Food Anal. Methods (2011) 4:447–452
DOI 10.1007/s12161-010-9187-8

Screening of Toxic Inorganic Arsenic Species in Garlic (*Allium sativum* L.)

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Received: 29 July 2010 / Accepted: 30 November 2010 / Published online: 18 December 2010

Abstract It has been evidenced that arsenic in garlic is present in the most toxic inorganic species As(III) and As (V). A non-chromatographic speciation method has been developed for the screening of inorganic toxic species of As in garlic samples by hydride generation atomic fluorescence spectrometry. The determination of As(III) and As(V) was based on the different efficiencies of hydride generation with $NaBH_4$ with and without a previous reduction with ascorbic acid and KI using a system of two proportional equations corresponding to these two different measurement conditions. The extraction efficiency of total arsenic and the stability of As(III) and As(V) in different extraction media (sulphuric acid, perchloric acid, and methanol/water) were evaluated. Based on the extraction yield and the stability of extracted species, 1.0 mol L^{-1} H_2SO_4 was selected as the best extracting solution for speciation analysis. The methodology developed allows us a limit of detection of 0.8 and 0.6 ng g^{-1} for As(III) and As(V), respectively. The relative standard deviation values were 4% for As(III) and 7% for As(V). This method was applied to determine As(III), As(V), and total As in different Spanish garlic samples. The arsenic (III) content varied from 17.1 to 22.1 ng g^{-1} and As(V) from 54.7 to 67.6 ng g^{-1}. The accuracy of the method was confirmed by the analysis of a certified reference material of tomato leaves treated in the same way as the garlic samples.

Keywords Arsenic · Atomic fluorescence spectrometry · Risk assessment · Speciation · Extraction · Garlic

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Introduction

Arsenic has been classified by the International Agency for Research on Cancer as carcinogenic to humans. However, the bioavailability and toxicity of arsenic present in food depend largely on its chemical form, being As(III) and As (V) as the most toxic species. The toxicity of the organic arsenical species is lower than that of inorganic ones, and arsenobetaine (AB), a trimethylated specie, is recognized as non-toxic. The LD_{50} (expressed in grams per kilogram) are 0.0345, 1.1, 1.8, >10.0, and >6.5 for As(III), monomethylarsonic acid, dimethylarsinic acid, AB, and arsenocholine, respectively (Shiomi 1994). So, in order to assess the risk of As for human health, the determination of the inorganic arsenic present in foods is of great importance.

It is well-known that fish is the kind of food which contains the highest level of As. However, the main parts of As species in fish are non-toxic compounds. On the other hand, the concentration of arsenic in other foods, such as vegetables, is a good indicator of the level of As contamination in the environment in which they are cultivated (soil, irrigation water, and atmosphere), and high levels of As inorganic species can be found in crops treated with fertilizers and

pesticides (Yan-Chu 1994). Therefore, arsenic speciation in vegetables is an important analytical task.

Nowadays, it is well accepted that combinations of chromatography, liquid chromatography, or gas chromatography with atomic fluorescence spectroscopy (AFS) or inductively coupled plasma-mass spectrometry (ICP-MS) provide the best tools for trace element speciation in any kind of sample (Ebdon et al. 2001; Cornelis et al. 2003; Ciardullo et al. 2010). However, non-chromatographic speciation methods provide cheap and fast tools, thus, offering an excellent alternative for the screening of toxic compound in speciation analysis (Gonzalvez et al. 2010).

The speciation of arsenic in solid samples involves a previous extraction step which is quite critical since high recoveries as well as species preservation are required in order to obtain accurate results. Methods proposed in the literature to do species extraction from solids used microwave-assisted treatments (Heitkemper et al. 2009) and ultrasound-assisted extraction (Hirata and Toshimitsu 2007), enzymatic extraction (Reyes et al. 2009), and Soxhlet extraction (Lin et al. 2008). However, extraction efficiency varies widely depending on the sample matrix and the extractants used. Thus, various extractants have been investigated to maximize the efficiency of arsenic species extraction from solid food samples also avoiding the interconversion of species during this process (Francesconi and Kuehnelt 2004; Matos-Reyes et al. 2008; Milstein et al. 2003). There are also available non-chromatographic speciation analytical methods (Kumar and Riyazuddin 2007; Gonzalvez et al. 2009; Muñoz et al. 1999) which are in general faster and cheaper than the chromatographic ones. Non-chromatographic methods coupled to hydride generation (HG) have been of special interest for As determination due to the differences in HG of As species as a function of their chemical structure (Mirna and Horacio 2004; Matos-Reyes et al. 2008; Chen et al. 2009).

