

Determination of total arsenic in fish by hydride-generation atomic absorption spectrometry: method validation, traceability and uncertainty evaluation

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Abstract. Fish containing arsenic compound is one of the important indicators of arsenic contamination in water monitoring. The high level of arsenic in fish is due to absorption through food chain and accumulated in their habitat. Hydride generation (HG) coupled with atomic absorption spectrometric (AAS) detection is one of the most popular techniques employed for arsenic determination in a variety of matrices including fish. This study aimed to develop a method for the determination of total arsenic in fish by HG-AAS. The method for sample preparation from American of Analytical Chemistry (AOAC) Method 999.10-2005 was adopted for acid digestion using microwave digestion system and AOAC Method 986.15 – 2005 for dry ashing. The method was developed and validated using Certified Reference Material DORM 3 Fish Protein for trace metals for ensuring the accuracy and the traceability of the results. The sources of uncertainty of the method were also evaluated. By using the method, it was found that the total arsenic concentration in the fish was $45.6 \pm 1.22 \text{ mg.Kg}^{-1}$ with a coverage factor of equal to 2 at 95% of confidence level. Evaluation of uncertainty was highly influenced by the calibration curve. This result was also traceable to International Standard System through analysis of Certified Reference Material DORM 3 with 97.5% of recovery. In summary, it showed that method of preparation and HG-AAS technique for total arsenic determination in fish were valid and reliable.

1. Introduction

It is well recognized that arsenic is present in the environmental and in biological systems in various chemical forms, both organic and inorganic forms. The organic forms of arsenic, such as arsenobetaine found in ubiquitous of edible marine animals has no toxic effect. However, the inorganic forms, arsenite (As^{3+}) and arsenate (As^{5+}), are known to be the most toxic. Food and water are significant sources of arsenic intake, accounting 77.5% in children [1]. Levels of arsenic in most food are fairly low, as it is not added due to its toxicity, however the levels of arsenic in fish and seafood may be high because fish can absorb and accumulate arsenic from the water they live in. Inorganic arsenic typically accounts for one to three per cent of the total arsenic found in food [2]; it is found at a higher content in such marine organisms as fish, crab, shrimp, oyster [3].

Hydride generation (HG) coupled with atomic absorption spectrometric (AAS) detection is currently one of the most popular techniques used for the quantification of arsenic in a variety of matrices. HG is a powerful technique of which utilizes chemical with typical properties of the metalloid group of elements such as As, Bi, Sb, Se, Te, Ge and Sn to form volatile hydrides (arsine in the case of As). These hydrides are carried using a stream of inert gas to the atomizer in Flame AAS [4]. The method offers several advantages, including improved detection limits for hydride-forming elements and reduced matrix effects. Although the method is simple, it is not totally free from interferences in the matrices. Several authors have reported that interferences such as transition metals [5-6], mutual hydride-forming elements [7-8], and conditioning of the quartz cell surface can interfere



in HGAAS technique [9]. Therefore, it is necessary to validate the method for the determination of total arsenic concentration in fish.

To achieve comparability of results over space and time, it is essential to compare all the individual measurement results to standards [10]. Traceability is accordingly intimately linked to uncertainty. Traceability provides the means of placing all related measurements to reference standards while an uncertainty characterizes the agreement to be expected between laboratories making similar measurements. The quoted uncertainty value combines all uncertainties, either arising from random variation or systematic effects [11-12]. Standard procedure to estimate the measurement uncertainty was adopted from the GUM (Guide to the expression uncertainty in measurement) and EURACHEM/CITAC Guide [10, 12].

In this study, determination of total arsenic in fish by HG-AAS was developed and validated. The sources of uncertainty of the method was also evaluated. Certified Reference Material DORM 3 Fish Protein for trace metals was used for ensuring the accuracy and the traceability of the results.

2. Experimental

2.1 Instruments

All samples were digested using a Milestone MLS 1200 microwave digestion system (Milestone, Bergamo, Italy) and dried ashing in a Vulcan Furnace model A550 (Dentsply International, York, United States of America). Arsenic concentrations were measured using a Hitachi Z-5000 Atomic Absorption Spectrometer (Hitachi Instruments Co. Tokyo, Japan) coupled with Hydride Generation system. The operating conditions of AAS are listed in Table 1.

