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Availability of arsenic in rice grains by *in vitro* and *in vivo* (humans) assays

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ABSTRACT

Background: Rice grains are consumed by approximately half of the world's population. This cereal has higher arsenic (As) concentrations in grains than wheat or barley. Arsenic determination in food and/or *in vitro* studies are important for risk assessment; however, it is not enough to assess the real human exposure.

Method: *In vitro* bioaccessibility was carried out in husked-rice using gastric and intestinal solutions similar to humans. Also, As naturally found in husked-rice was evaluated by *in vivo* bioavailability in humans. For this purpose, diets from the 1st and 2nd days were free of foods known to be high in As; 3rd and 4th days the diets were composed by rice and water and; 5th and 6th the diet was similar the 1st and 2nd days. During all experimentation, a representative aliquot of each meal, blood and urine were collected for total As (t-As) determination. Arsenic species were determined in the urine.

Results: t-As in husked rice varied from 157.3 ± 30.6 to 240.2 ± 85.2 μg kg^{-1}. The *in vitro*bioaccessible fractions ranged from 91 to 94%. Inorganic As (i-As) ranged from 99.7 ± 11.2 to 159.5 ± 29.4 μg kg^{-1}. For the *in vivo* assay, t-As concentrations in the woman and man blood were about 3 μg mL^{-1} from the 1st to 6th day. Arsenic from the rice ingested was excreted by urine about 72 h after ingestion. The t-As and dimethyl As (DMA) in urine ranged from 3.59 to 47.17 and 1.02 to 2.55 μg g^{-1} creatinine for the volunteers, indicating a two-fold DMA-increase in urine after ingestion of husked-rice.

Conclusion: After rice ingestion, As was quickly metabolized. The higher As concentrations were found in urine 72 h after rice ingestion. The main As-specie found in urine was DMA, indicating that methylation of As from rice followed by urine excretion is the main biological pathway for As excretion.

1. Introduction

Rice is among the cereals with the highest global production. World rice production in 2015 was 739.2 million tons being China and India the largest producers. These countries account for 366.4 million tons, that is, almost 50% of world production [1]. Rice plays an important role in society not only culturally and economically, but also in the nutrition. On the one hand, essential elements such as copper (Cu), zinc (Zn) and magnesium (Mg) are present in rice grains, being essentials for humans, as they are components of enzymes and related to homeostasis [2–4]. On the other hand, non-essential elements such as arsenic (As) are also present in grains of rice from different regions of the world, representing a risk to human health [5–7].

Ingestion and inhalation are the main routes for As exposure. In humans, about 90% of As^{3+} and As^{5+} (i-As) are absorbed by the gastrointestinal tract. The clearance of As in the blood occurs in 3 steps: i-) about 2–3 h, reaching up to 90%; ii-) about 3 h to 7 days and; iii-) about 200 h [8,9]. Although As kinetics in plasma and erythrocytes are very similar, As in erythrocytes are 3 times higher than plasma after 10 h of exposure [10]. Once in the bloodstream, As is methylated in the liver and excreted in the urine. Inorganic As is methylated to monomethyl As (MMA) and dimethyl As (DMA) by arsenite methyltransferase (As3MT) using methyl S-adenosylmethionine (SAM) and glutathione (GSH). After methylated, As is excreted by proximal tubules in kidneys [11,12].

The genotoxicity of As is directly associated with its chemical species and methylation. Trivalent compounds are considered reactive thiols, interacting with proteins and enzymes. In mitochondria, they

Abbreviations: Cu, copper; Zn, zinc; Mg, magnesium; SOD, superoxide dismutase; As, arsenic; i-As, inorganic As; MMA, monomethyl As; DMA, dimethyl As; SAM, methyl S-adenosylmethionine; GSH, glutathione; As3MT, arsenite methyltransferase; o-As, organic As; t-As, total As; LOD, Limit of detection

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can alter the redox reactions of the cells, promoting the uncontrolled formation of superoxide anion radicals, generating deleterious effects [13,14]. Associated to this effect, the presence of As in the organism can promote genotoxic effects such as simple DNA breaks, chromosomal aberrations and increased oxidative stress of DNA bases [13]. All these toxic effects are associated to the carcinogenic ability of As for lung, bladder and kidneys, as well as other diseases that are not directly linked to carcinogenicity such as vascular, skin and diabetes mellitus diseases [15].

Previous studies in the literature showed the nutritional and toxicological properties of rice by determining chemical elements in grains [57]. However, this is not enough to understand the release and absorption of these elements in the human digestive system. Bioaccessibility (*in vivo* or *in vitro*) provides information about the fraction of the elements and/or compounds that are released from the matrix (food) in the gastrointestinal tract, making it available for intestinal absorption [16]. *In vitro* bioaccessibility allows a better evaluation of oral exposure to health-related elements and risks, generally simulating the two phases of gastrointestinal physiology: stomach and intestine [17]. Absorption in humans occurs in the small intestine, which is formed per villi (microvilli), which have blood and lymphatic capillaries for absorption. Once in the blood, the chemical elements (nutrients and/or contaminants) are transported to the liver through the portal vein and thus carried by the bloodstream to other organs and tissues. In the liver, the elements can be metabolized by reactions such as hydroxylation and methylation. It is worth noting that there are also intestinal microorganisms with potential methylation, another important mechanism of biotransformation of toxic and nutritional compounds [18–20].

