$ 20.47 > $F_{critical}$ 7.71, and Pb: $F_{calculated}$ 17.87 > $F_{critical}$ 7.71, and for As with mice $F_{calculated}$ 95.20 > $F_{critical}$ 7.71, concluding that NOM-147 method is statistically different with respect to in vivo results.

The Pb bioaccessibility percentage in the intestinal phase (GSRSB-PBET) was 45.8 %. It is lower than the swine range values (73-79 %) (**Table III**, **Fig. 3**). Statistical one way ANOVA tests for both elements between the intestinal phase (GSRSB-PBET method) with swine (As: $F_{calculated}$ 166.35 > $F_{critical}$ 7.71, Pb: $F_{calculated}$ 289.24 > $F_{critical}$ 7.71) and for As with mice ($F_{calculated}$ 119.43 > $F_{critical}$ 7.71), indicate that intestinal phases are statistical different for in vivo results.

**TABLE II.** TOTAL CONCENTRATION AND GASTRIC AND INTESTINAL BIOACCESSIBILITY OF As, Pb, Fe, Cu, Ca AND Zn IN COMPOSITE SOILS AND NIST 2710 BY GSRSB-PBET AND NOM-147 METHODS.

| Determination | Method | Phase | Unit | C1 | | C2 | | C3 | | NIST 2710 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | As | Pb | As | Pb | As | Pb | As | Pb |
| Total | XRF | solid | (mg/kg) [a] | 32 490 ± 418 | 760 ± 17 | 10 648 ± 137 | 1 027 ± 19 | 4 964 ± 31 | 236 ± 5 | 626 ± 38 | 5 532 ± 80 |
| Bioaccessibility | GSRSB-PBET | Gastric | (mg/kg) [a] | 11 655±117 | 470 ± 40 | 4 004±80 | 612 ± 20 | 2 735±164 | 227 ± 5 | 290±17 | 3 981 ± 183 |
| | | | RSD % [b] | 1.3 | 8.5 | 2.2 | 3.3 | 6.2 | 2.2 | 5.8 | 4.6 |
| | | | n% | 35.9 | 61.9 | 37.6 | 59.6 | 55.1 | 96.1 | 46.3 | 72.0 |
| | | Intestinal | (mg/kg) [a] | 15 225±1,218 | 240 ± 2 | 4 211±126 | 205 ± 18 | 2 216±22 | 58 ± 8 | 144±23 | 2 024 ± 295 |
| | | | RSD % [b] | 8.5 | 0.9 | 2.9 | 8.6 | 1.4 | 13.1 | 15.8 | 14.6 |
| | | | n% | 46.9 | 31.5 | 39.5 | 19.9 | 44.6 | 24.5 | 22.9 | 36.6 |
| | NOM-147 | Gastric | (mg/kg) [a] | 12 283±540 | 493 ± 15 | 3 816±15 | 627 ± 6 | 3 000±105 | 167 ± 6 | 384±42 | 5 022 ± 344 |
| | | | RSD % [b] | 4.4 | 3.1 | 0.4 | 0.9 | 3.5 | 3.5 | 10.8 | 6.9 |
| | | | n% | 37.8 | 64.9 | 35.8 | 61.0 | 60.4 | 70.7 | 61.3 | 90.8 |

| Element | C1 | | | | C2 | | | | C3 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Total | Gastric bioaccesibility (NOM-147) | | | Total | Gastric bioaccesibility (NOM-147) | | | Total | Gastric bioaccesibility (NOM-147) | | |
| | (mg/kg) [a] | (mg/kg) [a] | RSD % [b] | % | (mg/kg) [a] | (mg/kg) [a] | RSD % [b] | % | (mg/kg) [a] | (mg/kg) [a] | RSD % [b] | % |
| Fe | 20,474±332 | 370±35 | 9.4 | 1.8 | 24,520±219 | 350±10 | 2.9 | 1.4 | 23,034±246 | 200±10 | 5.0 | 0.9 |
| Cu | 254±21 | 103±15 | 14.8 | 40.6 | 666±24 | 357±6 | 1.6 | 53.6 | 199±19 | 90±0 | 0.0 | 45.2 |
| Ca | 12,364±235 | 3,980±389 | 9.8 | 32.2 | 15,170±440 | 5,807±47 | 0.8 | 38.3 | 26,776±454 | 14,217±200 | 1.4 | 53.1 |
| Zn | 1,004±25 | 417±6 | 1.4 | 41.5 | 1,411±31 | 823±49 | 6.0 | 58.3 | 330±8 | 203±6 | 2.8 | 61.5 |

