



## On-line speciation and quantification of four arsenical species in rice samples collected in Argentina using a HPLC–HG–AFS coupling



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### ARTICLE INFO

#### Article history:

Received 23 December 2014

Accepted 29 December 2014

Available online 5 January 2015

#### Keywords:

Rice

Mild extraction

Speciation analysis of As

HPLC–HG–AFS

Method validation

### ABSTRACT

An investigation was carried out to explore further the analytical capabilities of the coupling high performance liquid chromatography (HPLC)–hydride generation (HG)–atomic fluorescence spectrometry (AFS) for the reliable determination of the four toxicologically relevant arsenical species namely, As(III), As(V), monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) in rice. Fifty nine samples of rice of five different cultivars were collected in province of Entre Ríos (Argentina). Samples were dried at  $103 \pm 2$  °C and mild conditions were adopted for species extraction ( $0.28 \text{ mol L}^{-1} \text{ HNO}_3$  at  $95 \pm 3$  °C for 90 min). Method validation included, evaluation of linearity; limits of detection,  $0.020 \mu\text{g g}^{-1}$  for As(III) and  $0.025 \mu\text{g g}^{-1}$  for the other three species; precision (intermediate precision), 4% for As(III) and DMA, and 6% for the other two species; trueness (bias), 9% for As(III) and As(V) and 11% for the other two species, and uncertainty (obtained from validation data) varying from 9.5% for As(III) to 19% for As(V). Total arsenic concentrations ranged from 0.08 to  $1.39 \text{ mg kg}^{-1}$ . The mean concentrations of sum of the As species extracted and determined by HPLC–HG–AFS was approximately 99.7% of certified value for total As in NIST SRM “Rice flour”. Levels of the four species in the analyzed samples were in the order  $\text{DMA} > \text{As(III)} > \text{As(V)} > \text{MMA}$ . Inorganic As (iAs) accounted for 28% of the As detected. DMA exhibited the highest levels, representing a 72% of total grain As on average.

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### 1. Introduction

Arsenic is an element that raises much concern from the environmental and human health standpoints. Arsenic is present in the environment as a consequence of both anthropogenic and natural processes. Anthropogenic uses of As are wide and diverse. Arsenic and its compounds are employed in the chemical industry for producing pharmaceuticals, agricultural chemicals including, herbicides, fungicides, wood preservatives, insecticides, veterinary drugs and rodenticides [1]. There are also natural sources responsible for potential As contamination namely, minerals (more than 200 minerals contain As), volcanoes (As is emitted as particulate matter) and some kind of waters. The natural contamination of As in ground waters from Tertiary and Quaternary loess aquifers is a serious problem in some areas of Argentina and it has been, and still is, extensively studied from different points of view [2].

Arsenic toxicity largely depends on the chemical forms under which it can occur, with adverse effects ranging from minor disorders to cancer and death. Among such species worth mentioning are the

inorganic forms (in order of toxicity)  $\text{As(III)} > \text{As(V)}$ . In contrast, the organoarsenicals namely, monomethylarsonic acid (MMA)  $>$  dimethylarsinic acid (DMA) are much less toxic and indeed are well tolerated by living organisms. Arsenobetaine (AsB) and arsenocholine (AsCh) are other organic compounds practically detoxified [3].

Ingestion of As with food is one major route for this metalloid to enter the human organism. Drinking water, cereals, seafood and algae-based products are among the commodities with the highest levels of As [4]. Unlike seafood where As is dominantly present as organoarsenicals, in rice it is mainly found as inorganic species [5]. Rice (*Oryza sativa*) is foodstuff for three billion people all over the world. It is mainly cultivated in tropical and sub-tropical areas in flooded soils where As is liberated by anaerobic microbial activity and leads to As accumulation at least of ten fold higher concentrations than other grain crops [5,6]. For this reason, in comparison to other plants As content in rice is higher. Taking into account the facts described above, it is becoming increasingly important that the various forms of As be quantitatively determined in matrices of nutritional interest.