Regarding bioavailability, it can be defined as the fraction of the contaminant present in the food that reaches the circulatory system, promoting toxic effects in the body. Therefore, bioavailability (absorption) is intensely associated to bioaccessibility (release), both directly influencing the risk [21]. *In vivo* bioavailability assays are commonly performed using models involving animals (generally rodents and swine) and determination of analytes in blood, urine, feces and organs such as liver and kidneys [22–24]. However, although there are some similar structures among mammals, the morphology of the gastrointestinal tract is considerably different between species. For example, small intestine diameter in humans is 5 cm while and in swine is 2.5–3.5 cm. [25,26]. According to Drobná et al., the primary hepatocytes from dog, rat and monkey are more efficient in i-As methylation than humans' primary hepatocytes [27]. Studies showed that marmoset monkey and chimpanzee are not able of methylating i-As [28]. Musther et al. [29] demonstrated that there is no strong linear association of bioavailability in animals and humans. The authors stated that bioavailability studies should be constructed according to the physiology of each species, and direct human studies provide more accurate and reliable results [29].

Bioavailability tests involving humans are scarce. It is still unclear what is the proportion of As metabolized after rice ingestion [30,31]. Assays with humans are important to understand the interaction of As with the human body. Several factors affect the physiological, metabolic and transport associated to As-bioavailability such as the As-specie, the amount ingested and nutritional status (fasting/fed) [32]. Therefore, this study aimed to investigate total As (t-As) and As-species in fractions of *in vitro* bioaccessible study of husked rice. Moreover, it was evaluated the *in vivo* bioavailability of As in human organism after husked rice intake by determining t-As in blood, plasma and urine as well as the As-species in the urine.

2. Material and methods

2.1. Reagents and instruments

Deionized water (resistivity of 18.2 MΩ cm) was used throughout the experiments (Master System All, Gehaka, Brazil). Plastic bottles used for storing solutions were cleaned for 24 h in acid bath at 15% v v^{-1} HNO_3 (65% m m^{-1}, Synth, São Paulo, SP, Brazil), rinsed five time with deionized water and dried in class 100 laminar flow (FilterFlux, São Paulo, SP, Brazil). The calibration standards were daily prepared by diluting 10 mg L^{-1} of stock solution multi-elemental (PerkinElmer, Shelton, Connecticut, EUA) in 2% v v^{-1} HNO_3. Yttrium (10 µg L^{-1}) was used as an internal standard. Certified reference materials of rice flour (National Institute of Standard and Technology - NIST, SRM 1568b, USA), blood reference material Seronorm Trace Elements Blood L-2, ALS Scandinavian, AB, Lulea, Sweden), serum (UTAK Serum toxicology control, Canada) and urine (NIST, SRM 2670a, USA) were used to evaluate the performance of the method.

A 12 mol L^{-1} HCl (36% v v^{-1}, Synth, Brazil), pepsin, sodium glycooxycholate, sodium taurodeoxycholate, sodium taurocholate hydrate, pancreatin and $NaHCO_3$ (3% m v^{-1}) (Sigma-Aldrich, St. Louis, USA) were used for the preparation of the gastrointestinal solution. Creatinine concentration was determined by using a kinetic creatinine test (Bioclin, Minas Gerais, Brazil) in a UV–vis spectrophotometer (Agilent 8453, CA, USA).

All acid digestions were performed using a microwave oven system (Ethos Easy, Milestone, Italy). Operating conditions were according to Paniz et al. [33]. The determination of t-As was performed by inductively coupled plasma mass spectrometer (ICP-MS) Agilent 7900 (Hachioji, Japan). Stock solutions of As^{3+} (As_2O_3, Aldrich, St. Louis, EUA), As^{5+} ($As_2O_5.H_2O$, Aldrich, St. Louis, EUA), DMA ($C_2H_7AsO_2$, Fluka, St. Louis, EUA) and MMA ($Na_2CH_3O_3.As_6.H_2O$, Fluka, St. Louis, EUA) were used for As speciation in a high-performance liquid chromatography (HPLC, Agilent 1290 Infinity II, Waldbronn, Germany) hyphenated to an ICP-MS (HPLC-ICP-MS). For speciation, all conditions and the preparation of standard solutions are described elsewhere [6]. Operating conditions for ICP-MS and HPLC-ICP-MS are in the supplementary Table S1.

2.2. Husked rice samples and total arsenic determination

Samples (5 kg plastic bag) of husked rice (n = 6 samples) were randomly purchased from markets localized in several Brazilian states (São Paulo, Minas Gerais, Rio Grande do Sul and Brasília). The samples were named as R1, R2, R3, R4, R5 and R6. These locals of sampling were selected due to the availability for sampling (once Brazil is a continental country) and because the population from those states present high rice consumption.

Microwave digestion was performed according to Paniz et al. [33]. The samples were milled and sieved (< 250 µm). After homogenization, all samples (in triplicate) were weighed (˜ 200 mg) into 100 mL polytetrafluoroethylene (PTFE) vessels (35 bar of maximum pressure). Then, 4 mL of HNO_3 20% v v−1 + 1 mL of H_2O_2 30% v v^{-1} were added and the tubes were closed and placed in the microwave. After cooling, the volume was made up to 50 mL with deionized water and analyzed by ICP-MS according to Paniz et al. [33]. The methodological limit of detection was 0.017 µg kg^{-1}. For method accuracy, the NIST certified reference material rice flour 1568b was analyzed, showing a recovery of 97 ± 11% for As.

