International Food Research Journal 28(6): 1164 - 1170 (December 2021)
Journal homepage: *http://www.ifrj.upm.edu.my*

Validation of an analytical method for the determination of inorganic, organic, and total arsenic in fish sauce based on hydride generation atomic absorption spectrometry

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Article history

Received: 18 June 2020
Received in revised form: 17 March 2021
Accepted: 3 May 2021

Keywords

HG-AAS, inorganic arsenic, organic arsenic, fish sauce, seafood

Abstract

An atomic absorption spectrometric (AAS) method was performed to determine the total, inorganic, and organic arsenic in fish sauce. The total organic arsenic was calculated from the total and inorganic arsenic values quantified using the hydride generation AAS (HG-AAS). Under optimal experimental conditions at the absorbance wavelength of 193.7 nm, the concentration of inorganic arsenic in fish sauce ranged from 0.05 to 1.2 mg/L, with a limit of detection (LOD) of 0.015 mg/L. The detectable total arsenic concentrations varied widely, ranging from 0.03 to 2.5 mg/L with the LOD of 0.01 mg/L. The practical applicability of the method was demonstrated with the recovery in the range from 97 to 102% for inorganic arsenic, and 97 to 101% for organic arsenic. The method was applied to the analysis of commercial products from Nha Trang, Phan Thiet, and Phu Quoc City, Vietnam. The total organic arsenic in fish sauce samples determined by HG-AAS was compared with the results of liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC-ICP/MS). The The *f*-test and *t*-test showed null hypothesis acceptable for variance and mean at a confidence level of 95%. The results showed that the HG-AAS method had high efficiency, accuracy, and sensitivity in quantifying inorganic and total organic arsenic in fish sauce using simple instrumentation.

Introduction

Arsenic is a hazardous chemical element causing significant health complications such as cancers (Martinez *et al.,* 2011), liver diseases (Hsu *et al.,* 2016), cardiovascular diseases (Navas-Acien *et al.,* 2019), respiratory diseases (Sancheza *et al.,* 2016), and diabetes mellitus type 2 (Beck *et al.,* 2017). In foods, arsenic accumulates at much higher levels in marine organisms than in terrestrial organisms. Arsenic is present in two forms: inorganic arsenic (iAs) and organic arsenic in seafoods. The high toxic forms include inorganic arsenic species (arsenate, arsenide), but their concentrations are very low (< 0.01 µg/kg) (Buchet *et al.,* 1996; Julshamn *et al.,* 2012). The predominant organic forms of arsenic are arsenobetaine, arsenosugars, and arsenolipids (Mania *et al.,* 2015). Dimethylarsenate (DMA) and methylarsenate (MA) are the most toxic organic arsenic compounds in seafoods, but their concentrations are as low as those of iAs (Lorenzana *et al.,* 2009). Although other organic arsenic species in seafoods are known to be non-toxic, recent findings have reported the cytotoxicity of some organic arsenic species and their intermediate metabolites in cell cultures (Meyer *et al.,* 2014; 2015; Molin *et al.,* 2015; Taylor *et al.,* 2017).

Fish sauce is a clear brown liquid hydrolysate from salted fresh fish. It can be made from many fish species and a few of sea creatures; but the most common species is anchovies (Lopetcharat *et al.,* 2001). Fish sauce is essential in the diets of almost all Vietnamese and more than 250 million Southeast Asians. It provides a substantial portion of the protein requirements for humans (McIver *et al.,* 1982; Park *et al.,* 2002). It is believed that arsenic compounds from seafoods are transferred to fish sauce products during the manufacturing process. Therefore, it is essential to evaluate arsenic amounts in fish sauce.

The most common method for iAs

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DOI: https://doi.org/10.47836/ifrj.28.6.07

determination is atomic absorption spectrometry. Nevertheless, although this method is widely used for assessing the levels of arsenic in seafoods (Muñoz *et al.,* 1999; Rasmussen *et al.,* 2012), it can only detect iAs. More recently, HPLC-ICP/MS has been developed to determine both inorganic and organic arsenic compounds, especially in seafoods and fish sauces (Hirata and Toshimitsu, 2005; Rodriguez *et al.,* 2009; Wolle and Conklin, 2018). However, the need for many standards and expensive instruments makes this method almost inapplicable for routine analysis. Therefore, the development of a simple, cost-effective, and sensitive method for both inorganic and total organic arsenic forms is crucial in food analytical chemistry.