Because of the toxicity of iAs, many studies have focused on the determination of sum of As(III) and As(V) [7–9] and others focused on the selective determination of inorganic As forms [4,10,11]. However,

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in many studies only a limited amount of samples ( $\leq 10$ ) were analyzed. In a less extent, other studies reported the selective determination of inorganic and organic As species including different methodologies, analytical techniques and instrumental couplings [12–17]. Specific studies on volatile organoarsenicals resulted more effective when gas chromatography was used [18].

The motivation of this study was to develop a fully validated method for the selective determination of four As species namely, As(III), As(V), MMA and DMA in 59 field samples of rice produced in the province of Entre Rios (Argentina). To this end, the coupling high performance liquid chromatography–hydride generation–atomic fluorescence spectrometry (HPLC–HG–AFS) was employed because it allows a rapid and reproducible separation and quantification of the As species.

## 2. Experimental

### 2.1. Instrumentation and reagents

The separation of the four arsenical compounds was achieved on-line by coupling an HPLC system with HG–AFS equipment as detector. The HPLC module consisted of a Jasco (Easton, MD, USA) Model PU-2089 Quaternary Gradient HPLC Pump metal-free isocratic pump, a Rheodyne (Cotati, CA, USA) Model 7125 syringe injection valve, fitted with a 200  $\mu\text{L}$  loop that was used to inject the sample into the mobile phase carrier stream, and an analytical column. The column temperature was controlled by a Thermasphere TS-130 HPLC Column Temperature Controller (Phenomenex, Torrance, CA, USA). Separation of As(III), MMA, DMA and As(V) by HPLC was carried out on a Hamilton PRP-X100 ((Hamilton Company, Reno, Nevada, USA) column (25 cm  $\times$  4.1 mm i.d.), polymeric (polystyrene-divinylbenzene), 10  $\mu\text{m}$  particle size. The column ran at a temperature of 29  $^{\circ}\text{C}$  at a 0.57  $\text{mL min}^{-1}$  flow rate. The pH value and the ionic strength of the electrolyte in the mobile phase were optimized to achieve the best possible reproducibility and separation of the four species. A small guard column was placed directly before the column to prevent damage of the main column. PEEK tubing was used to connect the outlet of the column to a Y connector (sample + acid + reductant input). The tubing length was kept as short as possible.

Arsenical species were determined by hydride generation–atomic fluorescence spectrometry (HG–AFS). The HG–AFS setup consists of a continuous flow hydride generator coupled to an As–boosted discharge hollow cathode lamp (BDHCL) (Millenium Excalibur, PS Analytical Ltd., Orpington, Kent, England). The pre-fixed wavelength of 193.7 nm was used to monitor the fluorescence of As. Peak area was used for quantifying instrument response. Full details on the experimental conditions adopted are listed in Table 1.

**Table 1**  
Experiment conditions for HPLC–HG–AFS.

<i>HPLC</i>	
Column and guard column	Hamilton PRP-X100 column 4.1 $\times$ 250 mm SGE Analytical 10GLC4-SAX guard column 4.0 $\times$ 10 mm
Column temperature	29 $^{\circ}\text{C}$
Mobile phase	Buffer $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ 20 mM; pH 5.8
Flow rate of mobile phase	0.57 $\text{mL min}^{-1}$
Injection volume	200 $\mu\text{L}$
<i>Hydride generation</i>	
Reductant concentration	0.9% (w/v) $\text{NaBH}_4$ in $\text{NaOH}$ 0.1 $\text{mol L}^{-1}$
Reductant flow rate	4.80 $\text{mL min}^{-1}$
HCl concentration	15.0% (v/v)
Acid flow rate	2.50 $\text{mL min}^{-1}$
<i>AFS</i>	
Wavelength	193.7 nm
Primary current of As BDHCL	27.5 mA
Boost current of As BDHCL	35.0 mA

