Video Article

Determination of Inorganic Arsenic in a Wide Range of Food Matrices using Hydride Generation - Atomic Absorption Spectrometry.

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Abstract

The European Food Safety Authority (EFSA) underlined in its Scientific Opinion on Arsenic in Food that in order to support a sound exposure assessment to inorganic arsenic through diet, information about distribution of arsenic species in various food types must be generated. A method, previously validated in a collaborative trial, has been applied to determine inorganic arsenic in a wide variety of food matrices, covering grains, mushrooms and food of marine origin (31 samples in total). The method is based on detection by flow injection-hydride generation-atomic absorption spectrometry of the iAs selectively extracted into chloroform after digestion of the proteins with concentrated HCl. The method is characterized by a limit of quantification of 10 µg/kg dry weight, which allowed quantification of inorganic arsenic in a large amount of food matrices. Information is provided about performance scores given to results obtained with this method and which were reported by different laboratories in several proficiency tests. The percentage of satisfactory results obtained with the discussed method is higher than that of the results obtained with other analytical approaches.

Video Link

The video component of this article can be found at https://www.jove.com/video/55953/

Introduction

Since January 2016 maximum levels for inorganic arsenic (iAs) in several rice commodities have been included in Commission Regulation (EC) 1881/2006 setting maximum levels for certain contaminants in foodstuffs[1] with 0.10 µg/L for rice destined for the production of food for infants and young children, 0.20 µg/L for non-parboiled milled rice (polished or white rice), 0.25 µg/L for parboiled rice and husked rice and 0.30 µg/L for rice waffles, rice wafers, rice crackers and rice cakes. This update of the European legislation for contaminants in food followed the Scientific Opinion on Arsenic in Food of the European Food Safety Authority (EFSA)[2] in which it is estimated that the exposure through diet to iAs for average and high consumers in Europe is such that can pose a risk to some consumers, keeping in mind that chronic exposure to iAs causes cancer of the lung, skin and bladder, and skin lesions. In the scientific report of EFSA on Dietary exposure to inorganic arsenic in the European population[3], published in 2014, it is concluded that the main contributors to iAs in the diet for consumers of all ages are processed products made of cereals other than rice and that also rice, milk, dairy products and drinking water contribute significantly to iAs intake, with milk and dairy products being the main contributors for toddlers and infants.

In 2010 the European Union Reference Laboratory for Heavy Metals in Feed and food, EURL-HM, ran a proficiency test, IMEP-107, for the determination of iAs in rice, demonstrating that it was possible to determine iAs in rice with sufficient accuracy, regardless of the analytical method used[4].

Several analytical methods have been validated for the determination of iAs in foodstuffs. China was the first country to introduce in its legislation a maximum level for iAs in rice. To make the implementation of legislation possible, a standard method was published in 2003 for the determination of what in the standard is called "abio-arsenic"[5]. The European Committee for Standardisation (CEN), published in 2008 a standardized method, EN 15517:2008, for the determination of iAs in seaweed[6]. The two methods are based on the use of optimized conditions to generate arsine only from iAs. In that way separation of iAs from other arsenic species that can also generate arsenic hydride is not needed. The final determination is done by atomic fluorescence[5] or by hydride generation atomic absorption spectrometry, HG-AAS[6]. However, it is difficult to set the exact conditions to generate arsenic hydride without suffering from interference of other arsenic compounds and all the iAs mass fractions in algae reported in IMEP-112 (PT organized by the EURL-HM) obtained with those two methods, were scored as unsatisfactory[7]. Organoarsenic species, such as monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), and arsenosugars present in algae samples, can generate volatile hydrides too and could interfere in the determination of iAs leading to a positive bias in the results[8].

Recently, CEN published a new standard method, EN 16802:2016, for the determination of iAs in foodstuffs of marine and plant origin using HPLC-ICP-MS[9]. Not all laboratories are equipped with that type of instrumentation and non-expensive, straight-forward methods are needed, in particular in countries with less developed laboratory infrastructures.

In 2012 CEN standardized a method for the determination of iAs in animal feeding stuffs by HG-AAS after microwave extraction and off-line separation of iAs by solid phase extraction (SPE), EN 16278:2012[10]. This method which proved to be fit for analyzing iAs in feed could lack the sensitivity required to determine iAs in food of non-marine origin, which according to EFSA seems to be the main dietary contributors in Europe[3]. However, the same group that developed and validated EN 16278:2012 tested and successfully applied and validated the method to determine iAs in seafood and rice in a collaborative trial[11,12].