[a] Mean ± SD (n=3). [b] RSD % = Relative Standard Deviation Percentage (N=3), calculated as (SD/mean) x 100.

**TABLE III.** As AND Pb IN VITRO BIOAVAILABILITY (BIOACCESSIBILITY =BA) PERCENTAGE OF NIST 2710, AND IN VITRO AND IN VIVO DATA REPORTED FOR THE SAME STANDARD BY KOCH ET AL. (2013).

| As Bioaccessibility of NIST 2710 (in vitro) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Data in this study | | | | | | Data reported (Koch et al. 2013) | | | |
| Method | GSRSB-PBET | | | | NOM-147 | | Central laboratory [a] | | Individual laboratories | |
| | Gastric phase | | Intestinal phase [f] | | Gastric phase | | Gastric phase | Intestinal phase [f] | Gastric phase | Intestinal phase [f] |
| N= Replicates M=no. methods | N=3 | | N=3 | | N=6 | | M=15 | M=10 | M=12 | M=8 |
| Determination | Absolute (BA) | Relative | Absolute (BA) | Relative | Absolute (BA) | Relative | - | - | - | - |
| Mean % | 46.3 | 46.7 | 22.9 | 23.9 | 61.3 | 57.3 | 42 | 31 | 48 | 34 |
| Range (%) | 43.6-49.0 [b] | 43.5-49.9 [c] | 19.3-26.5 [b] | 21.5-26.3 [c] | 54.7-67.9 [b] | 55.9-58.7 [c] | 8.8-70 | 2.8-59 | 23-79 | 24-52 |
| RSD % | 5.8 [d] | - | 15.8 [d] | - | 10.8 [d] | - | 4.7 [e] | 7.1 [e] | 3.0 [e] | 5.1 [e] |
| Pb Bioaccessibility of NIST 2710 (in vitro) | | | | | | | | | | |
| Data in this study | | | | | | | Data reported (Koch et al. 2013) | | | |
| Method | GSRSB-PBET | | | | NOM-147 | | Central laboratory [a] | | Individual laboratories | |
| | Gastric phase | | Intestinal phase [f] | | Gastric phase | | Gastric phase | Intestinal phase [f] | Gastric phase | Intestinal phase [f] |
| N= Replicates M=no. methods | N=3 | | N=3 | | N=6 | | M=15 | M=10 | M=13 | M=9 |
| Determination | Absolute (BA) | Relative | Absolute (BA) | Relative | Absolute (BA) | Relative | - | - | - | - |
| Mean % | 72.0 | 75.4 | 36.6 | 45.8 | 90.8 | 95.7 | 59 | 17 | 61 | 19 |
| Range (%) | 68.7-75.3 [b] | 72.3-78.5 [c] | 31.3-41.9 [b] | 42.2-49.4 [c] | 84.5-97.1 [b] | 93.4-98.0 [c] | 13-101 | 0.4-51 | 20-106 | 2.2-49 |
| RSD % | 4.6 [d] | - | 14.6 [d] | - | 6.9 [d] | - | 6.1 [e] | 7.4 [e] | 3.2 [e] | 11 [e] |
| Relative bioavailability of NIST 2710 ( in vivo) reported by Koch et al. (2013) | | | | | | | | | | |
| Element | As | | | | | | Pb | | | |
| Data source | INERIS | | Bradham et al. (2011) | | | | INERIS | | | |
| Animal | Swine | | Mice | | | | Swine | | | |
| mean % | 49 | | 44 | | | | 76 | | | |
| range (%) | 46-52 | | 37-50 | | | | 73-79 | | | |