Herein, a sensitive method based on the use of HG-AAS was developed to quantify inorganic and total arsenic in fish sauce, in which the total organic arsenic can be calculated by subtracting the amount of inorganic from the total arsenic. This method permits for a highly sensitive indirect analysis of total organic arsenic with low LOD. The HPLC-ICP/MS technique was used in the present work to confirm the accuracy of the HG-AAS method in analysing arsenic forms in the fish sauce samples. Such a method using simple instrumentation and cheap standard reagent (As^V) may provide a simple, cost-effective, and sensitive analysis of both inorganic and total organic arsenic in fish sauce samples.

Materials and methods

Materials and reagents

All reagents used in the present work were of analytical grade. Arsenic standard solution, As^V 1000 mg/L, traceable to SRM from NIST H_3AsO_4 in HNO_3 0.5 mol/L; arsenobetaine (AB); arsenocholine (AC); trimethylarsine oxide (TMAO); and trimethylaseno-propionate acid were purchased from Merck (Germany). Potassium iodide (≥ 99.5% purity), L-acid ascorbic (99% purity), concentrated hydrochloric acid (HCl; 36.5 - 38%), hydrobromic acid 48% (HBr; d = 1.50 g/mL), and hydrazine sulphate salt ($NH_2NH_2.H_2SO_4$) were purchased from Sigma-Aldrich (Singapore). Chloroform (99.8% purity) was purchased from Avantor (USA). All solutions were prepared and diluted using ultra-pure water with an electrical resistivity of > 18.3 MΩ cm, and produced by the Barnstead water purification systems. The fish sauce samples were purchased from Nha Trang, Phan Thiet, and Phu Quoc City, Vietnam.

HG-ASS analysis

The samples were prepared according to previous studies (Muñoz *et al.,* 1999; Maria *et al.,* 2017). Briefly, 1 mL of fish sauce sample (or standard solution) was diluted with 4.0 mL of deionised water in a 50.0 mL polypropylene (PP) centrifuge tube with screw cap. Then, 20.0 mL of concentrated HCl (> 37% m/v) was added. The mixture was agitated for 3 min on a mechanical shaker, and incubated overnight (about 12 - 15 h) at room temperature. Thereafter, 1.0 mL of hydrazine sulphate salt (1.5% w/v) and 2.0 mL of HBr 48% (d = 1.50 g/mL) were added to the resultant mixture in order to reduce As^V to As^{III}. To extract the total As^{III}, 10.0 mL of chloroform was added, and the mixture was shaken for 5 min and centrifuged at 800 rpm for 5 min to separate the chloroform phase from the aqueous phase.

The chloroform was pipetted into another 50.0 mL polypropylene (PP) centrifuge tube. The extraction procedure was repeated twice to ensure that most of the As^{III} in the aqueous phase was extracted from the chloroform phase. Care was taken to avoid cross-contamination from the acid phase. The total arsenic in the chloroform phase was re-extracted to the acid phase in three consecutive times by agitating with 10.0 mL of HCl 1.0 mol/L for 3 min at a time.

After extraction, the obtained acid phase was added to 2.5 mL of a mixture containing $Mg(NO_3).6H_2O$ 20% m/v, MgO 2% m/v, and 10 mL of concentrated HNO_3 (> 62.5% m/v). Then, the mixture was evaporated to dryness in a sand bath or a hot plate hob, and placed in a muffle furnace at an initial temperature below 150°C. The temperature was progressively increased at a rate of 50°C/h to 425 ± 25°C, and the sample was dried for 12 h at 425°C. The obtained ash was dissolved in 10.0 mL of 1:1 HCl, and the arsenic in the mixture was reduced using 2.5 mL of a solution containing 5.0% KI (m/v), 5.0% ascorbic acid (m/v), and 15.0 mL of concentrated HCl (> 36.5% m/v) for 15 min prior to dilution to 50 mL of final volume with ultra-pure water. A total of 0.5 mL of this solution was injected into the flow injection hydride generator (Perkin Elmer FIAS 100 Flow Injection for AAS), and analysed by atomic absorption spectrometry (Perkin Elmer Analyst 800; USA) at the operating conditions. The sample preparation for determining total arsenic was conducted using the same sample preparation procedure as inorganic arsenic, but without the extraction steps.

