SPECIAL GUEST EDITOR SECTION

Determination and Uncertainty Analysis of Inorganic Arsenic in Husked Rice by Solid Phase Extraction and Atomic Absorption Spectrometry with Hydride Generation

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This study enables the selective determination of inorganic arsenic (iAs) with a low detection limit using an economical instrument [atomic absorption spectrometer with hydride generation (HG)] to meet the regulatory requirements as per European Commission (EC) and Codex guidelines. Dry rice samples (0.5 g) were diluted using 0.1 M HNO_3–3% H_2O_2 and heated in a water bath (90 ± 2°C) for 60 min. Through this process, all the iAs is solubilized and oxidized to arsenate [As(V)]. The centrifuged extract was loaded onto a preconditioned and equilibrated strong anion-exchange SPE column (silica-based Strata SAX 500 mg/6 mL), followed by selective and sequential elution of As(V), enabling the selective quantification of iAs using atomic absorption spectrometry with HG. In-house validation showed a mean recovery of 94% and an LOQ of 0.025 mg/kg. The repeatability ($HorRat_r$) and reproducibility ($HorRat_R$) values were <2, meeting the performance criteria mandated by the EC. The combined standard measurement uncertainty by this method was less than the maximum standard measurement uncertainty; thus, the method can be considered for official control purposes. The method was applied for the determination of iAs in husked rice samples and has potential applications in other food commodities.

Arsenic is a potentially toxic element and found naturally in different chemical forms. It is also abundant in a wide range of plants and animals. The harmful effects of arsenic are well established; however, its toxicity depends on its form (inorganic or organic) and oxidation state (1). The bioavailability of different forms of arsenic also varies. Inorganic arsenic (iAs) is more toxic than organic arsenic and more closely associated with the potential for long-term health effects (2). Most crops absorb arsenic from the associated soil and aquatic environment. iAs is widely distributed in the environment and found mainly in the +3 or +5 oxidation state, either bound in thio- complexes or as the oxyanions arsenite As(III) and arsenate As(V), which can interconvert during extraction (3). Although seafood is known to contain high levels of total arsenic, most of it is present as organic arsenic.

Generally, the total arsenic content in any food products of plant origin is low, but rice and rice-based foods are exceptions (4). Rice tends to take up more arsenic from the environment than other cereal crops, although this can vary according to variety, soil, method of production, and type of rice (5, 6). The arsenic in rice also tends to be predominately in the more toxic, inorganic form, which has the potential to increase the risk of illnesses, including cancer, to human beings (7). Brown rice, especially, might contain high levels of arsenic, particularly in its inorganic forms. This can pose an increased risk for specific population groups for which brown rice is a staple food.

The maximum tolerable level of total arsenic in drinking water defined by the World Health Organization is 0.01 mg/L (8). The only country that has regulated the level of iAs in rice is China, where the maximum contaminant level permitted is 0.20 mg/kg (9). Recently, the limit for iAs in rice has been fixed as 0.20 and 0.25 mg/kg, respectively, by Codex (10) and the European Commission (EC; 11).

Speciation data for arsenic are strongly needed because of the large difference in toxicity to humans from the various chemical forms of arsenic. Recently, the European Food Safety Authority (Parma, Italy) stressed the need for more data on the levels of arsenic (inorganic and organic) in various foodstuffs and highlighted the need for robust validated analytical methods for the determination of iAs (12). Analytical data are mostly available for total arsenic. Analytical differentiation between the inorganic and organic forms of arsenic is not widely available. Quantification of iAs is difficult, and efficient methods have only recently become available. The most commonly applied analytical methods for arsenic speciation are based on HPLC with inductively coupled plasma MS (13–15). However, chemical separation of arsenic species with subsequent determination by AAS-HG is easier and more cost effective (16, 17). HG also brings about high selectivity due to the hydride formation of only few arsenic species (18, 19).

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Several methods (15, 16) have been published for the determination of iAs using AAS-HG; however, these methods have not elaborated the usage of buffers and chemicals, which are critical during the extraction, preconditioning, and elution steps in order to achieve sufficient recoveries and low detection limits. In this article, we describe a fully validated method as per Regulation No. 882/2004/EC. This method is applicable to meet the performance and numerical criteria considering the maximum levels fixed for iAs in rice as set by the EC and Codex.