2.3. Test of bioaccessibility

In vitro bioaccessibility assessment was performed according to Bertin et al. [34] and the United States Pharmacopoeia [35]. The husked rice samples were cooked as described below for volunteers' ingestion. The assay was performed in two steps: gastric solution and intestinal solution. For the gastric solution, 0.32 g of pepsin was weighed, solubilized in deionized water, added 0.7 mL HCl (36% v v^{-1}, Synth, Brazil) to 12 mol L^{-1} and the volume was made up to 100 mL with deionized water. The intestinal solution (100 mL) was composed of 0.2 g of bile salts (0.08 g of sodium glycooxycholate +0.05 g of sodium taurodeoxycholate +0.08 g of sodium hydrate taurocholate) and

0.5 g of pancreatin in 3% $NaHCO_3$ (m v $^{-1}$)

2.3.1. Gastric digestion

Samples (200 mg, in duplicate) were weighed into 50 mL conical tubes and 3 mL of the gastric solution was added. The pH was adjusted to 1.2 using a solution of HCl (0.1 mol L^{-1}). The samples were incubated in orbital shaking (SL-223, Solab, Piracicaba, Brazil) at 37 °C and 80 rpm for 2 h.

2.3.2. Intestinal digestion

After gastric digestion, the intestinal solution (3 mL) was added and $NaHCO_3$ (3% m v^{-1}) was used for pH adjustment in 6.8, followed by heating at 37 °C and 80 rpm for 2 h under orbital shaker. Finally, the samples were cooled to 25 °C and centrifuged (SL700, Solab, Brazil) at 1077 *g* for 20 min. The supernatant was separated from the precipitate, filtered (0.20 µm cellulose filter, Sartorius, Göttingen, Germany) and digested in the microwave as above mentioned [33].

2.4. Arsenic speciation in the bioaccessible fraction

For speciation analysis, 300 µL of the bioaccessible fraction was filtered (0.20 µm cellulose filter, Sartorius, Göttingen, Germany), acidified with 600 µL of HNO_3 (3% v v^{-1}) and analyzed by HPLC-ICP-MS. All the conditions of analysis are in supplementary Table S1. For the accuracy of the method, NIST rice flour 1568b certified reference material was analyzed, showing recoveries of 95% for i-As (As^{3+} + As^{5+}) and 81% for organic As (o-As: DMA + MMA).

2.5. Calculation of bioaccessible fraction

The bioaccessibility was calculated taking into account the mass of total As. The bioaccessible fraction in percentage (%) was obtained according to Eq. 1.

$$\text{Bioaccessible Fraction (\%)} = \frac{\text{mass of As solubilized in the } \textit{in vitro} \text{ extraction}}{\text{mass of As in the sample aliquot}} \times 100 \quad (1)$$

2.6. Estimated daily intake

The estimated daily intake of As was calculated using the following Eq. 2. EDI is the estimated daily intake of the toxic element (µg d^{-1} kg^{-1} body weight), C (µg kg^{-1}) is the concentration of the element in the food, FM is the daily mass of the food ingested (kg d^{-1}) and BM is the body mass (a person of 70 kg).

$$EDI = \frac{CxFM}{BM} \quad (2)$$

2.7. In vivo assay for arsenic bioavailability

Due to the difficulties of recruitment, only two Caucasian individuals, non-smokers and healthy (no medication intake, normal pressure and biochemical blood indices) participated of the experiment. Volunteer 1 was female, 33 years old, 1.68 m and 73 kg. Volunteer 2 was male, 35 years old, 1.78 m and 88 kg. The genders were selected trying to evaluate differences on As metabolism. Both volunteers were submitted to the same diet (Table 1), in which the volunteers understood the totality of the project and the experimental details. The project was approved by the Ethics and Research Committee of the Federal University of ABC (ethical process number 77067316.8.0000.5594). Throughout the experiment, fractions of food consumed (24 h total diet) were collected to determine the t-As concentration. Samples of blood and urine were also collected for determination of t-As and speciation (urine). The procedures for preparation and determination are described below.

2.8. The cooking of husked rice and portions consumed

After determination of t-As in the samples, only one bag of 5 kg of husked rice was selected for the consumption of the volunteers. Rice cooking was conducted in a glass pan according to the method of preparation provided by the manufacturer. For each 150 g of rice, 480 mL of mineral water and sodium chloride (˜ 5 g) were added and the cooking time of 10–15 min and/or until total water evaporation (about 98 °C). The portion consumed was according to daily calorie needs, considering gender, age and body mass index. The recommended daily caloric intake for men aged 31–40 years is 2400 kcal; for women in the same age group is 1800 kcal [36]. Based on the daily consumption of each gender and that 1 g of rice is ˜3.42 kcal, the man and the woman consumed 3 meals of 250 g and 3 meals of 170 g, respectively, in the 3rd and 4th days.