Table 1. HG-AAS operating condition.

System	Operation condition
Atomizer	Standard
Flame type	Air-acetylene
Fuel-flow	1.7 L min ⁻¹
Carrier gas flow	0.1 L min ⁻¹
Oxidant pressure	160 kPa (1.6 kg cm ⁻²)
Ar gas pressure	150 kPa (1.5 kg cm ⁻²)
Burner height	Cell position
Sampling time	40 s
Reaction time	30 s
Measurement time	15 s

2.2 Reagents and solutions

SRM 83d standard reference material of arsenic trioxide, As₂O₃ powder 99.9926 ± 0.0030 wt% was purchased from National Institute Standard and Technology (NIST), United States of America. A stock solution of arsenic was prepared by dissolving 0.132 g arsenic trioxide in water containing 0.4 g NaOH, and diluted to 100 g. An intermediate solution was prepared by diluting 1 g stock solution in 100 g of 0.1 M HCl. Accurate dilution 1 g intermediate solutions with 100 g of 0.01 M HNO₃ was carried out to prepare working solutions. Appropriate amount of working solution was diluted in 0.01 M HNO₃ to prepare calibration solution. Magnesium nitrate, Mg(NO₃)₂ and sodium borohydride, NaBH₄ powder with purity of ≥ 98.0 % was purchased from Sigma-Aldrich Inc. A solution of 7.5% (w/v) Mg(NO₃)₂ was prepared by dissolving 7.5 g Mg(NO₃)₂ in 100 mL ultrapure water, while sodium borohydride solution was prepared fresh daily by dissolving 2 g NaBH₄ in 200 mL 1 M NaOH.

Potassium Iodide for general purpose reagent was purchased from BDH Chemical Ltd, Poole, England. A pre-reductant solution with concentration of 10% (w/v) KI was prepared from 50 g KI dissolved in 500 mL water. Nitric acid and hydrochloric acid for poisonous metal analysis from Kanto Chemicals, Tokyo, Japan were diluted with ultrapure water to give a 0.01 M, a 0.1 M of nitric acid and a 2M, a 8M of hydrochloric acid solutions. Concentrated hydrogen peroxide and sodium hydroxide were purchased from E-Merck, Germany. All water used for analysis is ultrapure water ($18.3 \text{ M}\Omega \text{ cm}^{-1}$ resistivity) that was prepared by a Milli-Q Plus 185 from Millipore cooperation, Bedford, United State of America. DORM-3 Fish Protein Certified Reference Material for trace metals from National Research Council, Canada, was used for ensuring the accuracy and traceability of the method.

2.3 Method

2.3.1 Sample preparation

Sample preparation methods were adopted from American of Analytical Chemistry (AOAC) Method 999.10-2005 for acid digestion using microwave digestion system and AOAC Method 986.15 – 2005 for dry ashing [13].

- Solution A

An amount of 0.5 g sample was weighed accurately in a digestion vessel. Sample was added with 5 mL of HNO_3 14 M and 2 mL of concentrated H_2O_2 and then the vessel was closed. The closed vessel was kept for one night before run in a microwave digestion system. Sample was transferred to a 50 mL Nalgene bottle with a screw cup and diluted until 25 g with ultrapure water.

- Solution B

Accurate amount of 2 g solution A was transferred into the porcelain crucible and added with 1 mL 7.5% (w/v) $\text{Mg}(\text{NO}_3)_2$, and then heated on a hotplate at slow heat to dryness. The crucible was then placed in a furnace at $250\text{--}450^\circ\text{C}$ with maximum heating rate of 100°C/h . After being cooled, the residue was dissolved with 2 mL 8M HCl and diluted with ultrapure water to 20 g.

Accurate amount of 2.5 g solution B was added with 5 mL concentrated HCl and 5 mL 10% (w/v) KI, and then stand for 45 min before diluted 50 g with ultrapure water, and measured by HGAAS.