[a] Central Laboratory: single accredited commercial laboratory that analyzed all bioaccessibility extracts; [b] bioaccessible element concentration/total element concentration x 100 %, Total As concentration NIST 2710 = 626±38 mg/kg and total Pb concentration NIST 2710= 5,532±80 mg/kg (NIST 2003); [c] Relative bioaccessibility = calculated as reported by Juhasz et al. (2009); [d] RSD % = Relative Standard Deviation Percentage (N=3), calculated as (SD/mean) x 100; [e] RSD as repeatability of Central Laboratory or Individual laboratories; [f] Intestinal phase = Gastric + Intestinal phases

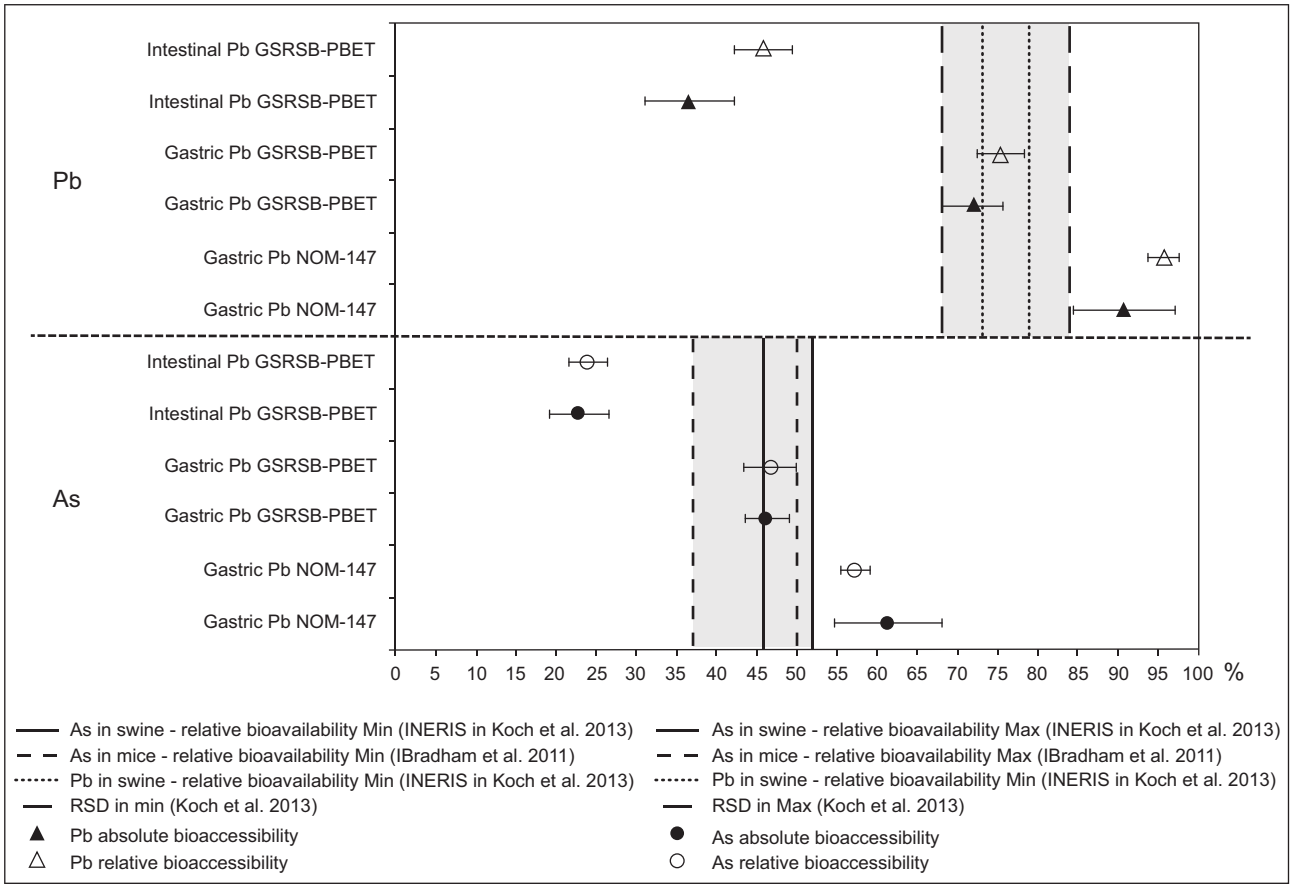



**Fig. 3.** As and Pb bioaccessibility results for the NIST 2710 and in vivo ranges reported by Koch et al. (2013). Note. The percentages of Relative bioavailability in swine (INERIS) and mice (Bradham et al. 2011) were taken from Koch et al. (2013). Relative bioaccessibility was calculated as reported by Juhasz et al. (2009). Intestinal phase = (gastric+intestinal).

Indeed, statistical tests of the results obtained in vitro between As and Pb with those measured in vivo reported to the same standard by Koch et al. (2013), indicates that only the results of the gastric phase of GSRSB-PBET have not statistically differences with the in vivo results, and fall in the range as can be seen in **figure 3**. As and Pb bioaccessibilities measured in the NIST 2710 (gastric phase) are more precise (less dispersion of results, reported as % Relative standard deviation) with the GSRSB-PBET method than the NOM-147 (**Table III**).

