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Method Article

Determination of arsenic chemical species in rice grains using high-performance liquid chromatography coupled to hydride generator with atomic fluorescence detector (HPLC-HG-AFS)

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A B S T R A C T

The objective of this work is the validation and implementation of an analytical method for the determination of arsenic chemical species in rice grain samples using High-performance liquid chromatography coupled to a hydride generator with atomic fluorescence detector (HPLC-HG-AFS). The extraction protocol developed was based on HNO_3 0.28 M (90 °C, 2 h), microwave-assisted. The results showed recovery percentages of arsenite (As (III)) (99–101%), arsenate (As (V)) (91–96%), dimethylarsinic acid (DMA) (92–102%) and monomethylarsonic acid (MMA) (94–97%). The precision of the method presented coefficients of variation lower than 7% and 8% for repeatability and reproducibility respectively. The detection limits were 2.5, 3.75, 7.5 and 4.0 μg kg^{-1} for As (III), As (V), DMA and MMA respectively. The proposed methodology is reliable for the quantification of As species, because they are conserved during the extraction.

- The extraction protocol developed was based on Microwave-assisted acid extraction.
- This methodology offers good sensitivity, precision, accuracy, detection and quantification limits.
- It was successfully applied to determination of arsenic chemical species in rice grains.

Abbreviations: As (III), arsenite; As (V), arsenate; DMA, dimethylarsinic acid; MMA, monomethylarsonic acid; HPLC-HG-AFS, High-performace liquid chromatography coupled to hydride generator with atomic fluorescence detector; LGS, liquid gas separator; MAE, microwave assisted extraction; (LOD), Limits of detection; (LOQ), Limits of quantification.

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Arsenic (As) is a metalloid present in the earth's crust in an average concentration of 2 mg kg^{-1} [1], being among the 20 most abundant elements on earth.

The presence of arsenic (As) in some foods such as rice has been of particular concern worldwide in recent years due to its toxicity and adverse health effects. Rice in particular can accumulate more arsenic than other foods, and because it is widely consumed it can contribute significantly to arsenic exposure [2]. In Colombia, it is estimated that 92% of the population consumes rice, and that weekly consumption is 1.6 pounds per person [3].

The As toxicity depends largely on its chemical species. In rice, the inorganic species As (III) and As (V) (the most toxic forms), and the organic DMA and MMA (less toxic forms) can be found [4].

Therefore, the measurement of the total content is an insufficient criterion to evaluate the true risk, because the mobility of As and its bioavailability for the plants depends on the chemical species in which they are associated.

Given the importance of knowing the contents of chemical species of As in rice, it is necessary to have analysis methodologies in order to accurately know its possible toxicity in the plant.

For the determination of the different chemical forms or species of As, it is necessary to separate them before their detection, for this purpose separative techniques are used as high performance liquid chromatography (HPLC).

This study presents a simple and fast method for the determination of As species in rice, using high performance liquid chromatography coupled to hydride generation with atomic fluorescence spectrometry (HPLC-HG-AFS) and microwave assisted extraction (MAE), which has been successfully applied to several food samples [5]. The extraction method followed was the one proposed by [6], because this method retains all the As speciation during the extraction. It also has advantages over existing methods due to the low cost, shorter analysis time and low infrastructure requirements, so it can be easily performed in any laboratory and is especially suitable for the analysis of large quantities of samples.

Equipment and reagents

The analytical method of speciation involves coupling with a modular interface (MI) chromatographic manufactured by PS Analytical (10.820), commercially available with a PSA Millennium Excalibur manufactured by PS Analytical (10.055).

It was used with a Hamilton PRP-X100 anion exchange column 250 mm long and 4.1 mm internal diameter (containing particles of 10 µm) at room temperature. The column output was connected to an in-line continuous flow hydride generation system using the collector shown in Fig. 1. The Excalibur atomic fluorescence detector was equipped with a powered discharge hollow cathode lamp (Photron) and the signal output was recorded or processed using a computer equipped with PSA SAMS+ chromatographic software, which was used to control Millennium Excalibur, as well as for