2.3.2 Standard preparation

Accurate amount of 2.5 g each calibration solution was added with 5 mL concentrated HCl and 5 mL 10% (w/v) KI, and then stand for 45 min before diluted into 50 g with ultrapure water, and measured by HGAAS.

2.3.3 Moisture content

Parallel with sample preparation, the moisture content of samples was examined by drying 1 g of sample at room temperature in desiccators over CaSO_4 anhydrous for 10 days to obtain constant weight.

3.Result and discussion

3.1 Method validation for arsenic determination by HGAAS

3.1.1 Optimizing HGAAS parameters

Some parameters of HGAAS such as molarity of hydrochloric acid or sodium tetrahydroborate may vary depending on the type of apparatus, therefore it should be optimized. Other parameters for reducing arsenate (pentavalent arsenic) to arsenite (trivalent arsenic) in the solution also have been optimized. The optimized values were marked with red bold line as shown in Figure 1.

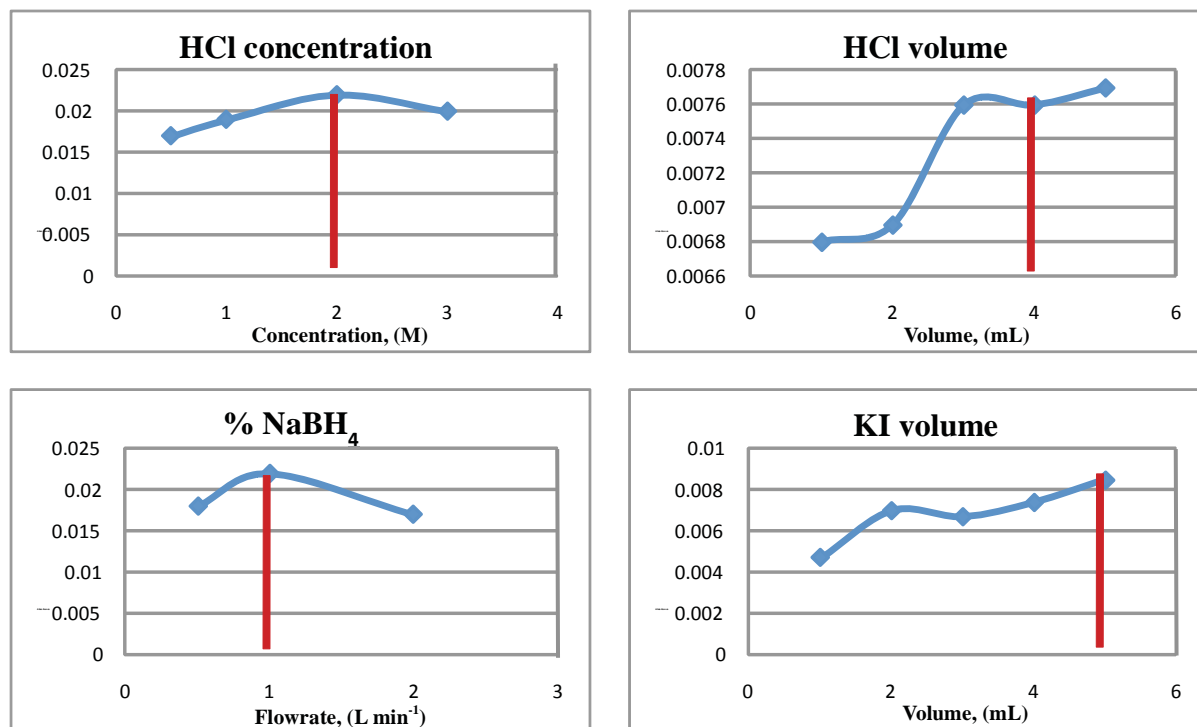


Figure 1. Effect of HGAAS parameters to the measured absorbance. The optimized values are shown by the red lines.

3.1.2 Method validation parameter

The optimized method validation parameters are shown in Table 2. The detection and quantitation limit of the proposed method were examined under the optimal experimental conditions.

Table 2. Optimization of validation parameters method.