All reagents were of analytical grade. Deionized distilled water (DDW), 18.3  $\text{M}\Omega$ , obtained from a NanoPure system (Barnstead, Boston, MA, USA) was used for the preparation of all standard and reagent solutions. Four species of As were employed in the chromatographic experiments. Stock solutions of 1.0  $\mu\text{g mL}^{-1}$  of As(III) and As(V) were prepared by dissolution of the corresponding oxides in 0.2% (w/v) NaOH solution. Monomethylarsonic acid, sodium form (Chem Service, West, PA, USA) and dimethylarsinic acid, sodium form (Sigma, St. Louis, MO, USA), both 100  $\mu\text{g mL}^{-1}$  (in As) solutions were prepared by dissolving the appropriate amounts of the reagents in water. All solutions were stored in polyethylene bottles, refrigerated at 4  $^{\circ}\text{C}$ . Dilutions were prepared daily.

Di-sodium hydrogen phosphate dodecahydrate ( $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ ) and  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  (Carlo Erba, Milan, Italy) were employed for preparation of mobile-phase buffer solutions. The resulting solutions were filtered through a 0.22  $\mu\text{m}$  Millipore filter before injection.

### 2.2. Sample collection

Fifty nine rice samples were collected from producing fields during harvesting in March of 2013 (summer at the Southern Hemisphere) in the province of Entre Rios, located in the Center-East of Argentina. Aliquots of 200 g of rice were packed in polyethylene bags and sent to the laboratory. Samples belonged to five cultivars: *Yerua*, *Camba*, *ZHE733*, *Puita* and *El Paso 144*.

### 2.3. Sample treatment

Rice must be necessarily subject to an extraction procedure before presenting samples to the HPLC–HG–AFS coupling for on-line species separation and determination. Since digestion is much quicker, easier and efficient if the rice samples are preliminarily ground and transformed into a fine powder, some tests were initially carried out to check whether grinding can cause significant contamination of the rice by As. To this end, the rice grains were ground in stainless still mill thoroughly decontaminated beforehand. For each of the 59 rice samples, three aliquots of approximately 10 g were subjected to the grinding procedure for  $\sim 30$  min. The first two fractions were discarded as these are thought to be more liable to contamination through grinding and only the third aliquot was forwarded to the digestion step.

#### 2.3.1. Sample digestion for total As determination

Three aliquots of each powdered sample were separated for subsequent digestion by acid-assisted microwave (MW) irradiation using a commercially available oven (MLS-1200 Mega FKV, Bergamo, Italy). Approximately 1.0 g of the rice powder was placed in a PTFE vessel and added with a mixture of 6 mL 65%  $\text{HNO}_3$  Suprapur (Merck, Darmstadt, Germany) and 1 mL 30%  $\text{H}_2\text{O}_2$  Suprapur (Merck) and 1 mL high purity deionized distilled water (DDW). The presence of water in the mineralization mixture helps to prevent the evolution of gases consequent to the high content of carbohydrates in the material under test. The vessels were sealed and then subjected to the MW digestion cycle. After cooling, the vessels were opened and the digestion solutions were filtered using a 0.45  $\mu\text{m}$  syringe type PVDF membrane filter to obtain a clear solution without any visible residue. After, DDW was added so as to adjust the final volume to 25 mL. The MW digestion program used was as follows: 2 min at 250 W; 2 min at 0 W; 5 min at 250 W; 5 min at 400 W and 5 min at 600 W. For checking accuracy, aliquots of the certified reference material from NIST 1568a, rice flour (NIST, Gaithersburg, MD, USA) were subject to the same treatment and included in the overall analytical process.

#### 2.3.2. Extraction of As species

Arsenic species were extracted from powdered rice grains using a heat assisted-extraction technique (sand bath). A portion of  $\sim 1.0$  g was accurately weighted into a glass beaker and 10 mL of 0.28  $\text{mol L}^{-1}$   $\text{HNO}_3$

were added. Samples were covered with a vapor recovery device (watch glass), placed in a sand bath at  $95 \pm 3$  °C and was left refluxing for 90 min without boiling. After cooling, samples were filtered through a 0.45  $\mu\text{m}$  syringe-type PVDF membrane filter. The filtrate was analyzed by HPLC–HG–AFS using an external calibration method. Blank tests were performed to investigate possible As contamination and none was detected. Even when the certified reference material NIST 1568a, rice flour (NIST, Gaithersburg, MD, USA) is not certified for species, aliquots of this material were subject to the same treatment and included in the overall analytical process for species identification and quantification.