Absolute and relative percentages for both elements are similar (**Table III**) because there is no significant statistically difference evaluating by a one-way ANOVA. Absolute and relative percentages for both elements are similar (**Table III**) because there is no significant statistical difference evaluating a one-way ANOVA. The comparison results concerning a Fcritical =7.71 for As bioaccesibilities in vivo and the gastric phases from GSRSB-PBET, and NOM-147; and the intestinal phase GSRSB-PBET method gave Fcalculated 0.03, 1.05, and 0.16, respectively. Also, Pb ANOVA comparisons for in vivo and the gastric phases from GSRSB-PBET method, NOM-147 method, and the intestinal phase for GSRSB-PBET gave Fcalculated 1.69, 1.60 6.19, respectively. In all cases concluding that results are not statistically different from in vivo results. All the statistical ANOVA tests were done with 95 % confidence.

## DISCUSSION

In general, the main elements As, Pb, Fe, Ca, Cd, Cu, Zn, Mn, and S (**Table I**) and the compounds

identified by XRD in soils match with the composition reported by Gutiérrez-Ruiz et al. (2003), Romero et al. (2008), Villalobos et al. (2010) and Martínez-Jardines (2018), for the same site. Ca and Pb arsenates (phamacolite and clinomimetite) were identified. They are secondary minerals that slowly form in soils of semi-arid regions. Anglesite ($PbSO_4$), arsenolite ($As_2O_3$), and clay with Fe, Ca, and Mg were also identified. $CaCO_3$ was detected indirectly in C3 through the reaction of carbonates with HCl. Nevertheless, other compounds reported in this terrain's soil samples were not identified, possibly due to low concentrations or crystallinity. Most compounds found in analyzed soils in the study site included traces of arsenopyrite (Pokrovski et al. 2002), PbS, $CaSO_4.2H_2O$, goethite (FeOOH), magnetite ($Fe_3O_4$), chalcopyrite ($CuFeS_2$), and pyrite ($FeS_2$).