No.	Parameter	Examined	Optimized value
1	Linearity ($\mu\text{g} \cdot \text{Kg}^{-1}$) (w/w)	0 – 20	0.5– 5
2	Detection limit ($\mu\text{g} \cdot \text{Kg}^{-1}$) (w/w)	3 sd of $1 \mu\text{g L}^{-1}$, 10 replicates	0.17
3	Quantitation limit ($\mu\text{g} \cdot \text{Kg}^{-1}$) (w/w)	10 sd of $1 \mu\text{g L}^{-1}$, 10 replicates	0.58
4	Precision (%RSD)	$2 \mu\text{g L}^{-1}$, 7 replicates	7.5
5	Accuracy (DORM 3*, mg/kg) (w/w)	$6.88 \pm 0.30^{**}$	$6.71 \pm 0.32^{***}$

* Fish Protein CRM for trace metals, National Research Council of Canada

** Value from certificate

*** Average and standard deviation from 2 replicates

The detection and quantitation limit were 0.17 and 0.58 $\mu\text{g L}^{-1}$, respectively, which were calculated as the three times and ten times of the standard deviation of ten replicates of 1 $\mu\text{g.L}^{-1}$ standard. Its precision was calculated as relative standard deviation (%RSD) from sample measurement with 7 replicates.

3.1.3 Effect of sample matrix

Since the method was developed from routine for food sample, the validity of the method was verified to be fit for fish sample by determining the matrix interference. In Figure 2, two calibration curves prepared by external and matrix matched calibration were compared to examine the effect of sample matrix.

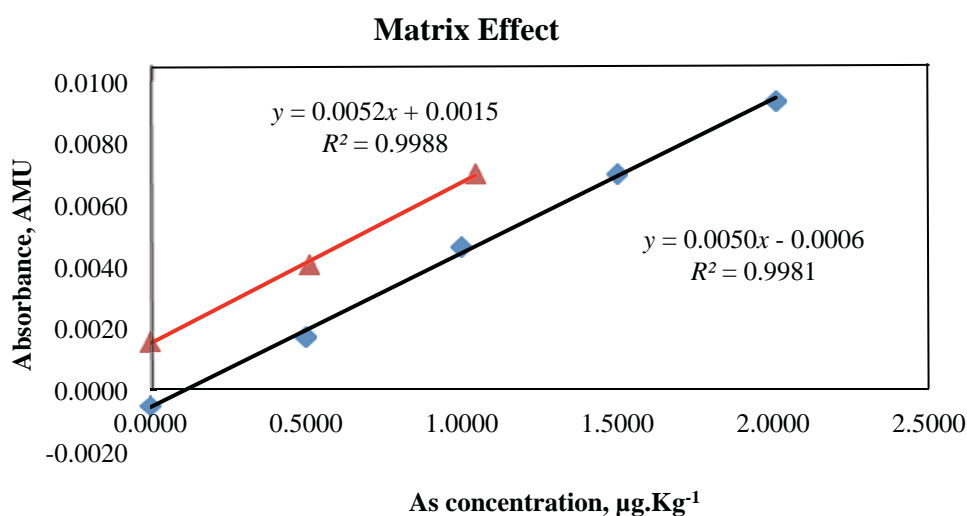


Figure 2. Effect of sample matrix. Red sign is sample spiked with arsenic standard; Blue sign is arsenic standard diluted in HNO_3 0.2 M.

Figure 2 shows that the slope of both calibration curves are similar (0,050 vs 0,052). The similarity indicates that sample matrix does not interfere with the measurement of arsenic and therefore HGAAS method is valid for fish sample.

3.2 Traceability and accuracy

Traceability can be obtained by reference measurement or certified reference materials. In this study, traceability was obtained by analyzing DORM-3 Fish Protein Certified Reference Material. As shown in Table 2, the result obtained by the method was in good agreement with the certified value. This result indicates that the present method was accurate and no bias.

3.2.1. Sources of uncertainty

Sources of uncertainty and the biggest uncertainty component for the determination of total arsenic in fish have been identified using bottom-up approach. This estimation evaluation was based on ISO/IEC Guide 98-3:2008, JCGM 100:2008 and Eurachem/Citac Guide 2012. There are four main steps to estimate the uncertainty components according to Eurachem/Citac Guide: (1) specification of the measurand, (2) identification of uncertainty sources, (3) quantification of uncertainty components and convert to standard uncertainty, and (4) combination of standard uncertainties.