### 2.3.3. Speciation analysis of As in rice

The solutions obtained after extraction were subject to chromatographic separation. The concentration and pH values of the  $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$  buffer were optimized through preliminary experiments as well as the best conditions for the hydride generation of arsenicals. The reproducibility of the heated PRP-X100 column under the experimental conditions selected were tested on different days and turned to be more than satisfactory. The operating conditions are listed in Table 1.

The As content of the calibrants employed namely, As(III), As(V), MMA and DMA was checked using two commercial As(V) calibrants for atomic spectrometry (Merck). In all cases, measurements were carried out by inductively coupled plasma optical emission spectrometry (ICP OES). This experiment let us verify that the chromatographic pattern of each calibrant did not evidence the presence of any other As containing impurity in the range of concentrations of the samples under study.

The chromatographic separation of the four As compounds is depicted in Fig. 1. The SRM 1568a was used to optimize the HPLC conditions. In general terms, the overall separation pattern of As(III), As(V), MMA and DMA in rice extracts actually coincided with that of the synthetic calibrant mixtures used. No unidentified peaks were observed in the 59 extracts analyzed. Rice samples were analyzed using a calibration curve constructed daily and prepared in the same buffer that the samples. Typically calibration curves obtained yielded  $\geq 0.99$  correlation coefficients. Analysis of residuals, a *t* test and *F* test ( $p > 0.05$  for both tests) were performed to check linearity.

## 3. Results and discussion

### 3.1. Optimization of the extraction of arsenical species

In order to arrive at reliable results, the quantitative recovery of inorganic and organic species of As from the organic matrix avoiding the transformation the organic arsenical species to inorganic forms is a

challenging task. In this direction, different procedures have been proposed including microwave apparatus or heating blocks. Yuan et al. [19] tested and compared the use of different solvents and mixtures including water, methanol, water–methanol, water–ethanol and water–acetonitrile. Nakurawa et al. [13] investigated the efficiencies of different reagents and concentrations as follows:  $\text{HNO}_3$ ,  $\text{HCl}$ ,  $\text{H}_2\text{SO}_4$ ,  $\text{HClO}_4$ ,  $\text{H}_2\text{O}_2$ ,  $\text{HBr}$ ,  $\text{H}_3\text{PO}_4$ , formic acid, methanesulfonate acid tetramethylammoniumhydroxide (TMAH) and  $\text{NH}_4\text{NO}_3$ . The authors reported that the use of  $0.15 \text{ mol L}^{-1}$   $\text{HNO}_3$ /heat block extraction prevented reduction of As(V) to As(III) and exhibited a high extraction efficiency. To prevent the interconversion of As(III) to As(V) the use of a strong extractant should be avoided [20]. Raber et al. [8] adopted the mixture trifluoroacetic acid (TFA)/ $\text{H}_2\text{O}_2$  because of the reasonable extraction yields obtained for rice samples. Alava et al. [21] reported that MW digestion using water was the best approachable and environmentally friendly method to assess both total and speciation content of As without species interconversion. In a comprehensive study, Huang et al. [20] demonstrated that an extraction using  $0.28 \text{ mol L}^{-1}$   $\text{HNO}_3$  at 95 °C for 90 min led to recover not only As quantitatively from rice grains independent of types but also preserved As speciation completely.