2.9. Scheme of rice ingestion and procedure for blood and urine sampling

The experiment occurred in 6 days, according to Table 1. In the first (1st and 2nd days) and third (5th and 6th days) periods, the diet consisted of a typical diet, without rice or foods known to be high in As (such as rice, fish, seafood and foods generally associated to Asiatic culinary). A 24 h diet method was used for monitoring. For this purpose, ˜50 g (solid + liquid foods) of each meal was collected for t-As determination. In the second period (3rd and 4th days), the diet was composed only by cooked husked rice and water. The period of rice-diet was due to the time of As metabolism and the difficult of volunteers' recruitment. Samples of blood were collected in all three periods. The As concentrations in diets, blood and urine were controlled analyzing each individual, separated: the 1st and 2nd days (Table 1) were the control of a typical diet of the volunteer before rice ingestion and the 5th and 6th days (Table 1) were the control after rice ingestion of the volunteer. The schedule of husked rice intakes (rice and water from 3rd and 4th days) and all blood collections are in Table 1. Urine was collected throughout the experiment.

2.10. Collection of blood and urine

Blood was collected in tubes for trace elements containing EDTA (Greiner Bio-One, Kremsmünster, Austria). Table 1 shows the time between blood collections. Aliquots of 4 mL of blood were stored in tubes of the Vacutainer® type at −80 °C until the determination of t-As.

Urine was collected throughout the experiment. For this purpose, a metal-free 50 mL plastic tube was used for urine collection. After despising the initial urine stream, each volunteer collected about 30 ml of urine for each bladder emptying and the time was recorded. Arsenic concentrations were related to creatinine concentration in urine. Creatinine is directly related to the variation of water reabsorption by the kidneys and the urinary concentrations of solutes. The analyte/creatinine ratio (expressed as µg analyte g^{-1} creatinine) minimizes the intra-individual variability that may occur by the urine dilution [37].

Creatinine was determined as described by Jaffe [38] by reacting creatinine and alkaline picric acid to produce a red/orange complex [38–41]. In a 15 mL conical tube, 250 µL of urine and 2 mL of alkaline reagent (sodium hydroxide 110 mmol L^{-1}, sodium carbonate 75 mmol L^{-1} and surfactant) were incubated in a water-bath (37 °C) for 10 min. The solution was analyzed by an UV–vis spectrophotometer at 510 nm (Abs1). Then, 100 µL of acetic acid 12.25 mol L^{-1} was added to the solution and homogenized. The sample was re-incubated in a water-bath (22.5 °C) for 5 min and analyzed by an UV–vis spectrophotometer at 510 nm (Abs2). Blanks were used for set zero the instrument. Creatinine standard (30 mg L^{-1}) was analyzed each run (Abs3). The concentration of urinary creatinine (mg L^{-1}) was calculated using Eq. 3.

Table 1
Schedule of meals (M: foods with known low As-concentrations; RM: only husked rice) and blood collection (B) for As kinetics after rice ingestion. The experiment was carried out for 6 days. Urine was collected throughout the experiment.

Day	Time (24 h)										
	7:00	7:30	8:00	8:30	9:00	13:00	19:00	19:30	20:00	20:30	21:00
1st	M*	B	–	–	–	M*	M*	–	–	–	–
2nd	M*	B	–	–	–	M*	M*	B	–	–	–
3rd	RM	B	B	B	B	RM	RM	B	B	B	B
4th	RM	B	B	B	B	RM	RM	B	B	B	B
5th	M*	B	–	–	–	M*	M*	–	–	–	–
6th	M*	B	–	–	–	M*	M*	B	–	–	–

* Foods known to be high in As (examples: seafood, fish, mushrooms, typical foods of Asiatic cuisine) were avoided.

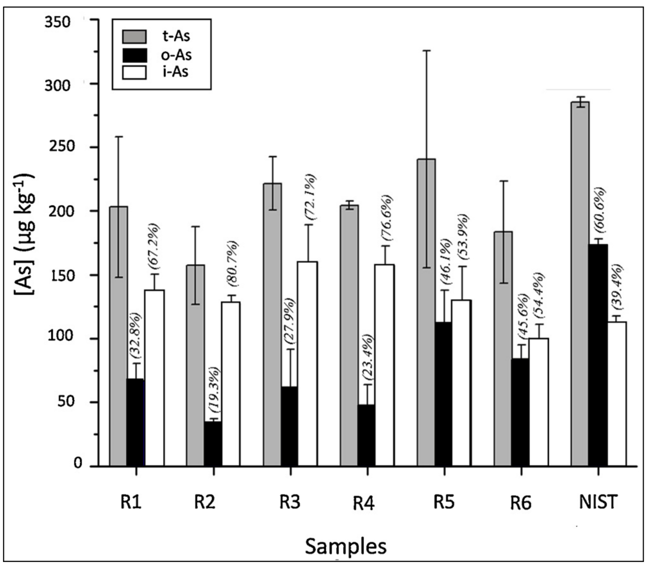

Fig. 1. Concentration of total As (t-As) and As species (i-As and o-As) in the bioaccessible fraction of husked rice (R1 to R6) purchased from Brazilian supermarkets and rice flour standard reference material 1568b from the National Institute of Standard and Technology (NIST). Above black and white bars are the percentage of the bioaccessible fraction of As species in relation to total As. Thicker bars in gray, black or white represent the mean; thinner lines in black are the standard deviation. The analysis were carried out in triplicate. Inorganic arsenic (i-As): sum of As^{3+} and As^{5+}; organic arsenic (o-As): sum of DMA + MMA.