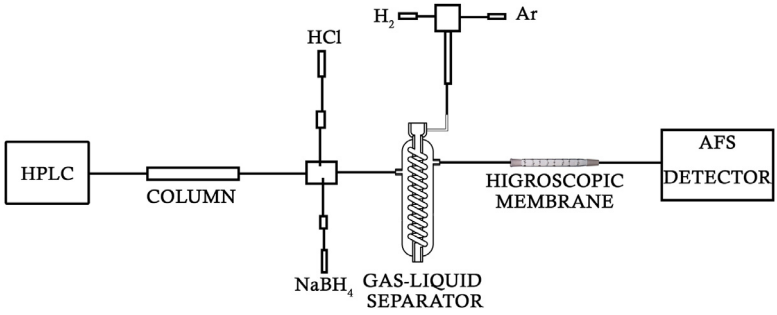

Fig. 1. Schematic diagram of the HPLC-HG-AFS system for As speciation.

data collection. In addition, a microwave digester, Milestone Ethos One was used for pretreatment of the sample.

Monosodium phosphate (NaH_2PO_4) and disodium phosphate (Na_2HPO_4) were obtained from Panreac. Sodium borohydride ($NaBH_4$) and nitric acid in the analytical grade were obtained from Merck. Arsenic (III) and (V) Standard, TraceCERT®, 1000 mg L^{-1} As were obtained from Supelco, Dimethylarsinic acid, analytical Standar Ampule of 500 mg Chem Service, Inc (PS51), and Disodium methyl arsenate Hexahydrate, Santa Cruz Biotechnology SC-257380. Standards of 10 mg L^{-1} of each species were prepared by reagent weighing or dilution of the standard solution as appropriate. The deionized water used throughout the all process was purified in a Milli-Q system (Thermo Scientific).

Preparation of the rice samples

Rice grain samples used for validation of the method were purchased in the local market. For the implementation of the method, the samples were obtained from the production fields found in an important rice region located in the northwest of Colombia. The samples were homogenized by maceration using a mortar. Fractions that passed through a 50 mesh (0.297 mm) were separated through sieving for analysis.

For the extraction, a mass of approximately 1.0 g of sample was weighed, 15 mL of extraction solution (HNO_3 0.28 M) was added to a 50 mL digestion tube and digested under controlled conditions at 90 °C for 2 h [5] in a microwave digester (Milestone Ethos One). After cooling, the extract is diluted to 25 mL with deionized water; the extract is filtered through a 0.45 µm nylon syringe filter prior to analysis by HPLC-HG-AFS.

Instrumental conditions

250 µL of sample solution were injected into a strong anion exchange column and an isocratic program was used to separate the arsenic species. As each of the species elutes from the column, they mix with a stream of hydrochloric acid and $NaBH_4$ / NaOH to form volatile hydrides that are removed from the liquid gas separator (LGS) in an argon stream. This stream flows through a hygroscopic membrane to remove moisture and then to the detector. Separation and detection of the four arsenic species take place in 8 minutes.

The conditions for the detection of HPLC-HG-AFS and the determination of As species are shown in Table 1.

Validation parameters

Grubbs' contrast was applied to verify that the series of data obtained during the validation process does not differ from the rest inexplicably (gross errors). The linearity was evaluated using 7 concentration levels (0.1–50 µg L^{-1} equivalent to 2–750 µg kg^{-1}) were prepared from the standard

Table 1
Instrumental conditions for HPLC-HG-AFS coupling.

HPLC conditions	
Anion exchange column	Hamilton PRP-X100 (4.1 × 250 mm), 10 µm particle size)
Mobile Phase	20 mM sodium phosphate buffer (NaH_2PO_4 /Na_2HPO_4) pH 6.25
Injection volume	250 µL
Mobile phase flow	0.8 mL min^{-1}
Pressure	700 kPa
HG Conditions	
Acid solution	20% v/v HCl (pump speed 100%; Flow 9.0 mL min^{-1})
Reducing agent	0.7% $NaBH_4$ at 0.1 M NaOH (pump speed 100%; Flow 4.2 mL min^{-1})
Argon - Carrier gas flow rate	250 mL min^{-1}
N_2 - Dryer gas flow rate	2.5 L min^{-1}
AFS Conditions	
Hydrogen flow rate	60 mL min^{-1}
Wavelengths	197.3 nm, 193.7 nm and 189.0 nm
Bandwidth	20 nm
Primary current	27.5 mA
Increased current	mA