Screening experiments carried out in our lab testing different extractants showed that diluted  $\text{HNO}_3$  was the most suitable reagent without decreasing the total extraction efficiency of As. Heating with a sand bath (diluted acid refluxed) was a simple alternative but the key point that affects the extraction is to fix the time necessary to break As(III)–thiolate complexes when  $0.28 \text{ mol L}^{-1}$  is employed for arsenical extraction. We observed that when the extraction time was  $\sim 120$  min, the reduction of As(V) was higher and as a consequence, the recovery resulted lower. In agreement with the findings of Huang et al. [20] an extraction time of  $\sim 90$  min resulted a good compromise to improve As recovery and to minimize As(V) reduction. For prevent interconversion of species, the mild oxidation conditions provided by hot diluted  $\text{HNO}_3$  (between 0.2 and  $0.7 \text{ mol L}^{-1}$ ) avoids As(V) reduction by thiolate compounds, at the same time, As(III) oxidation caused by the interaction between  $\text{HNO}_3$  and extraction in matrix was still negligible [20]. The effect of temperature on As extraction was also studied. In the conditions of our experiment, extraction efficiency increased with temperature till 95 °C, and higher values produced a decrease of extraction efficiency. We adopted a  $0.28 \text{ mol L}^{-1}$   $\text{HNO}_3$  solution at 95 °C for 90 min for the treatment of the 59 samples under study.

Grain size is another parameter that has to be taken into account to achieve effective extraction efficiency as well as species stability during the extraction process. Morphological characterization by scanning electron microscopy (SEM) of selected samples is shown in Fig. 2 (a and b). Particles exhibited similar shapes, fine solid with a range of

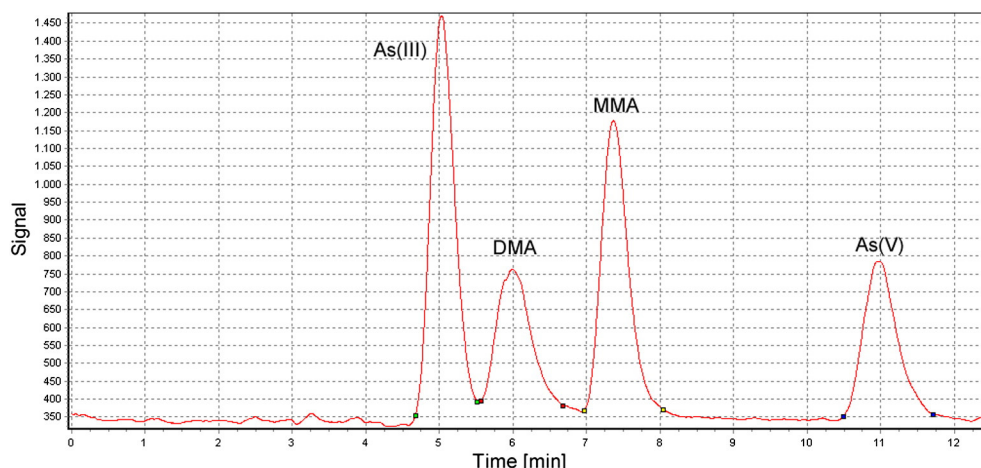


Fig. 1. Chromatographic separation of four As species in aqueous solutions ( $10 \mu\text{g L}^{-1}$  of each arsenical species, as As).



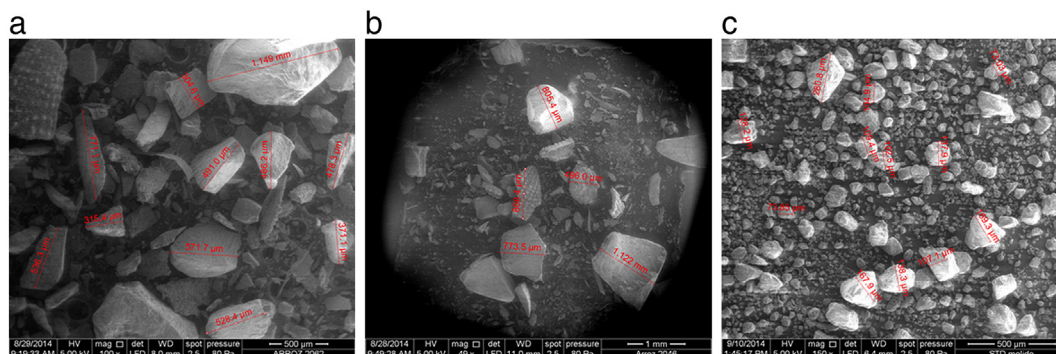


Fig. 2. Scanning electron microscopy (SEM) of selected rice samples (a and b). SRM NIST 1568a rice flour (c).