Garlic is a vegetable widely used throughout the world for both culinary and medicinal purposes. However, the literature offers not enough information about As content in this vegetable. Some researchers reported arsenic contents from 0.04 to 8 ng g^{-1} (Gonzalvez et al. 2008) and between 0.030 and 0.368 μg g^{-1} (Muñoz et al. 2002). Additionally, there is no recommended daily intake for garlic, but many experts agree that one to two cloves per day or 4 g of intact garlic may have health benefits (Amagase et al. 2001) and thus, it is clear that it would be of great interest to have a simple and easy method to be used for screening of toxic species of As in garlic. In the present paper, we proposed a simple and very easy procedure, and with a reduced cost for the speciation analysis of inorganic arsenic in garlic. The method has been validated and applied for the speciation of inorganic arsenic in garlic samples in order to verify the presence of toxic species.

Material and Methods

Instrumentation

A continuous flow hydride generation atomic fluorescence spectrometer model PSA Millenium Excalibur 10055 from PS Analytical (Kent, UK) was used for the analytical determinations. Arsenic boosted-discharge hollow cathode lamp from Photron (Victoria, Australia) was employed as excitation source. The instrumental parameters were adjusted according to the manufacturer recommendations, except the concentrations of HCl and $NaBH_4$ and the sample volume, which were optimized in previous studies (Cava-Montesinos et al., 2003). An ultrasound water bath Branson (Danbury, CT, USA) operated at 130 W and 40 KHz was used for sample pretreatment in order to extract inorganic arsenic. Other equipment included a Cryodos lyophilizer Telstar (Barcelona, Spain), a sand bath Selecta model 600709 (Abrera, Spain), and a Lenton ECF 12/45A muffle furnace equipped with a Eurotherm 2416 controller Biometa (Llanera, Spain) which were employed for the complete digestion of samples to determine the total concentration of arsenic in the samples.

Reagents, Solutions, and Samples

All reagents used were of analytical grade and all solutions were prepared in ultrapure water with a minimum resistivity of 18.0 MΩ cm obtained from a Milli-Q Millipore system (Bedford, MA, USA). The 1,000 mg L^{-1} As(III) was obtained from Scharlau Chemie (Barcelona, Spain) and the 1,000 mg L^{-1} As(V) standard solution was supplied by Merck (Darmstadt, Germany). For sample preparation, 37% (*w*/*w*) HCl from Merck to 65% (*w*/*w*) HNO_3 from J.T. Baker (Deventer, Holland) were used. A solution of 50% (*w*/*v*) KI prepared from the solid product Merck with 10% (*w*/*v*) ascorbic acid, prepared from the solid reagent Scharlau, was employed to reduce completely As(V) to As(III). The ashing agent used for dry ashing of samples before the total arsenic determination was a mixture of 20% (*w*/*v*) $Mg(NO_3)\,6H_2O$ and 2% (*w*/*v*) MgO. Both reagents were obtained from Scharlau Chemie. Sodium tetrahydroborate dissolved in 0.1 mol L^{-1} NaOH, both from Scharlau Chemie, was used to generate the corresponding hydrides before the AFS determination of As.

For the ultrasound-assisted extraction procedure, 1 mol L^{-1} H_2SO_4 from Scharlau Chemie pure product, 1 mol L^{-1} $HClO_4$ from Merck, and methanol (99.98%) from Scharlau were used. A 0.1% (*w*/*v*) solution of the disodium salt of ethylenediaminotetraacetic acid (Panreac, Barcelona, Spain) was also employed. Argon X505 with a purity higher than 99.9995% was employed as carrier gas and synthetic air was used in the Perma Pure drier system.

Both gasses were supplied by Carburos Metálicos (Barcelona, Spain).

Certified Reference Material and Samples

The certified reference material NIST 1573a (Tomato leaves) was obtained from the National Institute of Standards and Technology (Gaithersburg, MD, USA) and six different garlic samples from different origin: Las Pedroñeras-Cuenca, Sta Maria de los Llanos-Cuenca, Navarrés-Valencia, Alicante, Cuenca were purchased at the local market of Valencia city and prepared as indicated bellow.