An alternative method for the determination of iAs in food matrices after selective extraction of iAs into chloroform and further quantification by HG-AAS, was recently validated by the Joint Research Centre (JRC) in a collaborative trial[13]. The selectivity of the method is better than that of direct HG-AAS and is easy to implement not requiring the use of sophisticated instrumentation such as HPLC-ICP-MS. In this manuscript, the feasibility of using this method to determine iAs in a wide range of food matrices: vegetables, grains, mushrooms and food of marine origin, has been evaluated. Furthermore, the performance of laboratories that used the method in proficiency tests organized by the EURL-HM and the JRC covering several matrices is described.

Protocol

NOTE: All the material used needs to be decontaminated with 10% (m/v) HNO_3 and rinsed at least twice with deionized water.

1. Hydrolysis

1. Weigh accurately ca. 0.5 to 1 g of lyophilized sample (or the equivalent quantity of freshly homogenized sample *e.g.* 1 to 4 g) in a 50 mL polypropylene centrifuge tube with screw cap.
2. Add 4.1 mL of deionized water.
3. Agitate with a mechanical shaker for about 5 min until the sample is completely wet.
4. Add 18.4 mL of concentrated hydrochloric acid (HCl), not less than 37% m/v.
5. Agitate with a mechanical shaker for 15 min.
6. Let rest for 12-15 h (for instance overnight).

2. Extraction

1. Add 2 mL of hydrogen bromide (HBr) not less than 48% m/v and 1 mL of hydrazine sulfate ($N_2H_6SO_4$) solution (15 mg/mL) to the hydrolyzed sample.
2. Shake for 30 s with a mechanical shaker.
3. Add 10 mL of chloroform ($CHCl_3$).
4. Shake for 5 min with a mechanical shaker.
5. Centrifuge for 5 min at 800 x g.
6. Pipette the chloroform phase (lower phase) into another 50 mL polypropylene centrifuge tube.
7. Add again 10 mL chloroform to the remaining acid phase and repeat the extraction. At the end around 20 mL of chloroform should have been collected. Take care to avoid cross-contamination from the acid phase.

3. Clean-up of the Chloroform Phase

1. Centrifuge the pooled chloroform phases for 5 min at 800 x g. The centrifugation time or speed may be increased if needed to achieve a clear separation of the two phases.
2. Remove all acid phase residues remaining on the chloroform with a 1 mL pipette. This step is crucial. Any acid phase residues remaining in the chloroform phase will lead to overestimated iAs results because all other arsenic species in the sample are present in the acid phase.
3. Filter through a hydrophobic PTFE membrane (25 mm diameter) to remove the remaining solid or acid phase residues present in the chloroform phase and collect the chloroform phase in a 50 mL polypropylene centrifuge tube.

4. Back-extraction

1. Add 10 mL of 1 M HCl to back extract iAs from the chloroform phase collected after the filtration step.
2. Shake for 5 min with a mechanical shaker.
3. Centrifuge for 5 min at 800 x g.
4. Pipette the acid phase (upper phase) and pour it into a 250 mL glass beaker (*e.g.* Pyrex) for mineralization.
5. Repeat the back-extraction and combine the collected HCl phases.

5. Sample Mineralization

Note: This step allows elimination of interferences and pre-concentration in samples in which the iAs mass fraction is close to or below the quantification limit, and it is frequently omitted by laboratories which use this protocol with ICP-MS for final determination instead of HG-AAS .