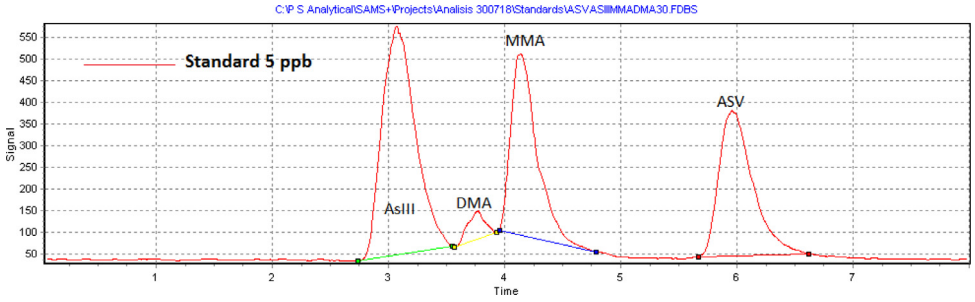

Fig. 2. Chromatogram of standards As(III), DMA, MMA and As(V), (5 µg L^{-1}), As(III): arsenite, DMA: dimethylarsinic acid, MMA: monomethylarsonic acid, As (V): arsenate.

of As (III), As (V), DMA and MMA of known concentration of 10 mg L^{-1} of each species respectively, each Concentration level was analyzed in triplicate by injecting 250 µL, under the same conditions previously established working chromatographs. A student test (calculated t_r with n-2 degrees of freedom at a confidence level of 95%) was applied to verify that the slope is significantly different from zero and to verify that the linear correlation is significant.

Repeatability, intermediate precision and accuracy were evaluated by analyzing three different levels of concentration 20%, 50% and 80% approximately of the working range and each concentration level was analyzed seven times independently. Repeatability and intermediate precision were evaluated as a coefficient of variation, while the accuracy was evaluated as recovery percentage with spiked rice grains samples at different concentrations. The Spiked samples were prepared by adding the appropriate volume of standard solution of arsenic species onto accurately weighted fraction of 10 g of rice grain sample, 24 h prior to MAE extraction protocol application. Additionally a Cochran test was applied to establish if the concentration does not affect the variability of the results.

Limits of detection (LOD) and quantification (LOQ) were determined using $LOD = \frac{Y_{bl}+3S_{bl}}{m}$ (Ec. 1) and $LOQ = \frac{Y_{bl}+10S_{bl}}{m}$ (Ec. 2). Where m is the slope of the calibration curve, S_{bl} = standard deviation of the blank replicates and Y_{bl} = average of the blank replicates.

Validation of the analytical methodology

During the chromatographic analysis, the species of As are adequately separated allowing an accurate calculation of the areas of their peaks. Fig. 2 shows a typical timeline indicating the

Table 2
Working range and limit concentrations of the analytical methodology for the determination of As species in rice grains.

Species of As	Working range (μg kg^{-1})	LOD (μg kg^{-1})	LOQ (μg kg^{-1})
As(III)	8–750	2.5	8.0
DMA	22–750	7.5	22
MMA	12–750	4.0	12
As(V)	12–750	3.75	12

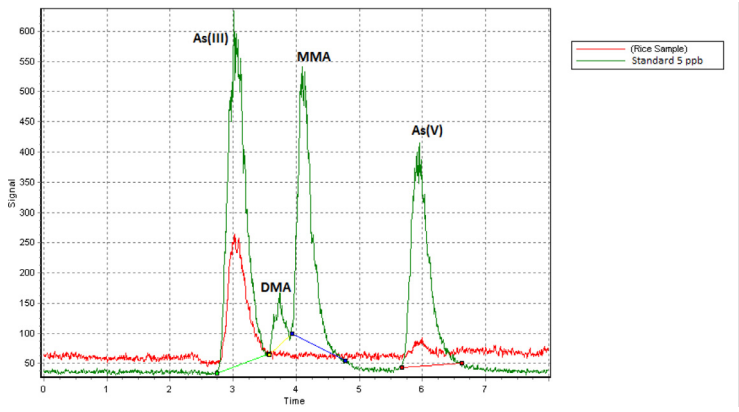

Fig. 3. Chromatogram of As(III), DMA, MMA and As(V) standards (5 μg L^{-1}) and real rice grain sample.

separation of As (III), DMA, MMA and As (V). The retention time for each species of As (As(III), DMA, MMA and As(V)), was 3.05, 3.75, 4.1 and 5.95 min respectively.

The identification of the As species is confirmed by comparing the retention times of a standard mixture of arsenic species with real rice grains extracts, as shown in Fig. 3.