Furnace and converter dust and black arsenic must be the primary sources of As and Pb in the soil for two reasons: they are very rich in these elements, and calcine could contribute to Pb concentration (**Table I**). Overall, composites composition is similar to that of Cu slag (Nazer et al. 2016). The low geoavailability of Ca and Fe with high total concentration, possibly can be explained considering that these elements are in the slag. Moreover, variability of the total and geoavailable concentrations of minor elements (**Table I** and **Fig. 2**) shows a possible mixture of slag with converter flue dust, smelting furnace flue dust, sulfates, and carbonates. The diverse geoavailability of all elements except Fe (that was always low) can be attributed to the varied composition of the residues and byproducts that polluted the soil as converter flue dust, smelting furnace flue dust, calcine, and black arsenic (**Table I**). The $CaCO_3$ in sample C3 explains the pH > 7. Pb, Cu, and Zn sulfates explain the pH < 7 of samples C1 and C2, as well as their low solubility in meteoric water (geoavailability) and high bioaccessibility in the gastric phase. These observations coincide with the data reported in other studies (Walraven et al. 2015, González-Grijalva et al. 2019). Despite this, not all of these metals must be sulfates. A portion of low concentrations may come from the acid lixiviation of slag in the gastric phase. Ca content in carbonate and plagioclase minerals could explain low geoavailability in meteoric water and high bioaccessibility under acidic conditions.

Arsenic geoavailability varies from 480 to 2855 mg/kg. Sample C3, with the lowest total As concentration and high geoavailability, emphasizes the great importance of the compound's behavior. The soils contain As (III) in addition to As (V) compounds, which have been identified in this and other studies (Martínez-Jardines 2018). They can coexist because of dry weather conditions on site, and because reduction from arsenate to arsenite is slow (ATSDR 2007). The geoavailable As fraction must be mainly related with arsenolite, since the As (V) compounds have limited solubility in meteoric water. Although a number of arsenates could be adsorbed in amorphous Fe compounds (Goldber 2002, Donahoe et al. 2005, Hernández et al. 2016), it is not likely in this case. Fe in these soils seems to mainly be in the slag, because the geoavailable and bioaccessible fractions are quite low (**Tables I**, **II**). Furthermore, supposing the existence of amorphous oxides, Fe should be released during gastric extraction by chelation with organic ligands (Sidhu et al. 1981). Nevertheless, gastric Fe bioaccessibility was very low.

Pb geoavailability at pH = 5.5 was negligible but not its bioaccessibility, suggesting that Pb is mainly identified in low water-soluble sulfates, arsenates, carbonates and slag, but not under acidic conditions. It could also be identified in sulfides (González-Grijalva et al. 2019). All the compounds reported above can be partially solubilized under the acid condition of the gastric phase, even the slag (Aoki et al. 1984). Pb adsorption in birnessite or other Mn oxides that cover clays should be considered, since Mn compounds present hydroxyl groups favoring adsorption (Yin et al. 2011) at pH = 4.5-5.5 (Lenoble et al. 2002), although it is unlikely under gastric conditions. González-Grijalva et al. (2019) reported a Pb range from 40.8 to 50.8 %, lower than those obtained in this study. Considering that particle homogenization, temperature, and extraction times were the same, a possible factor related to data variability and soil composition could be the agitation system. The end-over-end shaker used in the NOM-147 method causes particle collision, reduction in particle size, and can increase soil reactivity (Quiroz-Vivanco 2018). The GSRSB uses a gas system that avoids biases in the expected results related to mechanical agitation. However, evidence of agitation's influence is limited due to the insufficient number of analyzed samples.

Indeed, compound characteristics seem to be the most relevant factor explaining the variability. Even As lixiviated from slag can form soluble compounds under gastric conditions, depending on each reaction's kinetics. Arsenic can exist as $H_3AsO_3$ and $H_3AsO_4$, or possibly as arsenates adsorbed in clays due to slow reduction. Clays do not dissolve in HCl (Simon and Anderson 1990), but can be partially altered, producing amorphous solids with a positive charge (Smedley and Kinniburg 2002). Acidic conditions increase the sorption capacity of clays,

forming complexes with anions as arsenates at low pH (Simon and Anderson 1990, Magalhães 2002, Zhang and Selim 2005, Elsheikh et al. 2018). The soluble As (III) from arsenolite cannot be absorbed because the $H_3AsO_3$ is neutral and only acquires a negative charge at very high pH (Wang and Mulligan 2006).