HPLC-ICP/MS analysis

An Agilent 1260 Infinity HPLC system (Agilent, CA, USA) was used to separate arsenic species, and the products were quantified using an Agilent 7700 Series ICP-MS system (Agilent, CA, USA). The cation exchange chromatography was carried out on a Nucleosil100-5 SA column (250 × 4.6 mm i.d., Düren, Germany) at 25°C. The mobile phase was a solution containing 30 mM pyridine, 2% (v/v) MeOH, pH 3.0 in isocratic mode with a flow rate of 1.0 mL/min. The injection volume was 100 µL. Fish sauce samples were diluted 50-fold in distilled water prior to analysis using HPLC-ICP/MS.

The operating conditions for ICP-MS were as follows: RF power was set at 1550 W; the plasma, auxiliary, and nebuliser gas were argon at flowrates of 15.0, 1.0, and 1.0 L/min, respectively, with a double-pass spray chamber. The monitored ion for detection was As^+ (m/z = 75). The calibration curves of AB, TMAO, TMAP, and AC were plotted from the area of the peak at retention times of 4.95, 6.81, 7.75, and 9.32 min, respectively. The LOD for each compound was lower than 0.005 mg/L.

Results and discussion

The linearity of the method

In order to verify the HG-AAS method, the linearity, limit of detection (LOD), accuracy, and precision were sequentially evaluated according to EURACHEM (1998), Association of Official Analytical Chemists (AOAC, 2007), and International Organization for Standardization (ISO, 2017).

The linearity for the determination of inorganic and total arsenic in fish sauce samples was observed by recording the atomic absorption response at 193.7 nm with various concentrations of inorganic arsenic or total arsenic from 0.001 to 6 mg/L. The calibration curves obtained with concentrations of inorganic arsenic in a range from 0.05 to 1.2 mg/L with the coefficient correlation (R^2) of 0.996. The calibration liner for total arsenic was built in a concentration range of 0.03 to 2.5 mg/L (R^2 = 0.997). The stability of linearity was observed on three different days. The R^2 obtained for both inorganic and total arsenic was from 0.996 to 0.998 (Table 1), which was higher than the required value for a quantitative analysis method (≥ 0.995). The results showed that both calibration curves were available for the determination of arsenic species.

Limit of detection and limit of quantification

Limit of detection (LOD) and limit of quantification (LOQ) for the determination of inorganic arsenic and total arsenic were calculated based on the analysis of fish sauce samples. Eleven repetitive experiments were carried out on different fish sauce samples. The LOD was calculated as three times of the standard deviation (SD), and the LOQ was calculated as ten times of the SD. Because the commercial fish sauce samples usually have a high total arsenic concentration (> 0.68 mg/L) and a very low level of inorganic arsenic (< 0.005 mg/L) (Rodriguez *et al.,* 2009), the samples for LOD measurement of total arsenic were prepared by diluting fish sauce samples at 20-fold, while the spiked samples were used for LOD measurement of inorganic arsenic.

As shown in Table 2, the average LOD for

Table 1. The linearity for determination of inorganic arsenic and total organic arsenic in fish sauce.

Time	Concentration range (mg/L)	Abs = intercept + slope* (arsenic)		Correlation coefficient (R^2)
		Slope	Intercept	
Inorganic arsenic				
Day 1	0.005 - 1.2	0.6465 ± 0.0018	0.0185 ± 0.0013	0.997
Day 2	0.005 - 1.2	0.6119 ± 0.0034	0.0193 ± 0.0025	0.996
Day 3	0.005 - 1.2	0.6307 ± 0.0027	0.0199 ± 0.0031	0.996
Total organic arsenic				
Day 1	0.003 - 2.5	0.5733 ± 0.0029	0.0509 ± 0.0018	0.997
Day 2	0.003 - 2.5	0.6145 ± 0.0012	0.0504 ± 0.0019	0.998
Day 3	0.003 - 2.5	0.6100 ± 0.0024	0.0442 ± 0.0026	0.997

Table 2. The limits of detection (LOD) and quantification (LOQ) of arsenic determination in fish sauce.