Calibration curves provided a linear range of 0.5–30 μg L^{-1} for As(III), DMA and As(V); 1–30 μg L^{-1} for MMA, and a good correlation coefficient ($R^2 >$ 0.999). The t-student test to check the linear correlation showed for all the species of As studied that the $t_{exp.} > t_{t_{ablas}}$, so it was concluded that there is a significant correlation between the concentration and the area of each peak. On the other hand, for the four species of As a non-zero slope is verified using the t-student test, given that $t_{exp.} > t_{t_{ablas}}$. The detection (LOD) and the quantification (LOQ) limits of the method for As (III), DMA, MMA and As (V) are shown in Table 2.

The spiked rice grains samples at different concentrations (20%, 50% and 80% of the linear range) were analyzed by the developed method. a typical chromatogram can be seen in Fig. 4.

Table 3 shows the results obtained by HPLC-HG-AFS with microwave assisted extraction. The accuracy of the methodology is satisfactory because the results showed recovery percentages of As (III) (99–101%), DMA (92–102%), MMA (94–97%) and As (V) (91–96%) in the three fortified concentration levels, which indicated that the species of As were stable and did not transform during the preparation of the rice grain sample. These recovery percentages are within the limits established by the A.O.A.C. (60–115%) for the concentration levels evaluated [7]. The Cochran test shows that $G_{calculated} < G_{table}$ for the four species of As, which indicates that the variances of the evaluated concentrations are equivalent, demonstrating that the concentration factor does not influence the variability of the results.

Regarding the precision evaluated as repeatability and intermediate precision, it is good given that the As species present coefficients of variation lower than 7.3% and 8% respectively, in the three fortified concentration levels, which are within the limits established by the A.O.A.C. [7]. Similarly, for the repeatability tests the homogeneity of variances was verified, where for the Cochran test

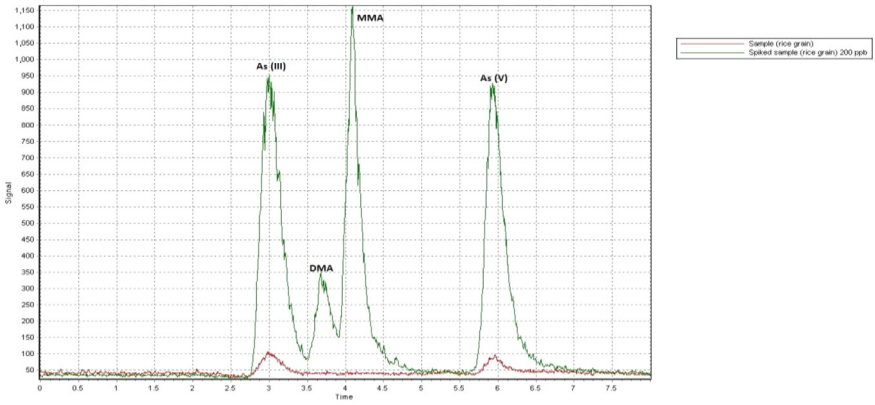

Fig. 4. Chromatogram of a rice grain sample and spiked sample at 200 μg kg^{-1}.

Table 3
Accuracy and precision for each species of As.

As Species	Level	Concentration (μg kg^{-1})	%Recovery n=3	Repetibility (%CV) n=3	Intermediate precision (%CV) n=9		
As(III)	Low	71.90	±	4.81	99.94	1.20	4.20
	Medium	381.32	±	22.37	100.35	5.48	3.70
	High	659.93	±	37.45	108.40	3.22	3.50
DMA	Low	154.21	±	9.08	99.38	7.21	7.10
	Medium	354.77	±	19.98	95.23	3.20	3.70
	High	597.89	±	33.28	99.73	2.79	2.50
MMA	Low	142.45	±	8.03	95.69	3.97	8.00
	Medium	382.97	±	20.86	100.49	1.92	5.00
	High	588.32	±	31.94	99.20	3.32	4.70
As(V)	Low	137.34	±	7.82	94.81	4.33	7.50
	Medium	358.53	±	19.53	97.06	1.92	4.00
	High	597.95	±	32.38	99.92	2.59	2.30

is observed that $G_{table} > G_{exp}$ for the four species of As. In intermediate precision the $t_{exp} < t_{table}$ at a confidence level of 95% and (n_1+n_2-2) free degrees, therefore there is no significant difference between two measures.