sizes between  $\sim 300$  and  $\sim 1200 \mu\text{m}$ . Fig. 2c shows a SEM image of the SRM NIST 1568a rice flour that was used for checking accuracy. As expected, particles are smaller (flour) and have a range of size between  $\sim 100$  and  $\sim 300 \mu\text{m}$ .

Using the SRM NIST 1568a as provided without further grinding, extraction efficiencies were calculated by comparing total As concentrations (MW digestion) with the sum of the species after applying the developed procedure. A good agreement with data published by Huang et al. [12] was obtained, reaching an extraction efficiency of 103%.

### 3.2. Method validation

To reach reliable results, method was fully validated. This validation included:

- Linearity: it was demonstrated through the evaluation of residuals (homogeneity of variances), determination coefficients ( $r^2$ : 0.9967 for As(III), 0.9970 for As(V), 0.9970 for MMA and 0.0072 for DMA) and tests of significance ( $t$  and  $F$ ). The working range of the optimized method was linear from levels close to the limit of quantification (LOQ) up to  $15 \mu\text{g L}^{-1}$ .
- Limit of detection (LOD) and LOQ: They were calculated as the concentration associated with three times the standard deviation of 10 independent measurements of the procedural blank ( $3\sigma$  criterion) resulted to be  $0.020 \mu\text{g g}^{-1}$  for As(III) and  $0.025 \mu\text{g g}^{-1}$  for the other three species. The LOQ ( $10\sigma$ ) resulted to be  $0.007 \mu\text{g g}^{-1}$  for As(III) and  $0.08 \mu\text{g g}^{-1}$  for As(V), MMA and DMA.
- Precision (repeatability, intermediate precision for two analysts): Repeatability was expressed as standard deviation and calculated by measurement of 10 independent replicates of each point of the calibration curve. Compliance with the Horwitz equation [22] was verified. Reference materials were used to evaluate intermediate precision. With the obtained results an ANOVA test was performed, the comparability of results was proved and intermediate precision resulted to be 4% for As(III) and DMA and 6% for the other two species.
- Trueness: It was expressed as bias and evaluated using the matrix certified reference material SRM NIST 1568a rice flour and included in the overall analytical procedure. Our results for total As were in good agreement with the certified values as follows:  $0.29 \pm 0.03 \text{ mg As kg}^{-1}$  (certified) vs  $0.30 \pm$

$0.06 \text{ mg As kg}^{-1}$  (found). The SRM was also subjected to the HPLC–HG–AFS procedure. Our results were compared (Table 2) with those reported by Huang et al. where mild conditions were used [20]. Since information of the individual species concentration is not defined for this SRM, we compared the concentrations of the four species of As obtained using the proposed method with those reported by Huang et al. [20] using the same digestion procedure. A good agreement was obtained and our results differed in 9% for As(III) and As(V) and up to 11% for the other two species (Table 2).

- Uncertainty: It was obtained from validation data and ranged from 9.5% for As(III) to 19% for As(V) [23].

### 3.3. Speciation analysis of As in rice

The fully-validated HPLC–HG–AFS method was applied to the determination of four arsenical species in 59 rice extracts. Total As in the 59 samples under study varied from 0.08 to  $1.29 \text{ mg kg}^{-1}$  with a mean concentration of  $0.38 \text{ mg kg}^{-1}$ .