Experimental

Instrumentation

(a) *Atomic absorption spectrometer with HG*.—A Varian 2005 280AS instrument was used for the determination of iAs. High-purity (99.998%) grade argon was used as the carrier gas, and high-purity (99.5%) acetylene and air (through an air compressor) were used for ignition. The optimum flow rate of gas, the wavelength, and the slit width used to achieve the desired sensitivity of arsenic standardized by this method are listed in Table 1. Sodium borohydride (0.6%) and sodium hydroxide (0.5%) were used as reducing agents for better sensitivity.

(b) *Other equipment*.—A 1.5 L capacity mixer grinder (Model PX74M; Bajaj India Ltd, Mumbai, India) was used for crushing the rice samples. The samples were predigested using a water bath (Cheminco, Kolkata, India). The extracts were centrifuged by using a high-speed refrigerated centrifuge (Sorvall Legend X1R; Thermo Scientific). The samples were eluted through an SPE system (Strata SAX, Phenomenex, 6 mL, 500 mg, 55 μm).

Reagents and Chemicals

Ultra-pure water and methanol were obtained from J.T. Baker (Deventer, The Netherlands). Nitric acid (69%), hydrochloric acid (37%), ammonium bicarbonate (powder), hydrogen peroxide (30%), acetic acid, sodium hydroxide, L-ascorbic acid, sodium borohydride, ammonium hydroxide, and potassium iodide were procured from Merck India (Mumbai, India). The 30% silicone antifoam emulsion in water was obtained from Loba Chemie (Mumbai, India). As(V) standard stock solution was obtained from Inorganic Ventures (Christiansburg, VA).

Preparation of Standard Solutions

Intermediate standard solutions (100 μg/mL) of As(V) were separately prepared from the stock standard solutions (1000 μg/mL) using ultra-pure water. Using these working standard solutions, subsequent successive dilutions of concentrations of 10 and 1 μg/mL were prepared.

Table 1. AAS-HG conditions for determination of iAs in rice samples

Parameter	Condition
Air flow, L/min	13.5
Acetylene flow, L/min	2.10
Wavelength, nm	193.7
Slit width, nm	0.5

Method Validation

Method validation was carried out as per Annex III of Regulation No. 882/2004/EC to meet the performance criteria set forth in Regulation No. 333/2007/EC (20) and Codex Standard 193-1995 (10). As(V) was spiked before the sample extraction step in order to achieve the desired spike levels and to estimate the recoveries. Husked rice samples free from iAs were used for method validation. Method validation was performed as three experiments on three different occasions by two different laboratory analysts by spiking in rice samples using As(V) at three levels (0.025, 0.05, and 0.25 mg/kg) along with one reagent blank and matrix blank. Applicability of the method was checked to meet the specified limit (maximum levels) set forth in Regulation No. 1881/2006/EC and its latest amendment, Regulation No. 2015/1006/EU (11).

Specificity

Specificity was estimated through teh analysis of blank matrixes. Linearity was assessed by external standard solutions (prereduced), matching the solvent composition of samples in triplicate from 0.01 to 0.175 μg/mL (six calibration points).

LOD and LOQ

LODs and LOQs were estimated by the equations LOD = $3\sigma/S$ and LOQ = $10\sigma/S$, where σ = the standard error of the *y*-intercepts of the regression analysis; and S = the slope of the standard curve.

Precision and Accuracy

The intra- and interday data were used for the respective determination of the repeatability (r) and within-laboratory reproducibility (R). Precision (intra- and interday) was established through the estimation of $HorRat_r$ and $HorRat_R$. The Horwitz equation (21) was used for estimation of precision at 0.25 mg/kg, and the modified equation (22) was used for precision at 0.025 and 0.05 mg/kg using the following equations: $HorRat_r$ = observed RSD_r/calculated RSD_R; and $HorRat_R$ = the observed RSD_R/calculated RSD_R. RSD_R was calculated by using the Horwitz equation ($2C^{-0.15}$), where C = the concentration ratio; and by using the modified Horwitz equation (22%) based on concentrations. Because no Certified Reference Material for iAs was available, the SPE-AAS-HG method was validated using spiked levels (0.025, 0.05, and 0.25 mg/kg) in rice samples free from arsenic for establishing the accuracy.

Statistical Analysis

Statistical evaluation was carried out in Microsoft Excel 2015.

