



Accuracy of a method based on atomic absorption spectrometry to determine inorganic arsenic in food: Outcome of the collaborative trial IMEP-41



I. Fiamegkos^a, F. Cordeiro^a, P. Robouch^a, D. Vélez^b, V. Devesa^b, G. Raber^c, J.J. Sloth^d, R.R. Rasmussen^d, T. Llorente-Mirandes^e, J.F. Lopez-Sanchez^e, R. Rubio^e, F. Cubadda^f, M. D'Amato^f, J. Feldmann^g, A. Raab^g, H. Emteborg^a, M.B. de la Calle^{a,*}

^a European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, 111 Retieseweg, 2440 Geel, Belgium

^b Metal Contamination Laboratory (IATA-CSIC), Avd. Agustín Escardino 7, 46980 Paterna, Valencia, Spain

^c Institute of Chemistry, University of Graz, Universitätsplatz 1, 8010 Graz, Austria

^d Technical University of Denmark, National Food Institute, Division of Food Chemistry, Mørkhøj Bygade 19, 2860 Søborg, Denmark

^e Department of Analytical Chemistry, University of Barcelona, Martí I Franque's 1-11, 08028 Barcelona, Spain

^f Department of Food Safety and Veterinary Public Health, Istituto Superiore di Sanità-Italian National Health Institute, Viale Regina Elena 299, 00161 Rome, Italy

^g Trace Element Speciation Laboratory (TESLA), University of Aberdeen, College of Physical Science, Chemistry, Meston Walk, Aberdeen AB24 3UE, Scotland, United Kingdom

ARTICLE INFO

Article history:

Received 13 November 2015

Received in revised form 22 March 2016

Accepted 12 June 2016

Available online 14 June 2016

Keywords:

Inorganic arsenic

Collaborative trial

Cereals

Vegetables

Mushrooms

Mussels

Fish

Algae

ABSTRACT

A collaborative trial was conducted to determine the performance characteristics of an analytical method for the quantification of inorganic arsenic (iAs) in food.

The method is based on (i) solubilisation of the protein matrix with concentrated hydrochloric acid to denature proteins and allow the release of all arsenic species into solution, and (ii) subsequent extraction of the inorganic arsenic present in the acid medium using chloroform followed by back-extraction to acidic medium. The final detection and quantification is done by flow injection hydride generation atomic absorption spectrometry (FI-HG-AAS).

The seven test items used in this exercise were reference materials covering a broad range of matrices: mussels, cabbage, seaweed (hijiki), fish protein, rice, wheat, mushrooms, with concentrations ranging from 0.074 to 7.55 mg kg⁻¹.

The relative standard deviation for repeatability (RSD_r) ranged from 4.1 to 10.3%, while the relative standard deviation for reproducibility (RSD_R) ranged from 6.1 to 22.8%.

© 2016 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Arsenic (As) is a widely found contaminant, which occurs both naturally and as a result of human activities. Since the late 1960s, scientific evidence has been building up showing that exposure to high levels of inorganic As (iAs) may cause skin lesions with carcinogenic (EPA, 2010; Hughes, Beck, Chen, Lewis, & Thomas, 2011; National Research Council (NRC), 1999) or non-carcinogenic effects (U.S. Department of Health and human services, 2007; National Research Council, 2013). Based on their chemical properties, arsenic species can be categorized as lipid-soluble or water-soluble arsenicals, the latter including both inorganic and organic compounds (Hajeb, Sloth, Shakibazadeh,

Mahyudin, & Afsah-Hejri, 2014). Inorganic arsenic species (As(III) and As(V)) seem to be the most toxic and carcinogenic forms (Muñoz, Vélez, & Montoro, 1999).

There is a worldwide concern about dietary iAs exposure and the associated health risks have been emphasized in recent toxicological evaluations by the European Food Safety Authority (EFSA) and the Joint Expert Committee on Food Additives (JECFA) of the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) (EFSA, 2014; FAO JECFA MONOGRAPHS 8, 2011; WHO, 2011).

Until recently, most studies focused on the determination of total As in the diet (FDA, 2004). However, the recognition that the chemical form of As is critical for assessing risk, coupled with advances in analytical methods, has resulted in a significant expansion of the amount of published scientific studies on As speciation (U.S. Department of Health and human services, 2007). The

* Corresponding author.