Fig. 3 shows box plots with the median, minimum and maximum concentrations of As(III), As(V), DMA and total As. The speciation analysis showed the predominance of DMA (73% of total grain As on average) with concentrations ranging from 0.07 to  $1.21 \text{ mg kg}^{-1}$ . Fig. 4a corroborates that the rice cultivated in Entre Ríos (Argentina) is clearly a DMA type where DMA increased linearly with increasing total As concentration. On the other hand, As(III) is fairly constant as can be observed in Fig. 4b. Zavala et al. [24] suggested that methylation of As occurs within rice and genetic differences lead to the two rice type (DMA and inorganic As types).

Mean iAs concentration resulted to be  $0.10 \text{ mg kg}^{-1}$  with a wide spread of concentrations varying from 0.02 to  $0.28 \text{ mg kg}^{-1}$ . MMA was not detected in any of the samples analyzed.

According to the Codex Alimentarius commission (FAO/WHO Food Standards Programme) [25] the maximum allowed limit for total As (in polished and husked rice) is  $0.30 \text{ mg kg}^{-1}$ . When higher values are detected, further investigations are necessary regarding the presence of iAs (no higher than  $0.20 \text{ mg kg}^{-1}$ ). Mean As concentrations detected in the 59 samples analyzed resulted to be  $0.38 \text{ mg kg}^{-1}$  which means a  $\sim 25\%$  above the recommended values. With respect to the recommended value for iAs, our data shows a lower concentration

Table 2

Concentration of As(III), As(V), MMA and DMA in the SRM NIST 1568a, rice flour. Results are expressed in  $\text{mg kg}^{-1}$ .

Method	As(III)	As(V)	Inorganic As	MMA	DMA	Total As ( $\sum$ species)	
SRM NIST 1568a						<b>0.290 <math>\pm</math> 0.030</b>	Certified values
0.28 M HNO <sub>3</sub> , 95 °C	0.066 $\pm$ 0.017	0.033 $\pm$ 0.010	0.099 $\pm$ 0.025	<LQ	0.200 $\pm$ 0.030	<b>0.299 <math>\pm</math> 0.075</b>	This study
0.28 M HNO <sub>3</sub> , 95 °C	0.074 $\pm$ 0.007	0.030 $\pm$ 0.003	0.1044 $\pm$ 0.007	0.015 $\pm$ 0.007	0.165 $\pm$ 0.007	<b>0.285 <math>\pm</math> 0.010</b>	Huang et al. [12]

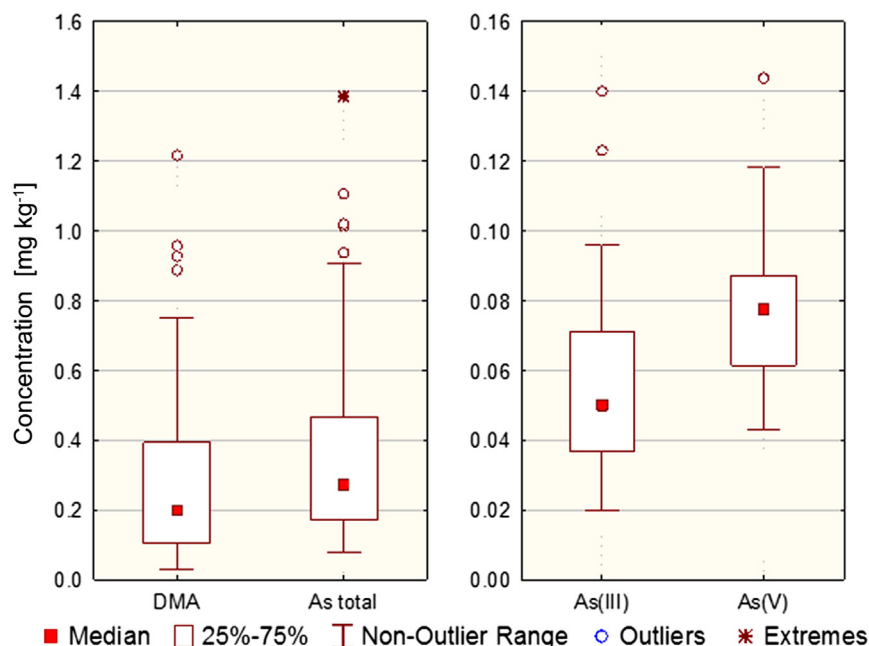


Fig. 3. Concentrations (expressed in  $\text{mg kg}^{-1}$ ) of the four species of As in the analyzed rice samples. Black dot represents the median, the box indicates the range 25–75% of the distribution and the whiskers represent minimum and maximum values.