Samples and Sample Preparation

Husked rice samples were obtained from a local retail market of Kolkata, India. Approximately 1 kg rice was homogenized

and ground to a fine powder and stored at room temperature (20°C) in polypropylene bottles until analysis. The sample preparation procedure was similar to the approach previously reported (15); however, the steps (extraction, preconditioning, elution, and collection) using chemicals and buffers have been elaborated in the present study (Figure 1). Samples (0.5 ± 0.05 g dry weight) were extracted (Figure 1) with 10 mL diluted acidic mixture (0.1 M HNO_3–3% H_2O_2) in 15 mL conical-bottom polypropylene tubes and kept in a water bath at 90 ± 2°C for 1 h. The samples were centrifuged for 10 min at 2500 × *g* at 10 ± 1°C. The sample extracts were stored at 2–8°C until the preconditioning step.

iAs was selectively separated from the other arsenic compounds using a silica-based strong anion-exchange SPE cartridge (Strata SAX, Phenomenex, 6 mL, 500 mg, 55 µm). The SPE cartridge was preconditioned (Figure 1) with methanol (2 mL), followed by a 2 mL aliquot of a mixture of 35 mM $(NH_4)_2CO_3$–0.05 M HNO_3–1.5% H_2O_2. The sample extract (2 mL) was eluted (Figure 1) with 70 mM $(NH_4)_2CO_3$ buffer (2 mL) by maintaining pH in the range of 5–7 and flow rate at 0.5 mL/min. The SPE cartridge was further washed with 3 mL 0.5 M acetic acid to remove mono- and dimethylarsenic acids. Elution was carried out using 1.25 mL 0.4 M HNO_3. An 0.8 mL aliquot of the collected elute (Figure 1) was mixed with 5.6 mL reduction solution [30 mM KI–28 mM L-ascorbic acid–0.1% (v/v) silicone–3 M HCl] and kept to react for 60 min. Subsequently, 4.8 mL 3 M HCl was added and left to react for another 60 min at room temperature before the measurements by AAS-HG.

Measurement Uncertainty

The measurement uncertainty in estimation of iAs in rice corresponds to various sources (Figure 2). The type A source was the repeatability obtained through the method, and the type B sources included the calibration graph, standard stock solution preparation, sample weight, make-up volume, and water bath temperature.

The standard uncertainty due to type A source was calculated as

$$U_{Rep} = \sigma/\sqrt{p}$$

where *p* = the number of readings.

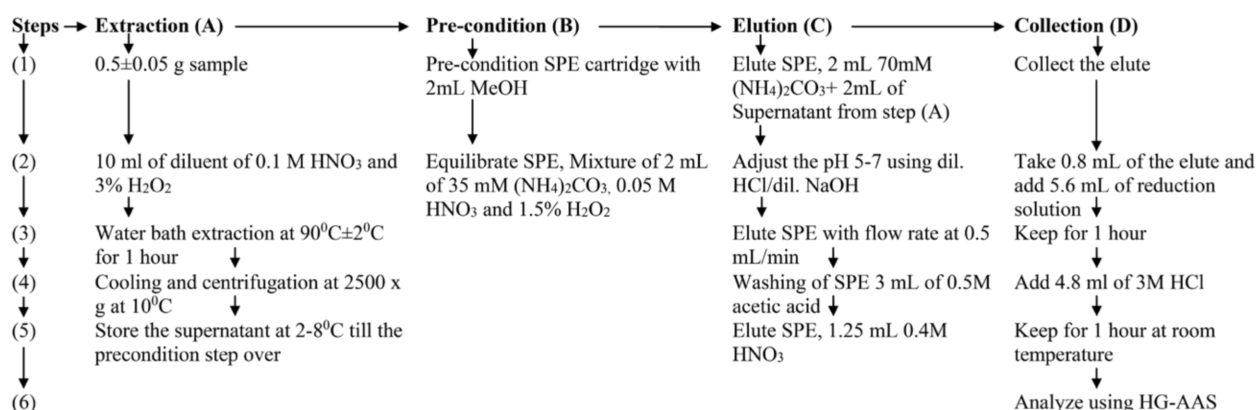

Figure 1. Schematic flow of sample preparation for the determination of iAs in rice samples.