E-mail address: Maria.de-la-calle@ec.europa.eu (M.B. de la Calle).

determination of the iAs levels in food and the calculation of typical intakes are critical to establish background exposure levels to iAs and to understand risks from excess intake of natural or anthropogenic sources (D'Amato et al., 2013; Lynch, Greenberg, Pollock, & Lewis, 2014). For this reason there has been an increasing interest in the development and validation of robust and reliable methods to determine iAs in a range of food commodities. Such methods should support the introduction of maximum levels for iAs in rice and rice-derived products in the forthcoming revision of Commission Regulation, (EC) 1881/2006 (2006).

The most commonly applied analytical methods for As speciation are based on high performance liquid chromatography hyphenated with inductively coupled plasma mass spectrometry (HPLC-ICP-MS) (Francesconi & Kuehnelt, 2004; Maher et al., 2012). However, some drawbacks are associated with those methods: HPLC-ICP-MS analytical platforms are expensive and not available in many routine control laboratories and furthermore, highly skilled analysts are needed, especially when matrices with a complex mixture of arsenic species are analysed as it is the case for food items of marine origin. Other methods of analysis are based on chemical separation of arsenic species with subsequent AAS determination (Rasmussen, Qian, & Sloth, 2013; Vieira et al., 2009). Hydride generation (HG) is one of the most straightforward approaches, with a high selectivity due to the formation of volatile hydrides of only few arsenic species (Musil et al., 2014; Pétursdóttir, Gunnlaugsdóttir, Krupp, & Feldmann, 2014). In general, methods based on HG-AAS are easier to implement and less costly than those based on HPLC-ICP-MS in terms of the analytical instrumentation needed.

In 2012 the European Committee for Standardization (CEN TC 327/WG 4) standardised a method (EN 16278:2012) for the determination of iAs in animal feeding stuffs by HG-AAS after microwave extraction and off-line separation of inorganic arsenic by solid phase extraction (SPE) (BS EN-16278, 2012). This method was validated in a collaborative trial in the frame of the IMEP-32 project (Sloth et al., 2011) and has furthermore been used in studies on inorganic arsenic content in seafood and rice (Rasmussen, Hedegaard, Larsen, & Sloth, 2012; Rasmussen et al., 2013). Currently, CEN TC 275/WG 10 is validating a method for the selective determination of iAs in food based on HPLC-ICP-MS. Two other standard methods have been published, GB/T 5009.11-2003 (in China) (EN 15517, 2008; GB/T5009.11, 2003), for the determination of abio-arsenic in food and of iAs and in seaweed, respectively. Both methods are based on the selective determination of arsenic from iAs under specific conditions without any previous separation of species and with final determination by atomic fluorescence (Chines standard) and by HG-AAS (CEN standard), respectively. However, IMEP-112 (de la Calle et al., 2012) (a proficiency test for the determination of total and iAs in wheat, vegetable food and algae) showed that the results obtained with those two standards were strongly biased when applied to algae, a matrix with a complex pattern of arsenic species. Methylated species, such as dimethyl arsenic acid (DMA), abundant in samples of marine origin, can also generate volatile hydrides and could, therefore, interfere in the determination of iAs and lead to positively biased results (Schmeisser, Goessler, Kienzl, & Francesconi, 2004).

Recently, the International Measurement Evaluation Program (IMEP), which is operated by the Joint Research Centre (JRC), a Directorate General of the European Commission, organised a collaborative trial (IMEP-41) for the validation of a method to determine iAs in several foodstuffs. This method, which is based on the selective extraction of iAs into chloroform and further determination by HG-AAS, should serve as inexpensive complement to the method being validated by CEN based on HPLC-ICP-MS. The standard operating procedure (SOP) had been previously developed, in-house validated (Muñoz et al., 1999) and applied to the

determination of iAs in marine samples (Muñoz et al., 2000) by the Trace Elements Group of the Institute of Agrochemistry and Food Technology (IATA) of the Spanish National Research Council (CSIC).

This manuscript summarises the outcome of IMEP-41 and includes a discussion of problems associated with the selective determination of iAs in food. The validated method will support the implementation of Regulation (EC) 1881/2006 setting maximum levels for certain contaminants in foodstuffs, which in its next revision will include maximum levels (MLs) for iAs in rice and rice-derived products.