1. Suspend 20 g of magnesium nitrate hexahydrate [$Mg(NO_3)_26H_2O$] and 2 g of magnesium oxide (MgO) in 100 mL of deionized water. Add 2.5 mL of this suspension to the glass beaker. Shake the suspension while adding it to avoid precipitation.
2. Add 10 mL of concentrated HNO_3 of at least 65% m/v and evaporate to dryness in a sand bath (or a thermal plate), avoiding any projections. To verify that the samples are totally dry, place a watch glass on top of the glass beaker and check that no condensation is formed.
3. Cover the beakers with watch glasses and place them in a muffle furnace at an initial temperature not exceeding 150 ºC and progressively increase the temperature to 425 ± 25 °C at a rate of 50 °C/h. Maintain at 425 °C for 12 h. This step is critical. To avoid any projections the rate of increase in temperature must be strictly implemented.
4. Allow the ashes to cool down to room temperature.
5. Add 0.5 mL of deionized water to wet the ash and add then 5 mL of 6 M HCL. Take care to recover all the ash from the walls of the glass beaker. Dissolve the ash completely, shaking if necessary.
6. Add 5 mL of pre-reducing agent, prepared by dissolving 5 g of potassium iodide (KI), and 5 g of ascorbic acid in 100 mL of deionized water, and wait 30 min to achieve a quantitative reduction of iAs to As(III).
7. Filter the solution through a Whatman number 1 paper or equivalent and collect it in a 50 mL polypropylene centrifuge tube. Rinse the glass beaker twice with 6 M HCl. Collect the rinsing liquids in a 25 mL tube and make it up to a final volume with 6 M HCl
   NOTE: When the iAs concentration in a sample is expected to be close or below the quantification limit of the method (0.010 mg/kg), or on the contrary, high, the mineralization steps 5.5-5.7 should be modified using the volumes given in **Table 1**, which would provide a lower quantification limit. Re-dissolved and pre-reduced samples are stable for 24 h at 4 °C. At least two reagent blanks should be used for the whole analytical process.

6. Calibration

NOTE: For quantification purposes use an external calibration curve of As(III) in the range 0.5 - 10 µg/L. Use a 1000 mg/L As(V) commercially available certified standard solution to construct the calibration curve applying subsequent dilutions.

1. Prepare a 10 mg/L As(V) standard solution by pipetting 1 mL of the 1,000 mg/L standard solution in a 100 mL volumetric flask and filling to the mark with 6 M HCl.
2. Prepare a 0.1 mg/L As(V) standard solution by pipetting 1 mL of the 10 mg/L As(V) standard solution in a 100 mL volumetric flask and filling to the mark with 6 M HCl.
3. Prepare a 25 µg/L As(V) standard solution pipetting 25 mL of the 0.1 mg/L As(V) standard solution in a 100 mL volumetric flask and filling to the mark with 6 M HCl.
4. Prepare the calibration curve of As(III) as follows: pipette from the 25 µg/L As(V) standard solution the volumes given in **Table 2** into 50 mL volumetric flask, add 10 mL of the pre-reducing solution in each volumetric flask, wait 30 min, then fill to the mark with 6 M HCl. Other volumes are suitable provided that they maintain the proportions described above.
5. Prepare a calibration blank as follows: pipette 10 mL 6 M HCl and 10 mL pre-reducing solution in a 50 mL volumetric flask. Wait 30 min and fill then to the mark with 6 M.
6. Use the standards marked as QC1 and QC2 in **Table 2** as quality control: QC1 ensures that the quantification at low concentration level is correct and QC2, ensures that the response is stable at high concentrations, with no significant drift in time.

7. Determination

1. Use an atomic absorption spectrometer equipped with an auto-sampler, a flow injection-hydride generation system, and an electro-thermally heated quartz cell for detection and quantification purposes, following the instrumental conditions for quantification of iAs by FI-HG-AAS as listed in **Table 3**.

8. Quantification

1. Calculate the iAs mass fraction in the samples analyzed (expressed in mg/kg), using the following equation:

   Where:
   C_x: Concentration in the extract (µg/L), calculated from the calibration curve
   C_{Bl}: Concentration in the reagent blank sample (µg/L), extrapolated from the calibration curve
   V: Final volume of the sample mineralization step (5.7), usually V= 25 mL
   w: Weight of sample (in grams)

Representative Results

The method was applied to determine the iAs mass fraction in several food commodities purchased from various Spanish markets. The results obtained with this method for a series of different matrices are classified in **Table 4** following the categories used by EFSA[3] in a report in which dietary exposure to inorganic arsenic in the European population is evaluated on the basis of data reported by Official Control Laboratories (OCL). The results in **Table 4** represent the mean of three replicates ± the reproducibility standard deviation (S_R) for the different food categories, calculated during the collaborative trial in which the present method was validated[13]. The results shown in **Table 4** are in good agreement with other previously published in similar matrices[11,12,14].