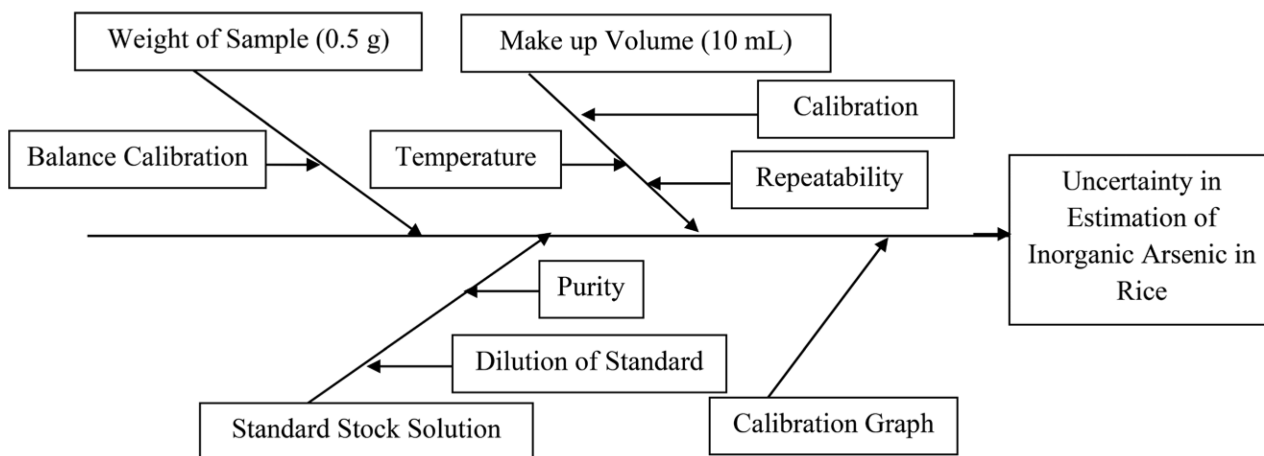

Figure 2. Cause-and-effect diagram of the measurement of uncertainty.

The standard uncertainty due to the calibration graph was calculated as

$$U(Co) = u(C_0) = \frac{SD_{xy}}{b} * \sqrt{\frac{1}{p} + \frac{1}{n} + \frac{(C_0 - C_m)^2}{S_{xx}}}$$

where C_0 = Mean concentration of readings (µg/kg); SD_{xy} = the residual SD; b = slope; p = Number of readings; n = the number of calibration concentrations; C_m = the mean value of calibration standards; $S_{xx} = \sum(C_i - C_m)^2$ where C_i = concentration of calibration standard at level i.

The standard uncertainty due to the standard stock solution was calculated as

$$U_{STD} = STD_{CONC} * \sqrt{(U_1^2 + U_2^2 + U_3^2 + U_4^2 + U_5^2)}$$

where U_1 to U_5 are the relative standard uncertainties due to purity and dilution of standards.

The standard uncertainty due to the weight of the sample was calculated as

$$U_{Sample_mass} = \sqrt{(2*(U_{SMF}/2)^2)}$$

where U_{SMF} = the uncertainty of the balance.

The standard uncertainty due to make-up volume was calculated as $U_{ext_vol} = \sqrt{(2*(U_{micropipette}/\sqrt{6})^2)}$.

The standard uncertainty due to water bath temperature was calculated by the equation $U_{Temp} = \sqrt{(2*(U_{water_bath,}\ °C/2)^2)}$.

The combined uncertainty was calculated as $u = C_0 * \sqrt{(U_{x1}^2 + U_{x2}^2 + U_{x3}^2 + U_{x4}^2 + U_{x5}^2 + U_{x6}^2)}$.

The expanded uncertainty was calculated at the 95% confidence level using a coverage factor of k = 2.

Measurement uncertainty was estimated by following the *EURACHEM/CITAC Guide CG4* (23). It adopted the approach of grouping the uncertainty components into two categories based on their method of evaluation, i.e., type A and type B. In this case, the type A uncertainty was the repeatability and the type B corresponded to the calibration graph, standard stock solution, sample weight, and make-up volume. The observation was made under the same conditions of measurement at ambient temperature, and the sample temperature was maintained at 25 ± 2°C. The standard uncertainty due to type A was 4.96 [e.g., the iAs in rice from eight different trials was measured as 0.254, 0.242, 0.242, 0.244, 0.236, 0.27, 0.267, and 0.234 mg/kg]. In the case of type B, the standard uncertainty due to the calibration graph was estimated as 3.11; the standard uncertainty due to standard stock solution was 2.58; the standard uncertainty due to sample weight was 0.000156; and the standard uncertainty due to make-up volume was 0.000061. The combined uncertainty was 0.0017 and the expanded uncertainty was 0.003 mg/kg. The final result of iAs was 0.25 ± 0.003 mg/kg. Similarly, uncertainty at 0.025 and 0.050 mg/kg was calculated, and the combined standard measurement uncertainties were 0.017 and 0.012 mg/kg, respectively. The expanded uncertainty for 0.025, 0.05, and 0.250 mg/kg was 0.003 mg/kg.