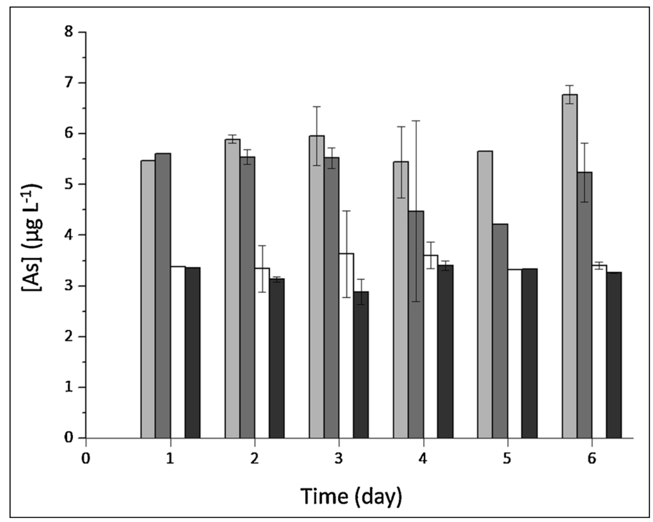

Fig. 2. Concentration of total As (µg L^{-1}) in plasma (light gray and dark gray bars) and whole blood (white and black bars), as a function of time (days). Diets in days 1, 2, 5 and 6 were without rice or other foods known to be high in As (such as rice, fish, seafood and foods generally associated to Asiatic culinary). Diets in days 3 and 4 were composed of cooked husked rice and water. Woman: light gray and white; Man: dark gray and black. Thicker bars in gray, black or white represent the mean; thinner black lines represent the standard deviation.

$$Creatinine = 30\,x\,\frac{Abs1 - Abs2}{Abs3} \quad (3)$$

2.11. Total determination of arsenic in whole blood, plasma and urine

Reference materials (blood and urine) were used to control the quality of the analysis. Recovery of blood reference material (Seronorm Trace Elements Blood L-2, ALS Scandinavia AB, Lulea, Sweden) was 100%. Concerning plasma (Serum toxicology control, UTAK, Canada) and urine (NIST 2670a), the recoveries were 111% and 102%, respectively. For blood, plasma and urine, the methodological limits of detection were 0.006, 0.02 and 0.036 µg L^{-1}.

The method described by Batista et al. [42] was used with modifications for the determination of As in blood. In a 15 mL conical tube were added 200 mL of blood + 9.8 mL of a solution containing 0.5% v $v^{-1}$of HNO_3 and 0.01 % v v^{-1} of Triton X-100. The samples were shaken and analyzed by ICP-MS.

Urine and plasma analysis were performed according to Freire et al. [43]. Urine or plasma (500 µL) were added in plastic conical tube (15 mL) and the volume made up to 10 mL with a diluent containing 0.4% v v^{-1} HNO_3 + 0.005% v v^{-1} Triton X-100. The concentrations of As in urine (µg L^{-1}) were adjusted by the concentration of creatinine (g L^{-1}).

2.12. Speciation of arsenic in urine

Due to the high number of urine samples, the speciation was performed in daily pools of urine of each individual. Also, in specific days, samples which showed higher t-As were individually investigated, to identify which species had the highest concentration. The urine samples were filtered (0.20 µm cellulose filter) and analyzed by HPLC-ICP-MS according to Batista et al. [6]. All instrumental parameters are in Table S1.

2.13. Statistical analysis

The one-way ANOVA was used to evaluate the statistical differences. The software used was OriginPro 8 version 8.0724 (Northampton, MA, USA). Statistical significance was set at 5%.