2. Collaborative study

2.1. Scope and principle of the method

The SOP (Fiamegkos et al. (2015)) can be downloaded from the webpage of the Institute for Reference Materials and Measurements of the Joint Research Centre (JRC-IRMM) (<https://ec.europa.eu/jrc/en/interlaboratory-comparisons>). iAs is separated from other arsenic species before being determined by flow injection-hydride generation-atomic absorption spectrometry (FI-HG-AAS). The extraction method is based on (i) solubilisation of the protein matrix with a high concentration of hydrochloric acid, which denatures the proteins and releases all the arsenic species, and (ii) the subsequent selective extraction with chloroform of the iAs present in the acid medium followed by back-extraction into acidic medium prior to analysis. The quantification limit of the method is 0.010 mg kg⁻¹ of iAs. When the method is run using the conditions described in the SOP, determination of iAs is free of the interferences of other known arsenic species with the exception of monomethyl arsonic acid (MA). However, this species is typically only found as a minor arsenic species (Schoof & Yager, 2007).

2.2. Matrices tested in IMEP-41

The seven food commodities used in this collaborative trial are listed in Table 1. Two of the distributed samples were identical (S1 and S3, both rice) to ensure that the analysis of one sample does not affect the subsequent measurement on another sample. A rice pre-test sample (ERM-BC211) was sent to participants allowing them to confirm the proper implementation of the method under investigation, before starting the collaborative trial.

2.3. Preparation of the test items

All the test items used in IMEP-41 were certified reference materials (CRMs) or reference materials (RMs) previously used in IMEP proficiency tests; for this reason the test items underwent little processing for the purpose of the collaborative trial. The bottles of the pre-test item and of the test items S1 (rice), S2 (wheat), S4 (mussels) and S6 (mushroom) were relabelled to avoid the

Table 1

List of reference materials used in the IMEP-41 exercise. S4, S7, S8 and pre-test are CRMs certified for total As. S7 and pre-test are also certified for iAs.

Sample ID	Reference material	Food commodity
S1 & S3	IMEP-107	Rice
S2	IMEP-112	Wheat
S4	ERM-CE278k	Mussels
S5	IAEA-359	Cabbage
S6	IMEP-116	Mushroom
S7	NMIJ-7405a	Seaweed
S8	DORM-4	Fish
Pre-test	ERM-BC211	Rice

identification of the test item by participants and expert laboratories. The new labels contained the appropriate code (IMEP-41, material number and sample number). For the test items S3 (rice), S7 (seaweed) and S8 (fish), the supplied units were opened, pooled into a 5 L acid-washed plastic drum and placed in a 3D-mixer for 30 min (Dynamix CM200, WAB, Basel, CH) for thorough mixing and re-homogenisation. The materials were then refilled in labelled vials using a vibrating feeder and a balance in a clean-cell equipped with a HEPA filter. For cabbage (S5), a handful of Teflon balls were added during mixing to break up agglomerates since the material was clogged upon delivery. In order to break the agglomerates the material was forced to go through a 500 µm mesh. It was then transferred into a 5 L acid-washed plastic drum and subsequently in a 3D-mixer for 30 min, before filling in vials using a vibrating feeder and a balance in the clean-cell.

Care was taken to avoid cross-contamination between the different materials and two powders were never handled at the same time. Every material was mixed and filled only after thorough cleaning of the whole equipment used.

2.4. Assigned values and associated uncertainties used to evaluate the trueness of the method

In order to assess the trueness of the method, assigned values for iAs mass fractions in all the test items were determined using methods of analysis different from the one under validation. The iAs certified values and uncertainties in the ERM-BC211 pre-test item and NMJJ-7405a (S7) (seaweed) were provided by the respective CRM producers. The assigned value for the mushroom test item (S6) was the one assigned during the PT IMEP-116, because that PT was run only some months before IMEP-41 and stability of the test item could be assumed. For the remaining samples the iAs mass fractions were determined by five expert laboratories, listed hereafter, selected on the basis of their demonstrated measurement capabilities in this field of analysis:

- Istituto Superiore di Sanità (Rome, Italy);
- Institut für Chemie, Bereich Analytische Chemie, Karl-Franzens Universität (Graz, Austria);

- Technical University of Denmark, National Food Institute – DTU (Søborg, Denmark);
- Department of Chemistry, University of Aberdeen (Aberdeen, UK);
- Department of Analytical Chemistry, Faculty of Chemistry, University of Barcelona (Barcelona, Spain)

Every expert laboratory received two bottles per test item with the exception of S4 (mussels). For S4, due to lack of samples only one bottle could be included meaning that the same bottle should be used for all analyses. Experts were requested to perform three independent measurements per bottle (under repeatability conditions) on two different days (one bottle/day) following the method of their choice. They had to report the values obtained for the six independent measurements, the corresponding mean and its associated expanded measurement uncertainty (corresponding to a 95% confidence interval). The five expert laboratories were informed about the type of food commodity contained in each bottle, because HPLC-based methods might need to be adapted depending on the matrix to be analysed.