General Procedures

Sample Pretreatment

Natural garlic samples (250 g) were peeled; the edible parts were cut into pieces and crushed, and finally frozen at −20 °C. Afterwards, they were freeze-dried for a minimum of 48 h at a chamber pressure of 0.05 mbar. The dried samples were crumbled and pulverized with a mill. The resulting fine powder was stored in polyethylene bottles and kept inside a desiccator until analysis. Other garlic samples, purchased as powder, were ground in a domestic mill Taurus (Lleida, Spain), and the powdered samples were stored in polyethylene bottles inside a dessicator till their analysis.

Dry Ashing Mineralization for Total Arsenic Determination

Approximately 1 g of sample was accurately weighed and treated with 2.5 mL of an ashing aid suspension containing 20% (*w*/*v*) $Mg(NO_3)_2$ $6H_2O$ plus 2% (*w*/*v*) MgO and 5 mL of 50% (*v*/*v*) HNO_3. The mixture was evaporated to dryness in a sand bath and mineralized in a muffle furnace at 450 °C with a gradual increase in temperature (Matos-Reyes et al. 2008). The white ashes were wetted with 1 mL water and dissolved with 9 mL of 10% (*v*/*v*) HCl. For As determination, 3 mL of this solution was transferred to a 50-mL polyethylene tube as well as 8.75 mL of concentrated HCl and 600 μL of the reducing solution, 50% (*w*/*v*) KI plus 10% (*w*/*v*) ascorbic acid. The final volume was made up to 30 mL with ultrapure water.

Ultrasound-Assisted Extraction for Inorganic As Speciation

Approximately 1 g of freeze-dried sample was accurately weighed inside a 50-mL polyethylene tube and 10 mL of 1 mol L^{-1} H_2SO_4 was added to the tube. The slurry was sonicated for 10 min, and the extract was separated by centrifugation at 3,500 rpm for 10 min. The solid residue was washed with 10 mL of 0.1% (*w*/*v*) EDTA; this suspension was centrifuged for additional 10 min, and the supernatant was mixed with the previous extract. Two aliquots were taken from the final extract. An aliquot was prepared in a medium containing 1% KI and 0.2% ascorbic acid, the solution was left to react for 30 min and another one was analyzed by hydride generation-atomic fluorescence spectrometry (HG-AFS) without a pre-reduction; the final HCl medium in both cases was adjusted to 3.5 mol L^{-1}. Recovery studies were made on commercial garlic samples spiked with 50 ng g^{-1} of both considered species added before the extraction process. The analytical figures of merit were evaluated as follows. Limit of detection (LOD) values of the proposed methodology were calculated by dividing three times the standard deviation of the fluorescence signal of 10 reagent blanks by the slope of the corresponding calibration line obtained in the best experimental conditions for each considered species. The LOD was also established for solid sample in nanograms per gram taking into account the original sample mass employed for the extraction and the dilution factor involved in the proposed methodology. Repeatability was established from the relative standard deviation (RSD) of 10 independent analyses of a sample containing 18.2 ng g^{-1} As(III) and 56.8 ng g^{-1} As(V).

Results and Discussion

Total Arsenic Determination in Garlic by HG-AFS

Table 1 shows the total content of As in garlic samples. In order to evaluate the mineralization step and the whole procedure, recovery studies were carried out by spiking the garlic samples with 50 ng g^{-1} of As before their dry ashing treatment. The recovery percentage found was 98.5±1.1. So, it can be concluded that no analyte loss and no contamination occurred during the different steps of the analytical procedure.

Table 1 Total arsenic content in garlic obtained after dry ashing by HG-AFS

Garlic sample	Origin	Total As (ng g^{-1})
1	Las Pedroñeras, Cuenca	73.9±1.1
2	Sta Maria de los Llanos, Cuenca	87.3±1.6
3	Navarrés, Valencia	76.2±1.8
4	Alicante	88.3±1.7
5	Cuenca	75.0±2.3
6	Las Pedroñeras, Cuenca	76.2±1.1

Total As was determined by HG-AFS after dry ashing of samples and the reduction of the dissolved ashes