Sample	Mean (mg/L)	SD (mg/L)	LOD (mg/L)	LOQ (mg/L)
Inorganic arsenic				
Sample 1	0.078	0.00467	0.0140	0.0467
Sample 2	0.071	0.00481	0.0144	0.0481
Sample 3	0.097	0.00493	0.0148	0.0493
Total organic arsenic				
Sample 4	0.064	0.00313	0.0097	0.0313
Sample 5	0.039	0.00322	0.0096	0.0322
Sample 6	0.081	0.00317	0.0095	0.0317

Fish sauce samples were from different commercial producers. Sample 1, 2, and 3 were spiked with 0.08, 0.07, and 0.1 mg/L As^V, respectively. Samples 4, 5, and 6 were 20-fold diluted from three commercial fish sauce samples.

inorganic arsenic determination ranged from 0.0140 to 0.0148 mg/L, the published LOD and LOQ of this method for inorganic arsenic determination were 0.015 and 0.05 mg/L, respectively. Further, because of the lack of extraction steps, the LOD and LOQ for total arsenic determination were lower by 0.01 and 0.03 mg/L, respectively. According to the maximum permissible limits as presented in the national technical regulation (USDA, 2011) and European Commission (EC, 2015), the values of LOD obtained in the present work proved that the method was highly sensitive for the determination of arsenic in fish sauce.

Accuracy and precision of the method

The recovery of the method was validated based on the analysis of spiked samples used to assess the accuracy of the method. The fish sauce samples were spiked with three different levels of arsenic prior to sample preparation. The spiked samples were analysed, and their arsenic concentrations were calculated and compared with the known value of added arsenic. The recovery of inorganic and total arsenic was directly determined, whereas the recovery of total organic arsenic was indirectly calculated. The recoveries varied from 97 to 102%, thus demonstrating the high accuracy of the quantification method for inorganic, organic, and total arsenic in fish sauce samples.

The repeatability of the method was also evaluated by analysing three different fish sauce samples. Six replicates of each sample were analysed. Because commercial fish sauce usually has a very low level of inorganic arsenic, the samples used for repeatability measurement were spiked with 0.50, 0.10, and 0.05 mg/L As^V. The maximum relative standard deviation (RSD) in inorganic and organic arsenic determination was 4.57%. According to the RSD Horwitch function or the Association of Official Analytical Chemists (AOAC, 2007), this value was much lower than the maximum RSD values and acceptable for As^V levels ranging from 0.1 to 1 mg/L (11 - 16%). Therefore, the method exhibited excellent repeatability precision for different forms of arsenic determination in fish sauce.

Comparison of the HG-AAS method with HPLC-ICP/MS in the determination of arsenic

The accuracy of the HG-AAS method in quantifying the total organic arsenic in fish sauce was tested by comparison with HPLC-ICP/MS results. Since AB, AC, TMAO, and TMAP were the predominant forms (more than 99%) in seafoods and fish sauce (Rodriguez *et al.,* 2009), the total organic arsenic determined by HG-AAS was compared using the sum of AB, AC, TMAO, and TMAP concentration determined by HPLC-ICP/MS. *f*-test and *t*-test for variances and means were used for the comparison (Lung *et al.,* 2003).

As shown in Table 3, most of the inorganic arsenic levels in the five commercial fish sauce samples were lower than the LOD (0.015 mg/L), thus indicating that organic arsenic was equivalent to the total arsenic. The organic arsenic concentrations in the five samples ranged from 1.68 to 2.34 mg/L, similar to the HPLC-ICP/MS results (from 1.66 to 2.36 mg/L) (Figure 1). The linear relation between the values obtained from the HG-AAS method and those of HPLC-ICP/MS method showed a strict correlation of $R^2 = 0.991$ (Figure 2). All statistical values (t_{stat} and f_{stat}) were less than the critical values (t_{crit} of 2.73

Table 3. The comparison of HG-AAS method with HPLC-ICP/MS in the real sample determination of total organic arsenic. The results were calculated on six repetitive experiments with confident level of 95%.

Fish sauce sample	HG-AAS		HPLC-ICP/MS			t_{stat}	t_{crit}	f_{stat}	f_{crit}
	Inorganic arsenic (mg/L)	Organic arsenic (mg/L)	Variance	Mean (mg/L)	Variance				
Sample 1	< 0.015	1.68	0.0013	1.66	0.0019	0.86	2.23	1.44	5.05
Sample 2	< 0.015	2.14	0.0023	2.09	0.0030	1.47	2.23	1,32	5.05
Sample 3	< 0.015	1.95	0.0043	1.98	0.0027	1.05	2.23	1.62	5.05
Sample 4	< 0.015	2.34	0.0037	2.36	0.0055	0.68	2.23	1.48	5.05
Sample 5	< 0.015	1.86	0.0036	1.81	0.0022	1.55	2.23	1.65	5.05

The total organic arsenic determined by HG-ASS was the total arsenic minus the inorganic arsenic. The total organic arsenic determined by HPLC-ICP/MS was the sum of AB, AC, TMAO, and TMAP. The results were calculated from six repetitive experiments with confident level of 95%.