The analytical methods used by the expert laboratories are summarized in Table 2. The order of these methods does not correspond to the list of expert laboratories given above. One of them analysed the test items using two different techniques, based on HG-ICP-MS and HPLC-ICP-MS (C4A and C4B), respectively.

The mean of the means provided by the expert laboratories was used, after removal of outliers, to derive the assigned values of the collaborative trial (X_{CT}), according to ISO Guide 35 (2006).

In all cases (except for S5 and S8) the expert laboratories reported values with overlapping expanded measurement uncertainties, Fig. 1A–C, E and F. The uncertainty contribution due to characterization (u_{char}) was calculated according to ISO Guide 35 (2006).

$$u_{char} = \frac{1}{p} \sqrt{\sum_{i=1}^p u_i^2} \quad (1)$$

where “ p ” refers to the number of expert laboratories used to assign the X_{CT} , while “ u_i ” is the associated combined standard measurement uncertainty reported by the experts.

Table 2

Analytical protocols, as described by the expert laboratories.

C1:

Microwave assisted extraction was used to solubilize iAs. Samples (0.35 g) were mixed with 10 mL of 1% (v/v) HNO₃ and 1% (v/v) H₂O₂ and left to stand overnight. Microwave irradiation was applied with the following temperature profile: 3 min ramp to 55 °C, 10 min at 55 °C, 2 min ramp to 75 °C, 10 min at 74 °C, 2 min ramp to 95 °C, 30 min at 95 °C. The extracts were centrifuged (10 min, 8000 rpm, 4 °C) and the supernatants filtered through a 0.22 µm filter. With the extraction procedure used, As(III) is converted to As(V), which appears as a well separated peak in the anion exchange HPLC-ICP-MS chromatogram. Therefore inorganic arsenic was measured as As(V), i.e., arsenate.

C2:

About 0.5 g of powder was weighed with a precision of 0.1 mg into 50 mL polypropylene tubes, and a solution (10 mL) of 20 mmol L⁻¹ trifluoroacetic acid containing 1% (v/v) of a 30% H₂O₂ solution was added. Samples were extracted with a GFL-1083 shaking water bath (Gesellschaft für Labortechnik, Burkwedel, Germany) at 95 °C for 60 min. After cooling to room temperature the extracts were centrifuged for 15 min at 4700 g. An aliquot of 1 mL was transferred to Eppendorf vials and centrifuged for 15 min at 8900 g. The supernatant was used directly for HPLC-ICP-MS analysis.

C3:

For the determination of iAs subsamples of approximate 0.200 g were weighed into plastic tubes and 10.00 mL of 0.1 mol L⁻¹ nitric acid (Merck) in 3% hydrogen peroxide (Merck) was added. The solutions were placed in a water bath at 90 °C for 60 min. Then the solutions were allowed to cool to room temperature and centrifuged at approximately 4000 rpm for 10 min and subsequently filtered (0.45 µm) prior to analysis. The determination of inorganic arsenic was done using anion exchange HPLC-ICP-MS. The method is currently being evaluated as a future European standard method by CEN.

C4A and C4B:

0.1 g sample (by weight) were diluted with 10 g extraction solution containing 2% (v/v) nitric acid and 3% (v/v) hydrogen peroxide in 50 mL Falcon tubes. The solution was mixed and heated (loosely capped) in a microwave oven for 50 min total (temperature program: ramp in 2 min to 50 °C, 5 min at 50 °C, ramp in 2 min to 75 °C, 5 min at 75 °C, ramp in 4 min to 95 °C, 30 min at 95 °C). The cooled solution was weighed and then centrifuged at 4200 rpm for 10 min and the supernatant separated from the residue. The supernatant for samples 4 and 8 was further diluted by a factor of 5 and sample 7 by a factor of 10 using extraction solution. The dilution of samples 4 and 8 was required due to excessive foaming of sample during hydride generation. The solutions were then analysed by (A) HG-ICP-MS and (B) HPLC-ICP-MS. Two sets of data were delivered.