3.2.2. Specification of the measurand

In this step, the estimation begins with making a summary of measurement which is shown in flow chart on fig 1. A dry-weight content of total arsenic (C_{As}) was calculated from the concentration of As in digested solution (C_o), the solvent mass (M), the mass of sample (m) and the dilution factor (DF) which is converted to method recovery (Rec) and moisture content (MC).

$$C_{As} = \frac{C_o \times M \times DF}{m} \times \frac{1}{Rec} \times \frac{100}{100 - MC} \times (Rep) \quad (1)$$

Precision of each uncertainty source was not calculated because it was included in all method precision (Rep).

3.3.3. Identification of uncertainty sources

All relevant sources of measurement uncertainty were identified through cause-effect diagram (Figure 3).

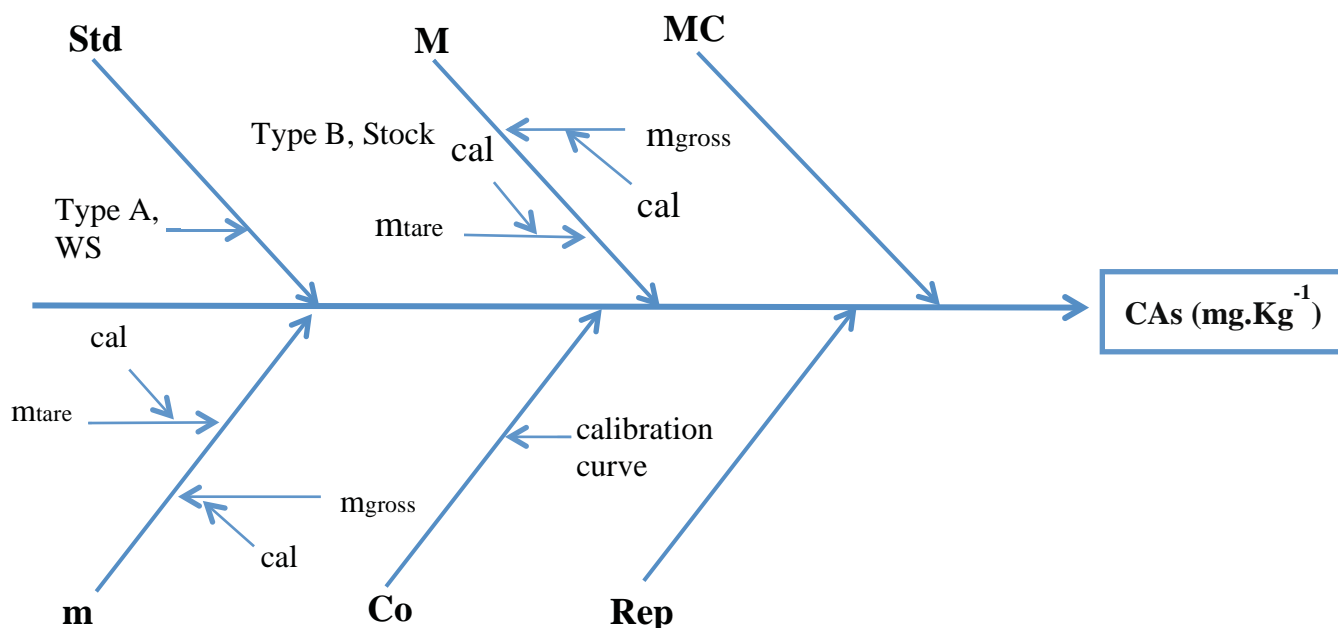


Figure 3. Cause and effect diagram for the determination of total arsenic concentration in fish.

The cause and effect diagram is a very convenient way of listing the uncertainty sources, showing the relation of each component and their influence on the uncertainty results.

3.3.4. Quantification of uncertainty components and convert to standard uncertainty

The quantification of all uncertainty sources are divided into each parameter and then converted into standard uncertainties.