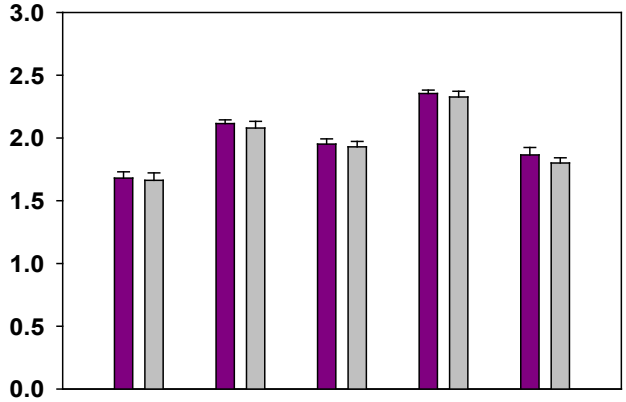

Figure 1. Practical method on five commercial fish sauce samples. The total organic arsenic determined by HG-ASS (dark pink) was the total arsenic minus the inorganic arsenic. The total organic arsenic determined by HPLC-ICP-MS (grey) was the sum of AB, AC, TMAO, and TMAP. Error bars are standard deviations from five repetitive experiments.

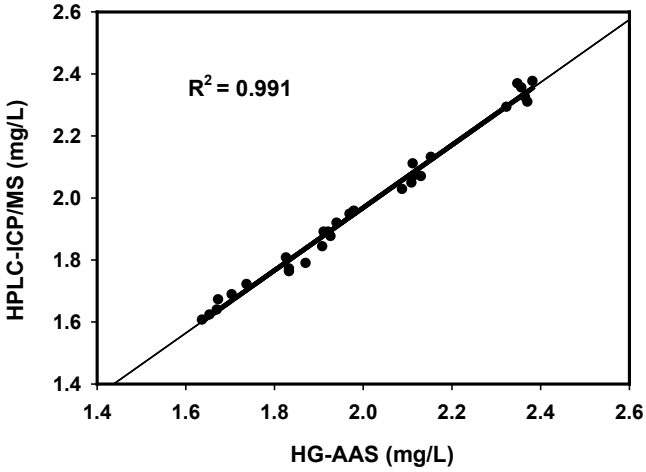

Figure 2. Liner correlation between HG-AAS and HPLC-ICP/MS methods of 31 experiments on five commercial fish sauce samples. The total organic arsenic determined by HG-ASS was the total arsenic minus the inorganic arsenic. The total organic arsenic determined by HPLC-ICP-MS was the sum of AB, AC, TMAO, and TMAP.

and f_{crit} of 5.05, $n = 6$, $p = 0.95$). These results showed that the current method had high accuracy and precision in the determination of arsenic forms in fish sauce.

Conclusion

An efficient and sensitive method based on hydride generation atomic absorption spectrometry was developed and validated for the determination of arsenic in fish sauce. This work represented the first example of determining total organic arsenic in fish sauce by HG-AAS, and the results exhibited excellent selectivity and sensitivity as expected. The calibration curves obtained by this method covered a wide range of arsenic concentrations with a very high correlation coefficient ($R^2 > 0.995$) and a low LOD. The method had desirable precision with RSD ranging from 1.24 to 4.57%, and its accuracy was comparable to that of HPLC-ICP/MS method. Furthermore, the method's practicality was proven by high recoveries in the range of 97 to 102%. In most fish sauce samples, inorganic arsenic was lower than the LOD (0.015 mg/L), and the total organic arsenic was in the range of 1.68 to 2.34 mg/L. The present work demonstrated that HG-AAS could be applied to the quantification of total organic arsenic in the fish sauce sample.

Acknowledgement

The authors would like to thank the Industrial University of Ho Chi Minh City for the financial supports received in the completion of the present work.

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