C5:

The samples were accurately weighed in PTFE vessels and then extracted by adding 10 mL of 0.2% (w/v) HNO₃ and 1% (w/v) H₂O₂ solution in a microwave digestion system. The temperature was raised first to 55 °C (and held for 10 min) then to 75 °C (and held for 10 min) and finally the digest was taken up to 95 °C and maintained for 30 min. Samples were cooled to room temperature and centrifuged at 3500 rpm for 12 min. The supernatant was filtered through PET filters (pore size 0.45 µm). Arsenic speciation was carried out in the extracts by LC-ICP-MS.

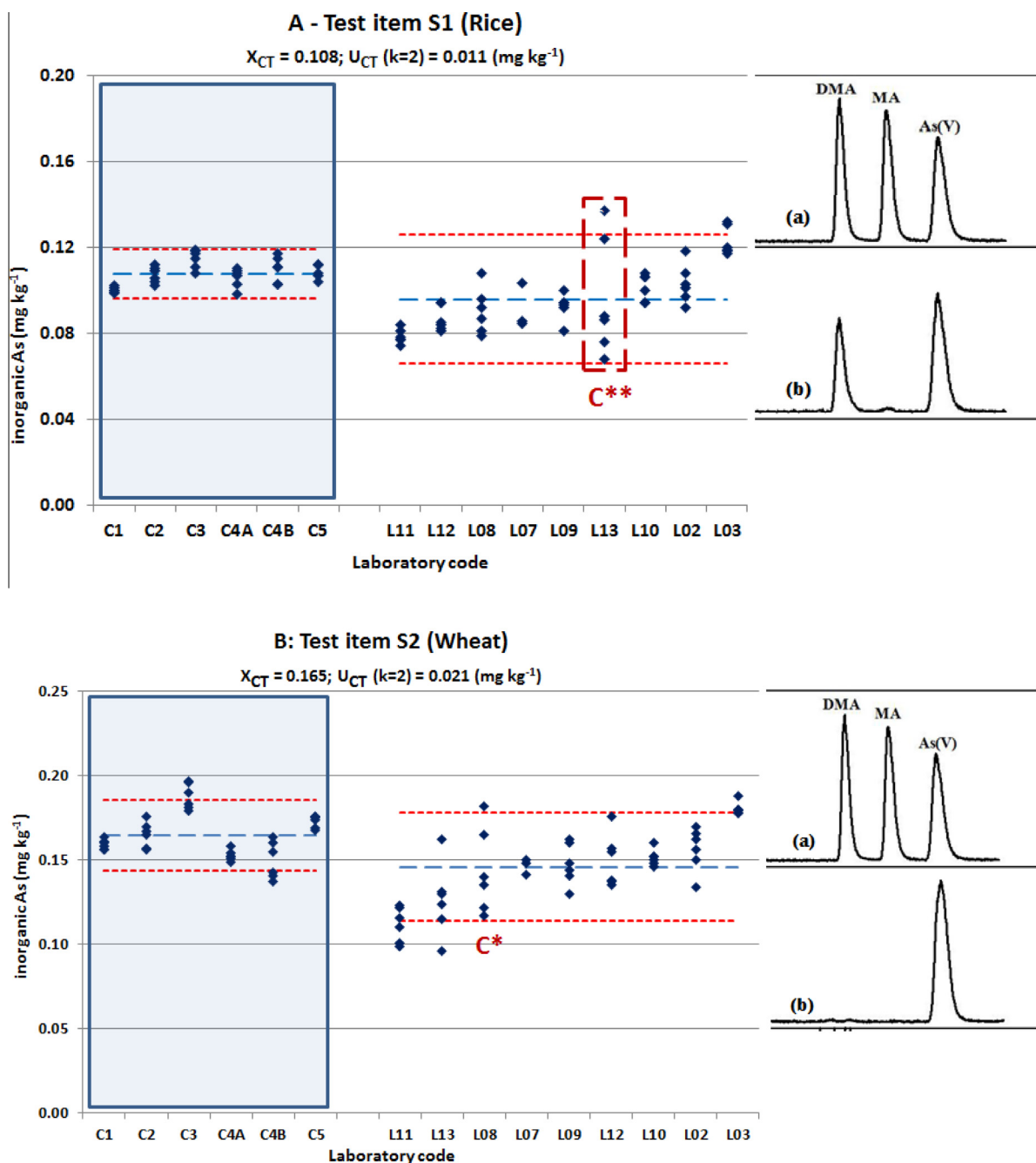


Fig. 1. Measurement results of the expert laboratories (blue frame) and the CT participants. The blue dashed line corresponds to the mean of the respective results (X_{CT} in the case of the experts) and the red dashed lines the boundaries of the mean ($X_{CT} \pm U_{CT}$ for the expert laboratories and $X_{obs} \pm 2S_R$ for the participants of the CT). C**, G** – Cochran and Grubbs outliers, C* – Cochran straggler, (a) HPLC-ICP-MS chromatogram of standards, (b) HPLC-ICP-MS chromatogram of the test item (Provided by expert C2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In the case of S5 (cabbage) and S8 (fish), expert