3.3.4.1. Arsenic concentration in digested solution (C_o)

This component was obtained from interpolated concentration in calibration curve equation, $y = Ax + B$, where y is the degree of absorption from the calibration standard, A is the angular coefficient, x is the concentration of the calibration standard, and B is the point of intersection with the y axis [14]. The calibration curve was made from five standard solutions that prepared manually (w/w), with

concentration 0.0, 0.5, 1.0, 2.5, and 5.0 $\mu\text{g.Kg}^{-1}$, respectively. The calibration standards were prepared from 1000 mg.Kg^{-1} arsenic stock solution and measured three times. The standard uncertainty from Co was calculated using equation (2):

$$\mu C_o = \frac{S}{B_1} x \sqrt{\frac{1}{p} + \frac{1}{n} + \frac{(C_o - \bar{C})^2}{S_{xx}}} \quad (2)$$

with the residual standard deviation S ($\mu\text{g.Kg}^{-1}$) and S_{xx} ($(\mu\text{g.Kg}^{-1})^2$) are given by

$$S = \sqrt{\frac{\sum_{j=1}^n [A_j - (B_o + B_1 x C_j)]^2}{n - 2}} \quad (3)$$

$$S_{xx} = \sum_{j=1}^n (C_j - \bar{C})^2 \quad (4)$$

Where

- A_j : measurement of absorbance
- B_o : intercept
- B_1 : Slope of calibration curve
- p : number of measurements to determine C_o
- n : number of measurements for the calibration
- \bar{C} : mean value of the different calibration standards (n number of measurements)
- i : index for the number of calibration standards
- j : index for the number of measurements to obtain the calibration curve

3.3.4.2. Mass

The standard uncertainty related to the mass of sample (m) and solvent (M) were identified from the uncertainty sources for the tare and gross weighing. Each source takes into account the contribution from the repeatability, the readability (digital resolution) of the balance scale, and the calibration function. There are two components that contributed to the calibration function, identified as the sensitivity and the linearity of the balance. The sensitivity component was neglected because the mass by difference is done on the same balance over a very narrow range [15]. The repeatability contribution from mass was considered as a whole method precision (Rep), therefore the standard uncertainty from this source was estimated using the data from analytical balance calibration certificate, as of 0.0001 g on 95% confidence level that accounted for twice, once for the tare and other for the gross mass.

$$u_{mass} = \sqrt{u_{gross}^2 + u_{tare}^2} \quad (5)$$

3.3.4.3. Dilution factor (DF)

In this method, the digested sample is diluted with water before measured using HG -AAS based on weight per weight calculation. The dilution factor (DF), is given by

$$DF = \frac{w_2}{w_1} x \frac{w_4}{w_3} \quad (6)$$

The weight is determined using the same calibrated analytical balance which has standard uncertainty 0.0001g on 95% confidence level. Therefore, total standard uncertainty μ (DF) from this sources is calculated taking into account all those weight uncertainties:

$$\frac{u(DF)}{DF} = \sqrt{\left(\frac{u(w_1)}{w_1}\right)^2 + \left(\frac{u(w_2)}{w_2}\right)^2 + \left(\frac{u(w_3)}{w_3}\right)^2 + \left(\frac{u(w_4)}{w_4}\right)^2} \quad (7)$$

The dilution factor of sample was 200 times which is from 2g (w_1) of solution A was diluted to 20g (w_2 , solution B) and then 2.5g (w_3) of solution B was diluted to 50g (w_4).

3.3.4.4. Method recovery (*Rec*)

The overall recovery, R , is an estimation of the “method recovery”, because it has been obtained by representatively varying the factors, such as matrix, concentration and analyte that can be affect to recovery. Therefore, proportional bias can be estimated in terms of the overall recovery [16]. The overall recovery is assessed using DORM-3 Fish Protein Certified Reference Material (CRM), from National Institute of Standard and Technology (NIST). The uncertainty estimation for this parameter was used for the comparison of obtained values with the certificates ones. The uncertainty associated with recovery, $u(R)$, is evaluated as follows:

$$u(R) = R \sqrt{\left(\frac{s_{obs}^2}{nC_{obs}^2}\right) + \left(\frac{u(C_{CRM})}{C_{CRM}}\right)^2} = 0.028 \quad (8)$$