3. Results and discussion

3.1. Concentration and total in vitrobioaccessible fraction of arsenic in husked rice

The average concentration of t-As found in husked rice ranged from

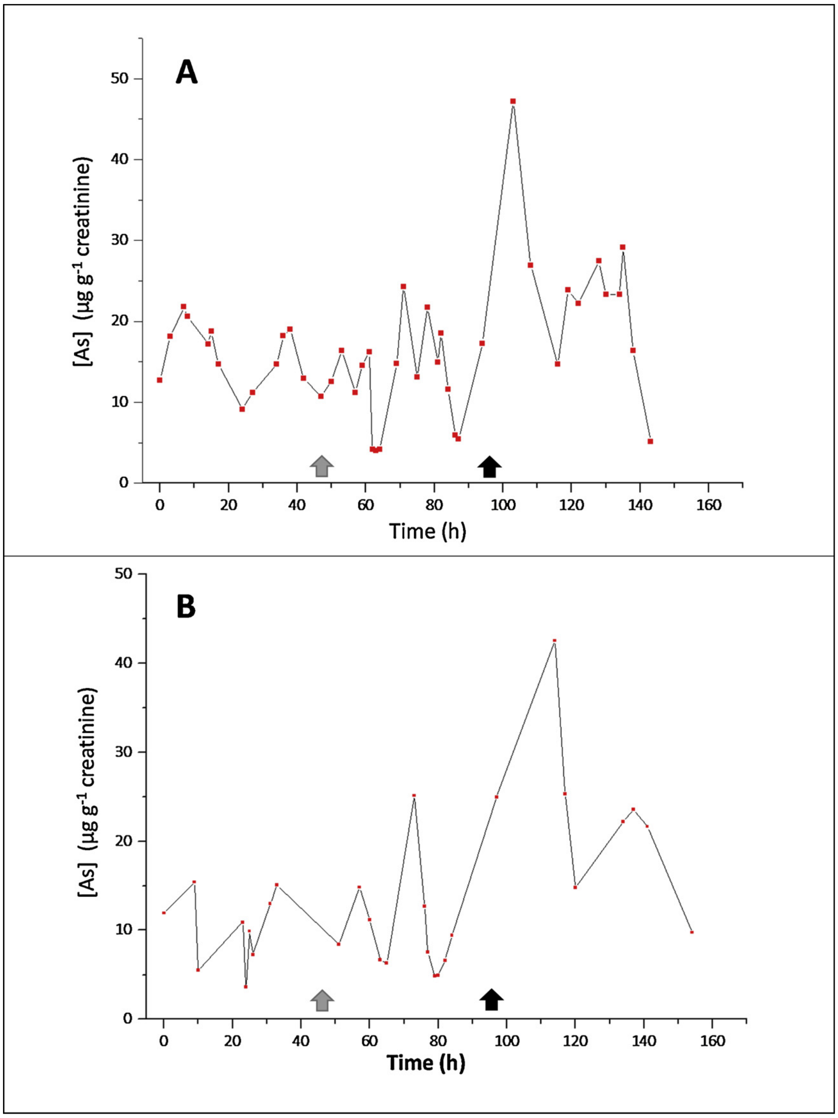

Fig. 3. Total As concentration in urine as function of time. A: man; B: woman. The concentrations in urine were corrected by the concentration of urinary creatinine. Arrows in gray and black indicate the first and the last husked rice meals, respectively. Diets in days 1, 2, 5 and 6 (0–48 h and 96–144 h) were without rice or other foods known to be high in As (such as rice, fish, seafood and foods generally associated to Asiatic culinary). Diets in days 3 and 4 (48–96 h) were composed of cooked husked rice and water.

157.3 ± 30.6 to 240.2 ± 85.2 µg kg^{-1} ($p < 0.05$) (Fig. 1). The *Codex* Committee on Contaminants in Foods established a maximum limit of As for husked rice of 350 µg kg^{-1} [44].

Food and beverages generally contribute between 20–300 µg day^{-1} As intake [45]. Rice, compared to other cereals such as wheat and barley, may have about 10 times more As [46]. Soil conditions, rice variety and irrigation are factors that may influence the uptake of As by rice [47,48].

Regarding bioaccessible fractions, the values ranged from 91 to 94% for t-As (Fig. 1). During digestion, the polypeptides and proteins can be denatured or broken by the action of HCl and pepsin, thus allowing access to peptide bonds. This mechanism provides more access to the As-thiol complex, making it more bioaccessible [49]. Chavez-Capello et al. [50] determined about 80% of the bioaccessible t-As coming from rice, in which 50% was present in the gastric fraction and 35% in the gastrointestinal fraction. Other studies presented similar results for bioaccessible fractions of t-As after different rice cooking processes. Regardless of the water ratio used and the type of cooking, the bioaccessible fractions of t-As from 70 to 100% and 72–80% according to Liao et al. [51] and Zhuang et al. [52], respectively. He et al. [53] determined the bioaccessible fraction of t-As from 71 to 97% in several types of grains (extra-long grains, long grains, long parboiled grains and husked grains) purchased in New York City. The authors attributed the lower concentrations to husked rice (71%) because of its external constitution. However, other authors show that the external composition presents a higher concentration of i-As than o-As [54,55]. Therefore, the As bioaccessibility is dependent on several variables such as rice type, As levels found, the proportions between As-species, and specific variations of the applied bioaccessibility test.

3.2. Species of arsenic in the bioaccessible fraction

Inorganic As was detected in all samples purchased from supermarkets. The variations of i-As ranged from 99.7 ± 11.2 to 159.5 ± 29.4 µg kg^{-1} ($P < 0.05$) and the maximum bioaccessible percentages for the species were 46% for o-As and 80% for i-As (Fig. 1). The standard reference material 1568b from the National Institute of Standard and Technology (NIST) was used for quality control purposes during the test of bioaccessibility and analysis (Fig. 1).

The found values are close to those reported by He et al. [53] who

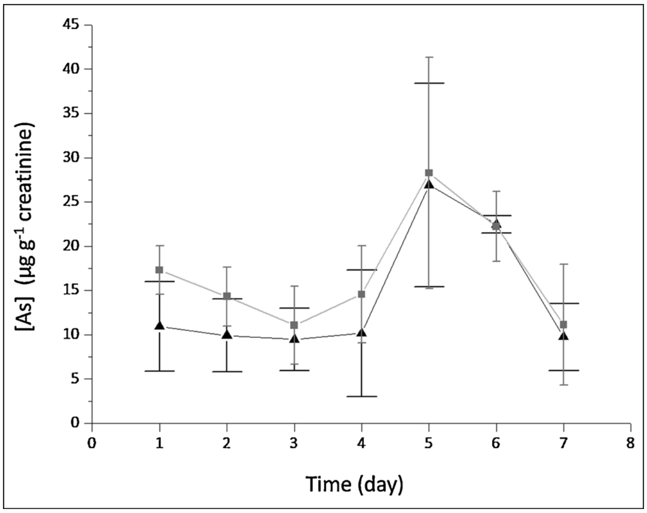

Fig. 4. Mean and standard deviation of total As concentration (creatinine-corrected) per day in urine. Black: woman (triangle); Gray: man (square). Diets in days 1, 2, 5 and 6 were without rice or other foods known to be high in As (such as rice, fish, seafood and foods generally associated to Asiatic culinary). Diets in days 3 and 4 were composed of cooked husked rice and water.