laboratories reported values, which did not overlap within their respective expanded measurement uncertainties (Fig. 1.d and 1.g). u_{char} was then calculated according to ISO Guide 35:

$$u_{char} = \frac{s}{\sqrt{p}} \quad (2)$$

where “s” refers to the standard deviation of the means obtained by the expert laboratories.

The uncertainties of the reference values (u_{CT}) were then estimated combining the standard uncertainty of the characterization (u_{char}) with the contributions for homogeneity (u_{bb}) and stability (u_{st}) in compliance with ISO Guide 35.

$$u_{CT} = \sqrt{u_{char}^2 + u_{bb}^2 + u_{st}^2} \quad (3)$$

For S7 (seaweed), u_{CT} was provided in the NMIJ certificate. For the former IMEP test items (S1 (rice), S2 (wheat), S6 (mushroom)) u_{bb} and u_{st} were extracted from the corresponding IMEP reports to participants. As for the remaining samples (S4 (mussels), S5 (cabbage), S8 (fish)) u_{bb} and u_{st} were derived from those reported for total As by the respective CRM producers.

The assigned values and their associated expanded uncertainties (X_{CT} and $U_{CT} = 2 u_{CT}$) are presented in Table 3. In the case of sample S7 (seaweed) the reference values of the CRM were used.