Fitness-for-Purpose

The fitness-for-purpose approach was used to assess the suitability of using the method for official control purposes. Fitness-for-purpose was calculated using the following formula:

$$Uf = \sqrt{(LOD/2)^2 + (\alpha C)^2}$$

where Uf = the maximum standard measurement uncertainty (mg/kg); C = the concentration of interest (mg/kg); and α = the numeric factor to be used depending on the value of C (i.e., 0.2 for concentrations ≤0.05 mg/kg, and 0.18 for concentrations 0.051–0.500 mg/kg).

Results and Discussion

Method

The extraction procedure was optimized to achieve a low LOQ by increasing the sample-to-solvent ratio by a factor of 2.5 (0.2–0.5 g), which is in agreement with other studies (15) conducted on iAs in rice using SPE-AAS-HG. The extraction, when conducted in a water bath (90°C, 60 min), enabled a higher sample throughput depending on the capacity of the water bath. The optimized flow rate was 0.5 mL/min, and elution at this rate through the SPE cartridge provided better recoveries. The use of a strong anion-exchange silica-based column assisted in the sequential elution and separation of iAs from organoarsenic species. The use of H_2O_2 (3%, v/v) facilitated quantitative oxidation of As(III) to As(V) for stronger retention on the anion SPE cartridge, which is in agreement with previous studies (16, 24). Studies have also reported the disadvantages of using carbonated buffers as eluent (13). However, irrespective of these problems, the current method could achieve good repeatability and reproducibility (Table 2). The absorption line of arsenic at 193.7 nm serves as a measure of the arsenic concentration. Because the use of AAS-HG reduced matrix interferences, this method could achieve a lower detection limit.

Specificity, LOD, LOQ, and Precision

A linear response was recorded for the external standard of As(V), which was treated as a sample and was obtained up

Table 2. Results of the in-house validation study in husked rice using SPE-AAS-HG[a]

As(V) spike levels, mg/kg	Mean recovery, %	Recovery range, %	Repeatability, mg/kg	Reproducibility, mg/kg	RSD_r, %	RSD_R, %	$HorRat_r$	$HorRat_R$	u, mg/kg[b]	Uf, mg/kg[c]
0.025	95	74–126	0.023	0.024	9	16	0.65	0.73	0.0017	0.007
0.05	95	77–110	0.047	0.048	10	11	0.60	0.48	0.0012	0.011
0.25	94	82–109	0.240	0.230	4	7	0.41	0.36	0.0017	0.045

[a] The number of readings was n = 18.

[b] u = Combined standard measurement uncertainty.

[c] Uf = Maximum standard measurement uncertainty.

to 175 µg/mL (corresponding to 350 µg/kg in samples), with correlation coefficients (r^2) ≥0.99. The analytical performance data of the method for the determination of iAs in rice, based on the in-house validation, are presented in Table 2. The LOD and LOQ values for iAs were 0.010 and 0.025 mg/kg, respectively, which were lower compared to previous studies (15) and, accordingly, met the performance criteria of the EC (20) and Codex (25). The mean recovery ranged from 74 to 126% (Table 2) at all three spiked levels. The intraday RSD_r varied from 4 to 10%. The intralaboratory RSD_R varied from 7 to 16%. The $HorRat_r$ and $HorRat_R$ values were in the range of 0.36–0.73, which met the performance criteria as per the EC (11) and Codex (25).

Measurement Uncertainty and Fitness-for-Purpose

The combined measurement uncertainty at 0.025 mg/kg was found to be 0.0017 mg/kg, which was below the maximum standard measurement uncertainty of 0.007 mg/kg (Table 2) and complies with the requirement of the EC (20).

Conclusions

The present study using SPE-AAS-HG for the determination of iAs in rice was optimized and validated successfully to comply with the method performance criteria of the EC and Codex and, accordingly, can be used for both official food control purposes and routine analysis. The optimization of the extraction procedure helped in achieving a lower LOD (0.01 mg/kg) and LOQ (0.025 mg/kg). The method proved to have adequate trueness and precision for the iAs in rice, ranging from 0.025 to 2.0 mg/kg. The uncertainty obtained by this method is below the standard measurement uncertainty, and it is evident that the method is fit for the intended purpose. The method was applied to the analysis of husked rice samples (n = 560) from various locations in India, and the data has been taken for fixing the limits for Codex. The method presented here for the determination of iAs in rice is selective, simple, fast, and inexpensive and can be applied to a variety of rice and rice-based products and other food commodities. The method will also be useful for risk assessment purposes in the future.

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