Selection of the Extraction Conditions for Toxic Arsenic Determination

Results obtained for the sum of As(III) and As(V) determination by HG-AFS in garlic samples sonicated with H_2SO_4, $HClO_4$, and methanol/H_2O (1:1) and reduced with KI are indicated in Table 2. Taking into account the total arsenic recovery, the most promising extraction agents were 1.0 mol L^{-1} H_2SO_4 and methanol/H_2O (1:1). However, no quantitative recovery was obtained for 3 mol L^{-1} H_2SO_4 and it could be due to the interaction of the excess of H_2SO_4 with KI during the reduction step generating a considerable amount of I_3^-, a brown compound that cannot be reduced by ascorbic acid and could interfere in the subsequent hydride generation, thus, reducing the AFS signals. Low recoveries obtained for 1 mol L^{-1} $HClO_4$ could be due also to interferences in the hydride generation additionally than problems to quantitatively extract As from the samples. In the present study, 1.0 mol L^{-1} H_2SO_4 was selected for further studies. Acid sulfuric concentrations below 1.0 mol L^{-1} were not enough for the quantitative extraction of arsenic for garlic samples. On the other hand, the good extraction efficiency for total As obtained by using a methanol/H_2O mixture provided interconversion of As species (results not shown) and thus, this extractant cannot be recommended.

Non-Chromatographic Speciation

As(V) reacts with $NaBH_4$ to form the volatile covalent hydride. However, the reaction kinetics is slower than that of As(III), and the signals produced by the pentavalent species depend strongly on hydride generation conditions. So, in order to determine the total As content, a pre-reduction is required to reduce As(V) to As(III). Based on the different sensitivities obtained for As(V), with and without a pre-reduction, it could be possible to make measurements on the same sample directly and after a quantitative reduction of As and to establish a system of two equations with two unknowns relating to the two oxidation states of As, as follows (Matos-Reyes et al. 2008).

$$IF_{\text{without reduction}} = 422C_{As(III)} + 263.8C_{As(V)} \quad (1)$$

$$IF_{\text{after reduction}} = 425.7C_{As(III)} + 418.0C_{As(V)} \quad (2)$$

Being $C_{As(III)}$ and $C_{As(V)}$ the concentration of both As species in the sample and $IF_{\text{without reduction}}$ and $IF_{\text{after reduction}}$ the fluorescence signals obtained for a same sample measured directly and after reduction with KI and ascorbic acid, respectively. The coefficients in Eqs. (1) and (2) correspond to the slope of the calibration lines obtained for As(III) and As(V) standards measured without reduction and after reduction in a same session, and based on the aforementioned strategy, As(III) and As(V) can be determined from two fluorescence sample data and using the reported coefficients.

Accuracy Studies

To evaluate the accuracy of the inorganic arsenic species determination in garlic, a recovery study was made on natural samples spiked with As(III) and As(V). The recovery was higher than 98% for both species (see Table 3) and these good recovery values evidence the stability of each species during the extraction process and the lack of interconversion of species.

The accuracy was also confirmed by the analysis of a CRM NIST 1573a (tomato leaves) for which As(V) concentration of 87.9±2.1 ng g^{-1} and As(III) concentration of 22.6±0.3 ng g^{-1} were obtained. The sum of the considered species, 110.2±1.7 ng g^{-1}, compares well with the total As certified value, 0.112±0.004 mg kg^{-1}, showing once again that As(III) and As(V) are the major species of arsenic in vegetables and that the proposed methodology could differentiate both oxidation states.

Analytical Figures of Merit

Table 4 summarizes the analytical features of the proposed method. Sensitivity values varied from 263.8 fluorescence

Table 2 Study of the extractant agents employed for the arsenic determination in garlic by HG-AFS after complete reduction with KI

Extractant	Total As (ng g^{-1})	Recovery (%)
H_2SO_4 3 mol L^{-1}	61.1±2.0	83
H_2SO_4 1 mol L^{-1}	71.6±1.3	97
H_2SO_4 0.5 mol L^{-1}	38.6±2.0	52
H_2SO_4 0.1 mol L^{-1}	29.7±2.1	40
$HClO_4$ 1 mol L^{-1}	59.2±1.7	80
Methanol/H_2O 1:1	71.1±0.9	96

Total As obtained after dry ashing was 73.9±1.1 ng g^{-1}. Results are expressed as interval confidence (at the 95% level). Observation number=3

Table 3 Recovery of As species from spiked garlic samples analyzed by HG-AFS after As extraction with 1.0 mol L^{-1} H_2SO_4

Added (ng g^{-1})	As(III) found	As(V) found
0	17.5±0.5	56.3±0.9
50 As(III)	66.8±0.9	55.9±1.1
50 As(V)	17.8±0.8	105.2±0.2
Recovery (%)	98.9±1.4	98.9±0.2

Table 4 Analytical at figures of merit of the developed method

Species	Slope[a]	R^2	LOD (ng L^{-1})	Working range (ng mL^{-1})	LOD (ng g^{-1})	RSD (%)
As(III)	422.0	0.9985	4.2	0–2.5	0.8	4.0
As(III)red	425.7	0.9994		0–2.5	0.9	
As(V)	263.8	0.9988	7.0	0–2.5	0.6	7.0
As(V)red	418.0	0.9991		0–2.5	0.8	