The results obtained for the validation parameters allow to demonstrate the capacity of the method for the determination of As species in rice grains using an improved extraction methodology that requires less time as well as the shorter chromatographic run compared to the methods reported by different authors [6,9–12], which is reflected in less reagent consumption and lower costs, with excellent detection and quantification limits for each of the studied species.

Extraction of As species in certified reference material of rice NIST-SRM-1568b

To ensure the quality of the analytical method, an appropriate certified rice reference material, rice flour NIST-SRM-1568b, was used, and a certified reference material is generally adopted to control the quality of the analytical methods applied. [8,9]. The concentrations of As (III), MMA DMA and As (V) were determined from our extraction method (0.28 M HNO_3, 90 °C) as shown in Table 4. The certified concentrations of As (III) and As (V) as inorganic As, DMA and MMA were 92 ±10, 180 ± 12 and 11.6 ± 3.5 μg kg^{-1}, respectively. In the rice flour NIST-SRM-1568b only species inorganic Arsenic (As(III) and As(V)) and DMA were found, with recovery percentages between 90% and 100%, demonstrating that there was no transformation of As species during their extraction.

Table 4
Extraction recoveries of As species (μg kg^{-1}), in certified reference material, NIST-SRM-1568b.

Method	AS (III)	AS (V)	Inorganic species	%Recovery inorganic SPECIES	DMA	% DMA recovery	MMA	*n*
0.28 M of HNO_3 (90°C, 2 hours)	41.9±4	50.7±4	92.6±8	100.6±9	167±13	92.8±8	*NQ	3
CERTIFIED CONCENTRATIONS	––	––	92±10	-	180±12	-	11.6±3, 5	-

*NQ= not quantified ND=not detected n= number of replicates.

Table 5
Determination of As species in rice grain samples, concentration values in μg kg^{-1}.

Samples	HPLC-HG-AFS					
	As (III)	As (V)	DMA	MMA	n	Total As Species
	249.1±14	23.7±1	ND	<LOD	3	272.0±14
2	102.7±6	31.2±1	12.2±0.7	<LOD	3	146.1±6
3	121.6±7	33.8±2	25.3±1	*NQ	3	179.0±7
4	123.06±7	<LOD	8.8±0.5	<LOD	3	131.8±8
5	128.2±8	20.9±1	33.1±2	*NQ	3	182.2±7
6	131.1±7	16.0±0.9	<LOD	<LOD	3	147.1±7
7	59.0±3	<LOD	8.6±0.5	*NQ	3	67.6±3
8	175.5±10	51.8±3	<LOD	*NQ	3	227.3±10
9	67.4±3	22.3±1	21.2±1	*NQ	3	110.9±3
10	173.3±10	38.6±2	*NQ	<LOD	3	211.9±10
11	77.9±4	35.0±2	<LOD	<LOD	3	112.9±4
12	24.2±1	<LOD	14.7±0.9	*NQ	3	38.9±1
13	199.2±11	27.5±1	17.4±1	*NQ	3	244.1±11

*NQ=not quantified <LOD=below the detection limit n=number of replicates

Speciation of As in rice grain samples

In the rice grains, obtained from an important rice region located in the northwest of Colombia, it was found that the total As concentration is in the range of 38 to 272 μg kg^{-1} with an average of 165 μg kg^{-1}. Table 5 shows the results obtained, in this speciation study, a clear predominance of As (III) species was observed in the rice grains as the main species, followed by As (V) and DMA, MMA was not found in the analyzed samples, this differs from a study conducted by Huang et al. [9], where it was the species with the lowest concentration (<6%) of the total As. The proportion of As (III) in the rice grains analyzed is on average 75%, while the proportion of As (V) was 14% and DMA 10%, but this pattern differs with the study conducted by Yang et al. [10], where the concentration of As (III) was the highest, which is consistent with our results, but followed by DMA, As (V) and MMA. In our study the inorganic As was predominant in all the investigated rice grains, representing an average > 90%, consequently, the inorganic As in the studied rice grains samples can be considered as As (IIII). This information is of great importance in assessing potential dietary risk, since rice is widely consumed.

The predominance of As (III) in rice grains in previous studies [7,9–12] are generally consistent with our finding. It is important to highlight that the confirmation of the general predominance of As (III) in the rice grain is valuable information to establish the limit of As concentrations in the rice grain in Colombia.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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