( $0.11 \text{ mg kg}^{-1}$  vs  $0.20 \text{ mg kg}^{-1}$ ). This is of prime importance taking into account the implication for human health that has the presence of iAs in food samples for its known carcinogenic characteristics.

#### 4. Conclusions

The extraction method adopted exhibited an extraction efficiency ranging from 92 to 103%. In addition, it is simple, cost-effective and

reliable for the extraction of inorganic and organic forms of As without altering species of As present in the sample.

The coupling HPLC–HG–AFS appeared to be a powerful and sensitive investigation tool from both a qualitative and quantitative point of view. AFS, adopted for As determination, accounts for an excellent specificity with a much lower instrumental and operative cost in comparison with other instrumental techniques such as inductively coupled plasma-mass spectrometry (ICP-MS).

The study evidenced that DMA (73% of total grain As on average) with concentrations ranging from  $0.07$  to  $1.21 \text{ mg kg}^{-1}$  is the dominant species indicating that the rice cultivated in the region under study is DMA type. In addition, for iAs, our data showed a lower concentration with respect of recommended values for FAO/WHO (found:  $0.11 \text{ mg kg}^{-1}$  vs recommended:  $0.20 \text{ mg kg}^{-1}$ ). On the basis of these findings, it is plausible to think that DMA rice type is likely to be less of a health risk than iAs rice type indicating that people with rice-based diet or those that consume rice with high amounts of As are exposed to potential toxic effects.

In conclusion, this study confirms that to gain information on As speciation in rice is crucial for accurate health risks assessment.

#### Acknowledgments

The authors thank ANPCyT through Project PICT no. 1955 (2011) “Presence of arsenic and toxic trace metals in Argentine rice. A multidisciplinary challenge for its minimization” for financial support.

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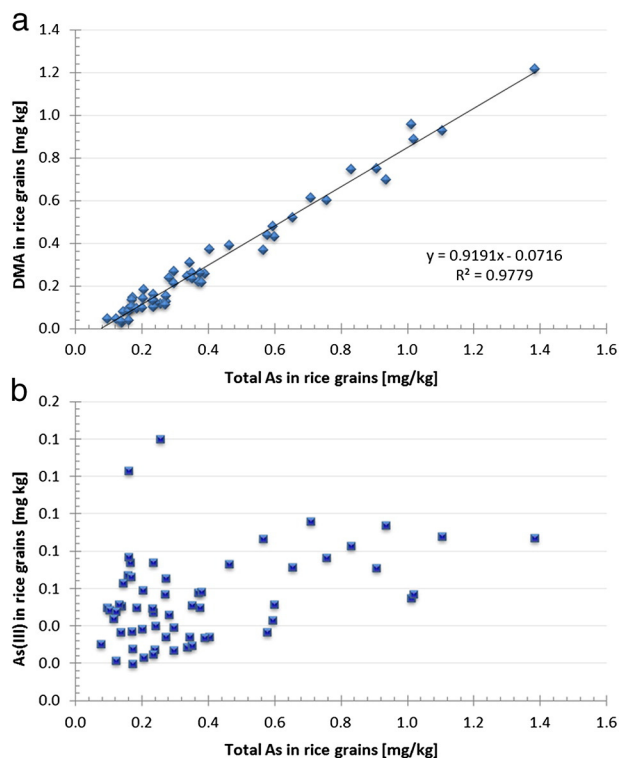


Fig. 4. Concentrations of DMA vs concentrations of total As (a). Concentrations of As(III) vs concentrations total As (b).

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