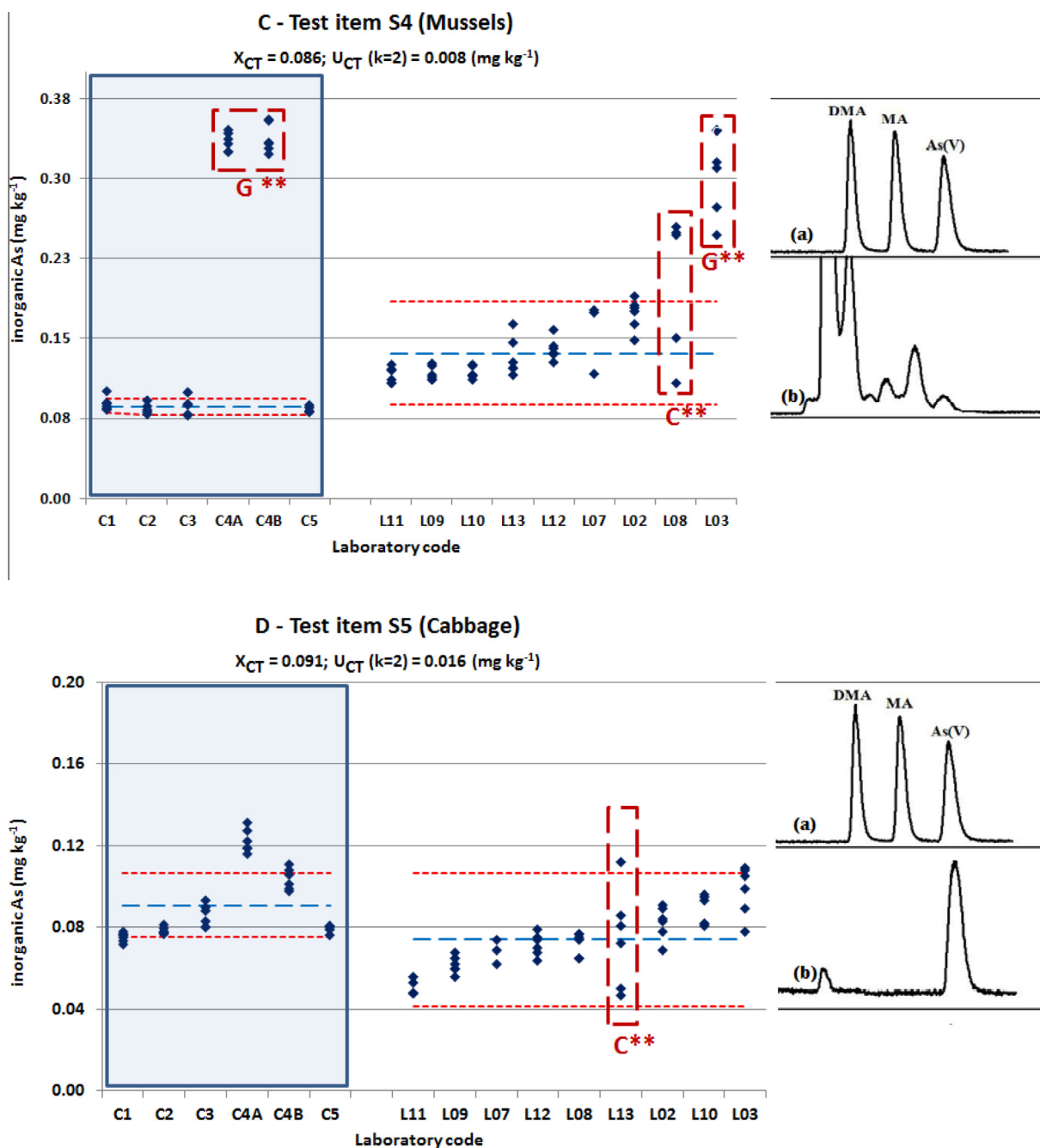


Fig. 1 (continued)

2.5. Organisation of the collaborative trial

A call for participants was published on the JRC-IRMM web site (<https://ec.europa.eu/jrc/en/interlaboratory-comparisons>) and via the network of National Reference Laboratories of the European Union Reference Laboratory for Heavy Metals in Feed and Food (EURL-HM). Thirteen laboratories from nine European countries registered to this collaborative trial. The letter accompanying the samples provided the general instructions for the participants, i.e., the measurand, type of samples, number of independent replicates required per bottle, detailed instructions on how to determine the moisture content of the test items and the description of the analytical method (SOP) to be used.

The measurand was defined as iAs in seven different food matrices. Laboratories were requested to perform three independent

measurements per bottle under repeatability conditions. This process was to be repeated on two different days (one bottle/day) following the SOP. Laboratories were informed in the letter accompanying the test item that the purpose of this collaborative trial was to evaluate the method, not the analytical capabilities of the laboratory and that the SOP needed to be followed strictly. Any deviation of the SOP had to be reported to the organisers.

L05 failed to analyse correctly the pre-test item and L04 did not report any results due to instrumentation failure. L06 modified the SOP and used ICP-MS instead of the prescribed HG-AAS; for this reason the data submitted by this laboratory were excluded from statistical calculations. Ten participants reported compliant results that were further evaluated.

Each participant received a package with sixteen bottles containing each approximately 20 g (S1 (rice) and S2 (wheat)), 8 g