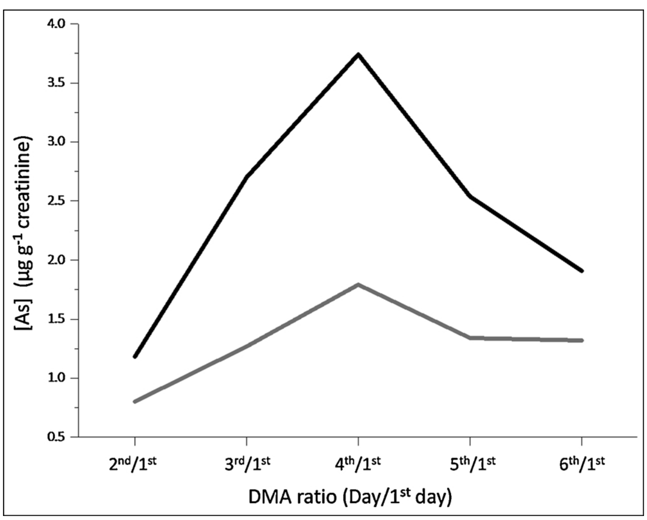

Fig. 5. Dimethyl arsenic (DMA) mean ratio from the 2nd to 6th day of experiment divided by the 1st day. Black line: woman; gray line: man. Diets in days 1, 2, 5 and 6 were without rice or other foods known to be high in As (such as rice, fish, seafood and foods generally associated to Asiatic culinary). Diets in days 3 and 4 were composed of cooked husked rice and water.

observed bioaccessibility in husked rice varying from 64 to 98%. Meharg et al. [54] found approximately 55 and 65% of bioaccessible i-As in polished and husked rice, respectively. The bran, a superficial layer of the grain present in husked rice, have higher amounts of i-As [54,55]. Concerning the conversion of species, generally, As species are stable during *in vitro* bioaccessibility procedures, except for As-sugars [56] that, as far as we know, are not commonly found in rice.

3.3. Estimated daily intake (EDI)

The sample used to estimate the daily intake by body weight was the husked rice used for ingestion (sample R5), in which the EDI was 0.027 µg As day $^{-1}$ kg^{-1} body weight. Lee et al. [57] estimated, on average, 0.02 µg day^{-1} kg^{-1} per body weight for i-As in Korean rice. However, the consumption of rice by Koreans is higher than that of Brazilians (about 22-fold higher). Nonetheless, the tolerable intake levels are under discussion by regulatory agencies. In 2009, the European Food Safety Authority concluded that the provisional tolerable weekly intake (PTWI) of 15 µg kg^{-1} per body weight is no longer safe [58]. In 2015, the European Commission established EU Regulation 2015/1006 concerning the maximum concentrations for i-As in husked rice (250 mg kg^{-1}) [59]. To better understand the mechanisms involving rice-As-humans, studies focusing on rice its derivatives in a non-restrictive diet are necessary [30,60,61].

3.4. Arsenic bioavailability in rice grains after ingestion by humans

3.4.1. Arsenic concentration in food consumed before and after rice diet

The t-As in the diet without foods known to be high in As (such as rice, fish, seafood and foods generally associated to Asiatic culinary) ranged from less than the limit of detection (< LOD) to 121.75 µg kg^{-1} (woman) and from < LOD to 67.05 µg kg^{-1} (man) (Supplementary Table S2 for the foods consumed during the experimentation). Table S2 shows the diet before (1st and 2nd days) and after (5th and 6th days) the rice intake (3rd and 4th days). The concentrations in the present study are close to those found by Schoof et al. [62] who analyzed the USA food basket in 1999. The authors found As concentrations ranging from 1.4 to 86 µg kg^{-1} to all kind of food except rice, seafood and fish. Recently, He and Zheng [30] reported concentrations in a controlled diet (containing some rice derivatives) ranging from 2 to 52 µg kg^{-1}. In general, foods that require more water during their cultivation have higher As concentrations. Foods such as tuberous vegetables have higher As concentration than leafy and fruity vegetables [63]. Vegetables and fruits, in general, may have concentrations ranging from 4 to 800 µg kg^{-1}. With regard to animal products, the concentrations may range from 33 to 38 µg kg^{-1} [64].