R regression coefficient, *LOD* limit of detection values were established from the calibration lines in terms of naograms per liter and in nanograms per gram of dry mass, taking into account the amount of sample employed for extraction. *LOQ* limit of quantification, *RSD* relative standard deviation

[a] Calibration lines were obtained for standards of As(III) and As(V) in the absence and in the presence of KI

units ng L^{-1} for As(V) measured directly till 425.7 fluorescence units ng L^{-1} for As(III) after reduction, being the LOD for real samples 0.8 ng g^{-1} As(III) and 0.6 ng g^{-1} As(V) with relative standard deviation values of 4% and 7%, respectively, for As(III) and As(V). So, it can be concluded that the aforementioned figures are suitable for the speciation of inorganic arsenic in market garlic samples.

Analysis of Spanish Market Samples

Samples of garlic, obtained from the Spanish market, were analyzed by the developed methodology, and results found for As species are summarized in Table 5, together with data found for total As determination based on dry ashing mineralization and HG-AFS. The total As content is higher in comparison with data reported by Gonzalvez et al. (2008) and in the same order than those reported by Muñoz et al. (2002).

As a difference of studies made on arsenic speciation in sea products, for which the main part of As is present as arsenobetaine, data in Table 5 clearly show that the sum of inorganic species extracted from garlic by sonication is coincident with the total content of arsenic (percentages from 98% to 100%) thus indicating the absence of organic species in garlic samples. These data are in a good agreement with the percentages of inorganic arsenic reported by Muñoz et al. (2002) which varied from 94% to 100%. Additionally, it can be seen that As(V) is the main component being its concentration between 73% and 78% of the total arsenic.

Conclusion

The procedure proposed for the determination of total and inorganic arsenic species in garlic samples by HG-AFS offers a simple and sensitive methodology with low LOD values. The need to measure the fluorescence of a same sample without reduction and after reduction with KI and ascorbic acid implies an additional pre-reduction step of 30 min which causes a delay in the acquisition of data. However, the fact that several samples could be reduced simultaneously provides a faster procedure than the chromatographic approach for which a delay is required for each chromatographic run.

No degradation of As(III) and As(V) or interconversion of species during extraction and hydride generation steps occurred, and the accuracy was supported by good recoveries for both total arsenic and inorganic species in garlic samples and in a certified reference material.

Regarding food safety against humans, it must be indicated that the As concentrations found in market garlic samples were in all cases lower than 90 ng g^{-1}, which is clearly under the maximum tolerated level by the Spanish

Table 5 Arsenic content in Spanish commercial garlic samples determined by HG-AFS

Sample	As III (ng g^{-1})	As(V) (ng g^{-1})	Sum (ng g^{-1})	Total As (ng g^{-1})	% As(III)	% As(V)
1	18.2±0.8	56.8±2.0	75.0±1.7	73.9±1.1	25	75
2	20.4±0.6	67.6±1.6	88.0±1.5	87.3±1.6	23	77
3	20.6±0.2	54.7±0.6	75.2±0.8	76.2±1.8	27	73
4	22.1±0.3	64.5±1.4	86.6±1.2	88.3±1.7	25	75
5	18.8±0.5	57.5±0.4	76.3±0.3	75.0±2.3	25	75
6	17.1±0.2	59.0±0.3	76.0±0.2	76.2±1.1	22	78

Total As content was determined by HG-AFS after dry ashing mineralization of samples. Results are expressed as interval confidence (at the 95% level). Observation number=3

law for As in foodstuffs, which varies from 1 mg kg^{-1} in biscuits, brandy, salt, sugar, or vinegar till 0.01 mg kg^{-1} in water, with 3 mg kg^{-1} being the maximum tolerated level in spices and condiments (The British Food Manufacturing Industries Research Association 1993). However, the extended use of garlic around the world and the fact that arsenic species in garlic are the most toxic ones must be taken into consideration to routinely control arsenic in garlic samples imported for foreign countries.

Acknowledgements Authors gratefully acknowledge the support of the Ministerio de Educación y Ciencia Project AGL2007-64567, Consellería de Sanidad Project PLAT-A-05/09, Conselleria d'Educació de la Generalitat Valenciana Project GV PROMETEO 2010-055, and National Council for Scientific and Technological Development (CNPq).

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