Of particular relevance are the results obtained for iAs in different types of rice because maximum limits are included for them in the European legislation for contaminants in food[1]. The highest values being obtained for brown rice and the lowest for white rice, in agreement with the findings of the OCLs[3]. The highest levels were found for the sea weed *Hizikia fusiforme*, whose consumption has been discouraged by several authorities as indicated in the report by EFSA.

The performance of laboratories that participated in PTs organized by the EURL-HM and the JRC and that used this method for the determination of iAs, has been compared to the performance of laboratories using other methods. Most of the other methods are based on HPLC-ICP-MS (around 50% of the evaluated results) and on HG-AAS without previous separation of iAs from other arsenic species (25% of the total), **Figure 1**. Other approaches used (around 15% of the evaluated results), were based on electrothermal atomization (ETAAS), fluorescence detection and ICP coupled to atomic emission spectroscopy (ICP-AES), with and without hydride generation, and are evaluated together under the name "Other methods" because the individual numbers would be too few to be of any statistical significance.

Some of the laboratories that have used the evaluated method introduced some variations to the original protocol and used ICP-MS instead of FI-HG-AAS. Frequently those laboratories did not apply the dry ashing step (Step 5 in the protocol) and just introduced the 1 M HCl phase into the ICP-MS. The PTs evaluated covered various matrices: rice[15,16], wheat, spinach, algae[17] and chocolate[18].

The performance of laboratories was expressed as z-score:

Where:
x_{lab} is the measurement result reported by a participant in a PT
X_{ref} is the assigned value (used to benchmark laboratories). In all PTs dealt within this paper the assigned value was established by a group of expert laboratories in the field of iAs analysis using different analytical methods.
σ is the standard deviation for proficiency assessment, fixed by the PT provider taking into consideration the state of the art in a certain area of analysis. In the PTs considered in this paper σ was 15% of the assigned value for rice and wheat, 22% in algae and 25% for spinach and chocolate.

The interpretation of the z score is done according to ISO 17043:2010[19]:
|score| ≤ 2 satisfactory (S) performance
2 < |score| < 3 questionable (Q) performance
|score| ≥ 3 unsatisfactory (U) performance

Seventy-five per cent of the results obtained with the method described above, got a satisfactory z-score. The determination of the iAs mass fraction in algae turned out to be challenging as expected, taking into consideration the complex distribution of arsenic species in matrices of marine origin. Two out of the three values reported in IMEP-112 for iAs in algae, using this method, got an unsatisfactory z-score. The same difficulty was observed among the results obtained with other methods. Excluding the results reported for iAs in algae, 85% of the results obtained with the evaluated method were satisfactory.

Figure 1: Comparison of Performances (expressed as z-scores) of Laboratories Taking Part in PTs (IMEP-107, IMEP-112, EURL-HM-20 and IRMM-PT-43) with the Method Described in this Paper and with Other Commonly Applied Methods. S: satisfactory, Q: questionable and U: unsatisfactory. Please click here to view a larger version of this figure.

	Expected iAs mass fraction lower than 0.010 mg/kg	**Expected iAs mass fraction higher than what is covered by the calibration curve**
6 mol L-1 HCL volume used to re-dissolve the ashes (mL)	2	10
Pre-reducing agent volume (mL)	2	10
Final volume (mL)	10	50

Table 1: Modifications of the Protocol when Analyzing Samples in which Very Low or Very High iAs Concentrations are Expected.

Concentration in the calibration curve (µg/L)	**Aliquot (mL)**
0.5	1
1	2 (QC1)
2.5	5
5	10 (QC2)
7.5	15
10	20
All As(III) calibration standard solutions shall be prepared freshly before each calibration.	

Table 2: Aliquots to be taken from the 25 µg/L As(V) standard solution to construct the As(III) calibration curve in a 50 mL final volume.

Flow injection Hydride generation	· Loop sample: 0.5 mL (To be adapted when the reconstitution volume of the final pre-reducing solution is different from 25 mL).
	· Reducing agent: 0.2 % (w/v) $NaBH_4$ in 0.05 % (w/v) NaOH; 5 mL/min flow rate.
	· HCl solution 10 % (v/v), 10 mL/min flow rate.
	· Carrier gas: Argon, 100 mL/min flow rate.
Atomic absorption spectrometer	· Wavelength: 193.7 nm
	· Spectral band-pass: 0.7 nm
	· Electrodeless discharge lamp system 2
	· Lamp current setting: 400 mA
	· Cell temperature: 900 °C

Table 3: Instrumental Conditions used for iAs Quantification by HG-AAS.