Pb could be soluble or coordinated with organic ligands in the gastric solution. The NOM-147 method uses glycine, which forms Pb $(NHCH_2COOH)_2$ (Zhang et al. 2011), a soluble compound that mobilizes Pb from bones (Alcaraz-Contreras et al. 2011). The PBET method uses citrates, acetates, and other organic chelates. All of these can form soluble compounds with metals, releasing arsenates (Ruby et al. 1993). For example, citrates are useful to recover Pb from batteries (Villa-Vargas 2017, Villa et al. 2018), and acetates leach Pb from calcines (García-Villa 2016).

The observed differences between gastric and intestinal bioaccessibility have been related to particle size, mineral solubility, sorption complexes, soil characteristics, and new compounds (Ruby et al. 1999, Walraven et al. 2015). In this case, in soils with acid pH and low Ca content, a higher As bioaccessibility in the intestinal phase than in the gastric phase was observed. One possible explanation is the adsorption of arsenates on clays under gastric conditions, being released in the intestinal phase when the pH increases to neutrality with $NaHCO_3$ and the clays lose their charge. Although Singh et al. (2011) reported that some abiotic or biotic oxidoreduction reactions could happen with the added reagents changing the solubility of As, García-Rodríguez (2017) reported that adding only $Na_2HCO_3$ also recovered lower As in the gastric phase than in the intestinal, reinforcing our hypothesis.

Pb in the intestinal phase was lower than in the gastric phase, as has been reported by Yan et al. (2016). This is possibly because the Pb released from organic complexes precipitate at the intestinal pH (Ruby et al. 1993, 1999, Li et al. 2014). However, Pb (II) can be bio-transformed, modifying its solubility and toxicity (Calatayud and Llopis 2015, Cangelosi et al. 2017).

An increase of Pb bioaccessibility in the intestinal phase can enhance its toxicity. According to Kan et al. (2017), the Pb carbonates' transformation to soluble organic Pb-complexes at neutral pH explain the increase of Pb bioaccessibility.

The bioaccessibility data for NIST 2710 in the gastric phase with the GSRSB- PBET method was more precise than with NOM-147, but this tendency is not clearly observed in the composite samples as mentioned above (**Table II**). Nevertheless, all the As and Pb values fall into the in vivo range reported by Koch et al. (2013) (**Fig. 3**), indicating that the reagents —and possibly the novel reactor system that reduces soil particle erosion and provides easier pH control— improve results.

In addition to those reported by several laboratories (Koch et al. 2013), all the data obtained in this study in the intestinal phase were lower than the in vivo range. It is worth mentioning that, according to Dabin et al. (2012), the in vitro methods with results similar to "in vivo" have longer agitation times or use more violent shakers. Therefore, it is possible that the intestinal phase results obtained with the GSRSB-PBET method can be improved by increasing agitation time.

## CONCLUSIONS AND RECOMMENDATIONS

The PBET method using the novel reactor system (GSRSB) provides easier pH control, and gastric phase data are more precise (% RSD) than the standard Mexican NOM-147 method.

The NIST 2710 results using the GSRSB-PBET method with the novel reactor show no statistical difference with respect to the in vivo measurements in the gastric phase.

The NOM-147 method measuring As and Pb bioaccessibility in the gastric phase does not simulate in vivo values and seems to overestimate the associated risk. Therefore, GSRSB-PBET method could be considered a precise alternative to measure As and Pb bioaccessibility in polluted soils modifying NOM-147 method and including the bioaccessibility measurement of other elements, mainly As.

The bioaccessibility of Pb measured during the intestinal phase (PBET) is always lower than in the gastric phase using both methods. Nevertheless, As behavior is the opposite except in C3, the only basic sample. It is recommended to include both phases and not only the gastric. It can also be possible to use the highest bioaccessibility value from both phases or perform in vivo bioavailability measurements.

Nevertheless, variability in soil results within the same metallurgical complex poses a challenge: to identify a general method with which to measure bioaccessibility in all types of contaminated soils, simulating in vivo data. Thus, it is essential to perform a complementary study to measure the bioaccessibility of a higher number of previously characterized soils. It must include at least the two studied methods and the two agitation systems with different agitation times and in vivo bioavailability determination.

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