The method recovery is calculated as the ratio

$$R = \frac{C_{obs}}{C_{CRM}} = 0.943 \quad (9)$$

Not all the recovery parameter have to used in calculation result, to determine whether the parameter is significantly different from one or not a student's t test is used. The t value is compared with the 2-tailed critical value t_{crit} for $n-1$ degrees of freedom at 95% confidence level. If the t value is greater or equal than the t_{crit} then the recovery parameter is significantly different from 1 and has to include in calculation result.^[15]

The equation to calculate the t value is given by:

$$t = \frac{|1 - R|}{u(R)} = 2.036 \quad (10)$$

This value is compared with the 2-tailed critical value t_{crit} , for $n-1$ degrees of freedom at 95 % confidence (where n is the number of results used to estimate *Rec*). If t is greater or equal than the critical value t_{crit} than *Rec* is significantly different from 1, therefore *Rec* is explicitly included in the calculation of the result. From the experimental, the t value is smaller than $t_{crit;5}$ (2.571), then it can be concluded that the procedure does not have any significant proportional bias. Therefore, the recovery parameter can be neglected in future results obtained with the procedure.

3.3.4.5. Moisture content (*MC*)

The moisture content of the sample is obtained by drying 1 g of sample at room temperature in desiccators over CaSO_4 anhydrous for 10 days. The percentage of moisture content in sample is quantified in the equation (11)

$$\% \text{ MC} = \frac{(M_a - M_b)}{M_a} \times 100\% \quad (11)$$

Sample mass before (M_a) and after (M_b) drying is weighted using the analytical balance which has standard uncertainty 0.0001 from calibration certificate on 95% confident level. This uncertainty sources has contributed to combined uncertainty which calculated as below

$$u_{MC} = MC \times \sqrt{\frac{(uM_a)^2 + (uM_b)^2}{(M_a - M_b)^2} + \frac{(uM_a)^2}{M_a^2}} \quad (12)$$

The result is shown in Table 3.

3.3.4.6. Method precision (Repeatability)

The results of the method validation experiments show repeatability for the arsenic determination from four kind samples using HGAAS, which is 0.35% as RSD. The entire individual sources precision included in this value. The value can be used directly for the combined standard uncertainty calculation associated with the different precision terms.^[16]

3.3.4.7. Standard

The uncertainty component from concentration of calibration standards are also contributed to combined uncertainty. There are two uncertainty sources of this component, uncertainty from standard stock solution (Type B Effect) and uncertainty of working solutions from dilution and random use of analytical balance (Type A Effect). The type B effect is considered the effect of the working standards in the same way and included shifting of calibration curve, such as purity, balance bias, and temperature whereas type A effect is considered the effect of each working solution differently.

3.3.4.8. Combination of standard uncertainties

The relative combined standard uncertainty for the method as whole is evaluated though all individual sources of uncertainty using the following equation

$$\left(\frac{u_{C(As)}}{C_{As}}\right)^2 = \left(\frac{u_{C_{Calstd}(type A)}}{C_{Calstd}(type A)}\right)^2 + \left(\frac{u_{C_{Stock}(type B)}}{C_{Stock}(type B)}\right)^2 + \left(\frac{u_{C_o}}{C_o}\right)^2 + \left(\frac{u_M}{M}\right)^2 + \left(\frac{u_m}{m}\right)^2 + \left(\frac{u_{DF}}{DF}\right)^2 + \left(\frac{u_{MC}}{MC}\right)^2 + \left(\frac{u_{Rep}}{Rep}\right)^2 \quad (13)$$

Detail of each uncertainty sources shown in table 4, where C_{As} represents the dry-weight content of total arsenic and calculated using eq. 1 without recovery factor.

Table 3. Uncertainties sources in total arsenic in fish determination.