3.4.2. Total As in blood and plasma

Arsenic concentrations in whole blood and plasma remained constant throughout the experiment for both volunteers (Fig. 2). The mean of t-As concentration in whole blood ranged from 3.38 to 3.63 and 2.88 to 3.36 µg L^{-1} for the woman and the man, respectively ($P < 0.05$). The highest concentrations in whole blood occurred on the 3rd and 4th days, respectively. The highest concentrations in plasma occurred in the 6th day for the woman and in the 4th day for man. The t-As concentrations in plasma ranged from 5.44 to 6.77 µg L^{-1} (woman) ($P < 0.05$) and 4.22 to 5.54 µg L^{-1} (man) ($P < 0.05$). Overall, As-concentration in plasma was 60% of whole blood.

Yoshino et al. [65] found that patients with acute promyelocytic leukemia treated with As trioxide (As_2O_3) presented 80–90% of As in red blood cells, suggesting that As binds to hemoglobin. Unfortunately, the author did not determine the As concentrations in plasma. The higher the presence of As in the plasma, the higher the toxic effects due to the increased availability. Besides, As in plasma can be metabolized by the liver and eliminated by the kidneys quickly, which explain the fast blood distribution and excretion [11]. Therefore, due to the fast half-life of As in the blood-stream, the higher amounts of As are found in urine once As accumulates in the bladder after the excretion by kidneys [66].

3.5. Total concentration of As and its species in urine

Crecelius [67] conducted an experiment with a 30-year-old man who ingested a controlled diet containing wine, water and crab. The author found a background of As in the urine of 15 µg L^{-1} and, 61 h after ingestion of 63 µg of As, ˜80% of As was excreted in the urine. In the present study, after ingestion of husked rice containing about 60 µg and 40 µg of As per meal for the man and the woman, respectively, As was eliminated by urine after approximately 72 h. The man eliminated about 70% and the woman 96% of the As ingested through of rice.

Concentrations of t-As per hour in urine were corrected by the concentration of urinary creatinine, as mentioned before. For the man, the As concentrations ranged from 3.98 to 47.17 μg g^{-1} creatinine ($P < 0.05$), the lowest As concentration occurred in the 3rd day and the highest in the 5th day (Fig. 3A). The As concentrations ranged from 3.59 to 42.53 μg g^{-1} creatinine for the woman ($P < 0.05$) (Fig. 3B), in which the lowest concentration occurred on the 2nd day and the highest on the 5th day. Therefore, for both volunteers, ingestion of husked rice significantly increased the As-concentrations in the urine.

He and Zheng et al. [30] observed higher urinary As excretion of people consuming a diet rice-containing. The authors found concentrations of 13 μg L^{-1} (diet containing rice) and 6 μg L^{-1} (diet without rice) for woman and 8 μg L^{-1} (rice diet) and 5 μg L^{-1} (diet without rice) to man. Karagas et al. [60] showed that t-As in the urine of children who ingested rice is 2-fold higher than those who do not consume rice.

The concentrations of t-As in urine was very similar between the two volunteers (Fig. 4). Regarding the As-species, among the six days of the experiment, the concentrations of As-species for the woman varied from 2.05 to 4.67, 1.02 to 2.45 and 3.47 to 13.00 μg g^{-1} creatinine for i-As, MMA and DMA respectively. For the man, the As-species concentrations varied from 1.74 to 8.22, 1.26 to 2.55 and 6.25 to 14.03 μg g^{-1} creatinine for i-As, MMA and DMA, respectively.

The rates of urinary excretion of DMA were calculated using the ratio between the 2nd, 3rd, 4th, 5th and 6th days and the 1st day (Fig. 5). The lowest and the highest ratios for the woman were 1.18 between the 2nd/1st days and 3.74 between the 4th/1st days, respectively (Fig. 5). For the man, the lowest and highest ratios were 1.45 between the 6th/1st days and 2.12 between the 3rd/1st days, respectively (Fig. 5). Therefore, the highest ratio of excretion occurred between the 3rd and 4th days.

Women of childbearing age and not adolescents have higher methylation capacity compared to men. Estrogen can promote methylation through the oxidation mechanism of phosphatidylcholines, which are regulated by estrogen [68], probably explaining the higher As methylated species present in woman urine (Fig. 5). These results are consistent with the study of He and Zheng [30] with two volunteers. The authors also found increased DMA concentrations from 4 μg L^{-1} (diet without rice) to 8 μg L^{-1} (diet containing rice) for woman and 2 μg L^{-1} (diet without rice) to 5 μg L^{-1} (diet containing rice) for man. Although several studies showed that women are more efficient than men in As-methylation [69–72], more studies are necessary for better understanding the As kinetics in the organism after rice ingestion.

4. Conclusions

The analyzed rice presented an *in vitro* bioaccessible fraction between 91–94%, indicating that practically all As present in rice can be absorbed by the human organism, in which 80% was i-As, the most toxic As-form. The *in vivo* assays improved our knowledge about the understanding of As toxicodynamic in the human body after the ingestion of husked rice naturally contaminated with As. The presence of As in blood (40%) was lower than plasma (60%). In both, the As-concentrations remained constant during the experiment. However, in urine, As-concentrations increased with rice ingestion. The presence of As in urine in the 5th day was twice as high compared to the days before, indicating an elimination varying from 70 to 96% of the As ingested through the rice. Higher methylation by the woman was evidenced; however, more studies with more volunteers are necessary to corroborate this observation. Therefore, although the concentration of As is a good indication for food risk consumption, *more in vivo* studies are required to improve the knowledge about rice risk assessment.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jtemb.2019.08.014.

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