Food		i-As (µg/kg fresh weight)
Grain and grain-based products		
Rice	White	113 ± 18
		73 ± 12
		56 ± 9
	Brown	197 ± 32
		125 ± 20
		275 ± 44
	Parboiled	134 ± 21
		159 ± 25
	Wafers	162 ± 26
		127 ± 20
Vegetable and vegetable products		
Dehydrated mushroom	Boletus edulis	174 ± 10
	Galocybe gambosa	74 ± 4
	Marasmius oreades	104 ± 6
	Cantharellus lutescens	16 ± 1
	Lentinula edodes	96 ± 6
Sea weed	Hizikia fusiforme	97000 ± 14550
		44943 ± 6742
	Fucus vesiculosus	288 ± 43
		433 ± 65
Fish and other seafood		
Fish meat	Flathead grey mullet	53 ± 12
		21 ± 5
	European eel	72 ± 16
		42 ± 9
	Crayfish	33 ± 7
		20 ± 4
	Tuna	11 ± 2
		5 ± 1
Molluscs	Clam	243 ± 54
		133 ± 29
	Mussel	32 ± 32
		139 ± 31

Table 4: Results Obtained for a Range of Different Matrices Applying the Described Method.

Discussion

A critical step in the described protocol is the clean-up of the chloroform phase (Step 3.2) because any acid phase residues remaining in the chloroform phase will lead to overestimated iAs results since all other arsenic species in the sample are present in the acid phase. This is of particular relevance when analyzing marine samples due to the presence of a plethora of organic species, which could account for most of the arsenic mass fraction present in the sample. The use of a hydrophobic PTFE (3.3) membrane is of paramount importance. If an emulsion is formed during the extraction of iAs into chloroform, the speed of centrifugation (3.1) can be increased. Other traditional approaches to eliminate emulsions can also be applied. Another critical step is the mineralization (Step 5.3). The rate of increase in temperature must be strictly implemented to avoid any projections which would reduce the iAs recovery leading to an uncontrolled negative bias and could be dangerous for the analyst.

As mentioned above some laboratories have used the evaluated method using ICP-MS instead of FI-HG-AAS. In such a case the dry ashing step (Step 5 in the protocol) is not needed and the 1 M HCl phase can be introduced into the ICP-MS. In the case of HG-AAS, due to its higher detection limit, a pre-concentration step which also eliminates possible interferences, is needed.

The percentage of satisfactory results obtained with the method described in this paper, both with and without the results reported for algae, is comparable to that of HPLC-ICP-MS and higher than that of HG-AAS. The latter technique (HG-AAS) is widely available but prone to interferences from organic arsenic species, especially in food commodities with a complex arsenic species distribution pattern. The lowest percentage of satisfactory results characterizes those obtained with "Other methods" but it must be kept in mind that it covers several analytical approaches, each one of them represented by a small amount of results, **Figure 1**. The method presented in this paper is an alternative to the more sophisticated/expensive HPLC-ICP-MS, still being characterized by a similar performance even in complex matrices. Frequently the use of hyphenated techniques, such as HPLC-ICP-MS, requires highly qualified operators and expensive infra-structures. The method presented in this paper can be implemented by any analyst trained in basic analytical chemistry.

There are some main drawbacks associated to the method. It is time-consuming since several steps must be followed to separate iAs from other arsenic species and to pre-concentrate iAs down to even sub-ppm levels. It implies the use of chloroform. There is a tendency to avoid the use of chlorinated compounds in the laboratories, due to the negative health effects that they could have. Nevertheless, if good laboratory practices are kept and samples are handled in fume hoods, those negative effects could be avoided. MMA will interfere in the determination of iAs. This must be kept in mind when analyzing samples in which MMA could be present, such as algae, fish and other seafood. However, MMA is normally present in small amounts which would be covered by the uncertainty associated to the results obtained for iAs.

Disclosures

The authors have nothing to disclose.

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