Uncertainty components	Value (Xi)	Standard uncertainty (u Xi)	Unit	RSU (u Xi/Xi)
Calibration standard type A	0.0010	0.000000159	mg.Kg ⁻¹	0.000159
Calibration standard type B	1865	0.00150	mg.Kg ⁻¹	0.000000804
Calibration curve (C_o)	0.00390	0.0000503	mg.Kg ⁻¹	0.0129
Mass of solvent (M)	25.9	0.0000707	g	0.00000273
Mass of sample (m)	0.517	0.0000707	g	0.000137
Dilution factor (DF)	200.0	0.00909	-	0.0000454
Moisture content in sample (MC)	14.4	0.000100	%	0.00000699
Repeatability of method (Rep)	-	-	-	0.00353
C_{As}	45.6		mg.Kg ⁻¹	
$u_c(As)$	0.611		mg.Kg ⁻¹	
U	1.22		mg.Kg ⁻¹	

Figure 4 shows the majority of uncertainty contribution generates from calibration curve and method precision, in contrast with uncertainties from calibration stock type B and solvent mass that have not significant contribution to overall standard uncertainty.

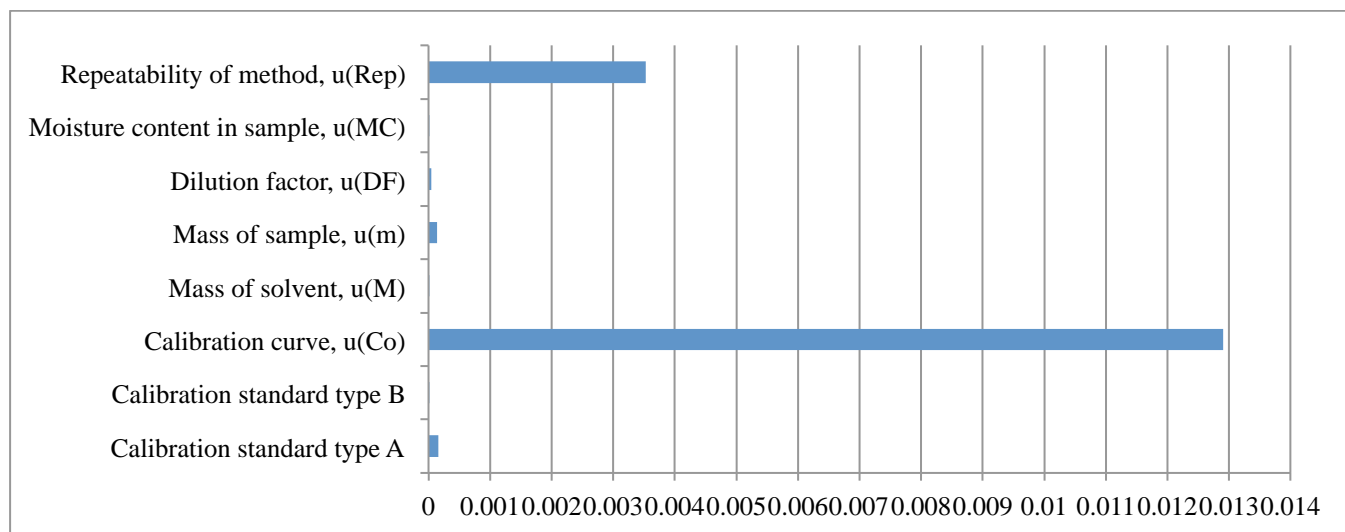


Figure 4. Uncertainty budget of measurement of arsenic in fish.

The expanded uncertainties, U_{As} , is obtained by multiplying the combined standard uncertainty by the coverage factor of $k=2$ which gives a confidence level of approximately 95%.

$$U_{As} = k \times u_{C(As)} = 2 \times 0.611 = 1.22 \text{ mg.Kg}^{-1}$$

Therefore, the measured total arsenic concentration in fish was $45.6 \pm 1.22 \text{ mg.Kg}^{-1}$ (w/w).

4. Conclusion

The developed method for determination of total arsenic in fish product was valid and traceable to International Standard. The result shows that the total arsenic concentration found in fish was $45.6 \pm 1.22 \text{ mg.Kg}^{-1}$, with a coverage factor of k equal to 2 with 95% confidence level and %recovery obtained was 97.5% which is in good agreement with analysis of Certified Reference Material DORM 3.

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