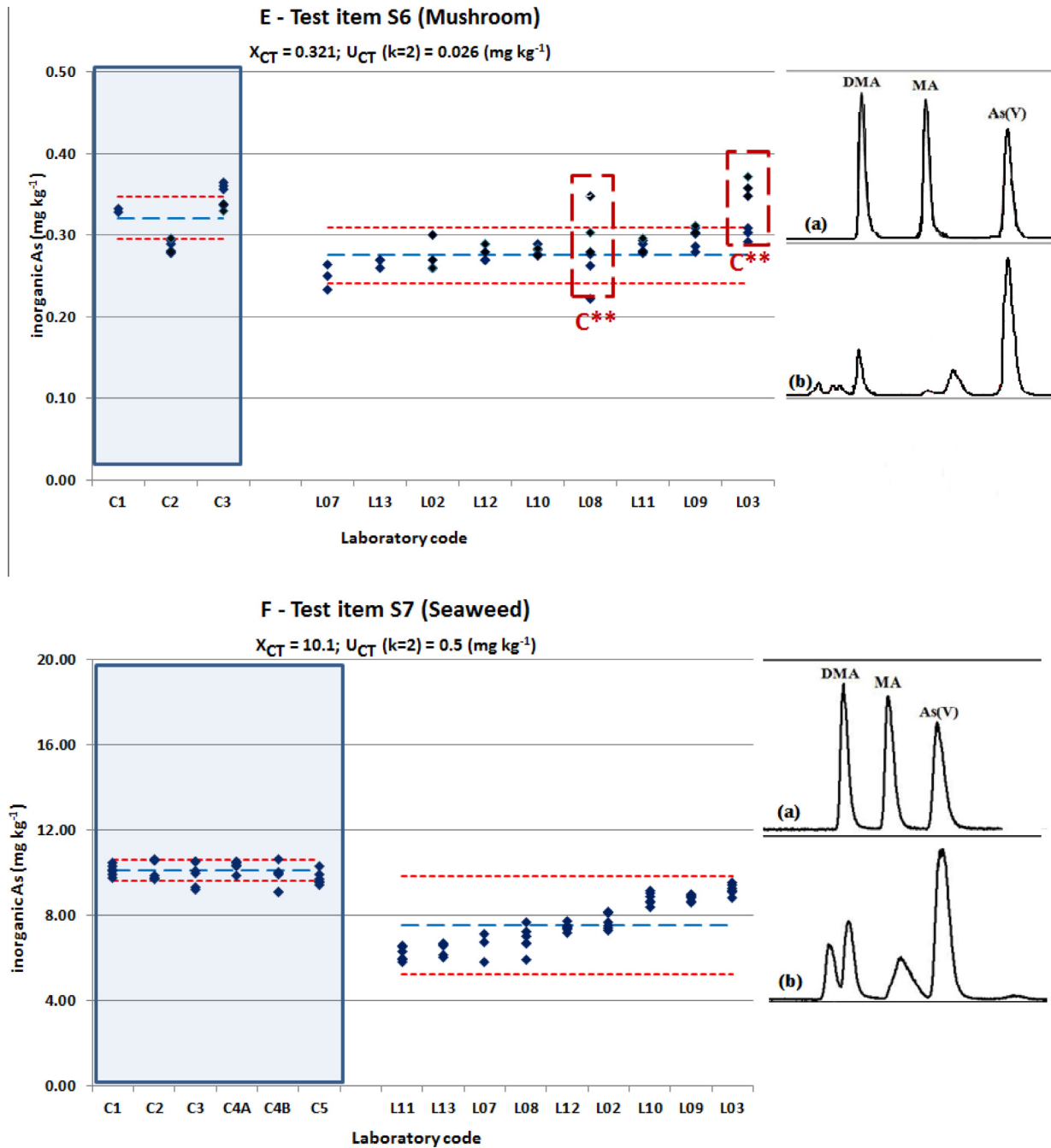


Fig. 1 (continued)

(S4 (mussels)), 5 g (S3 (rice), S5 (cabbage), S6 (mushroom), S7 (seaweed) and S8 (fish)) of the test items (two bottles from each test item), a bottle of the pre-test item (containing 15 g of material), a letter accompanying the samples, a “Confirmation of Receipts” and a copy of the SOP (Fiamegkos et al., 2015).

Dispatch was followed by the messenger's parcel tracking system on the internet. Participants received an individual code to access the on-line reporting interface, to report their measurement results and to complete the related questionnaire for collection of relevant information about the measurements and the laboratories.

2.6. Statistical analysis

The statistical evaluation of data was performed following the international standard recommendations set by ISO 5725-2

(1994). The same statistical approach was used for the evaluation of the results reported by the expert laboratories.

The following sequence of statistical tests was applied:

- Analysis of variance (ANOVA) to confirm that no statistically significant difference existed, for any of the test items, between the two individual bottles provided to the participants, analysed on different days. Since this was the case, all six replicated measurements were pooled for further calculations. This test could not be applied to the results of L07 because this laboratory analysed only one bottle on one single day.
- Check for outliers in the laboratory precision (variance) applying the Cochran test. This test compares (for each test item) the highest laboratory internal repeatability variance with the sum of reported variances from all the participants;

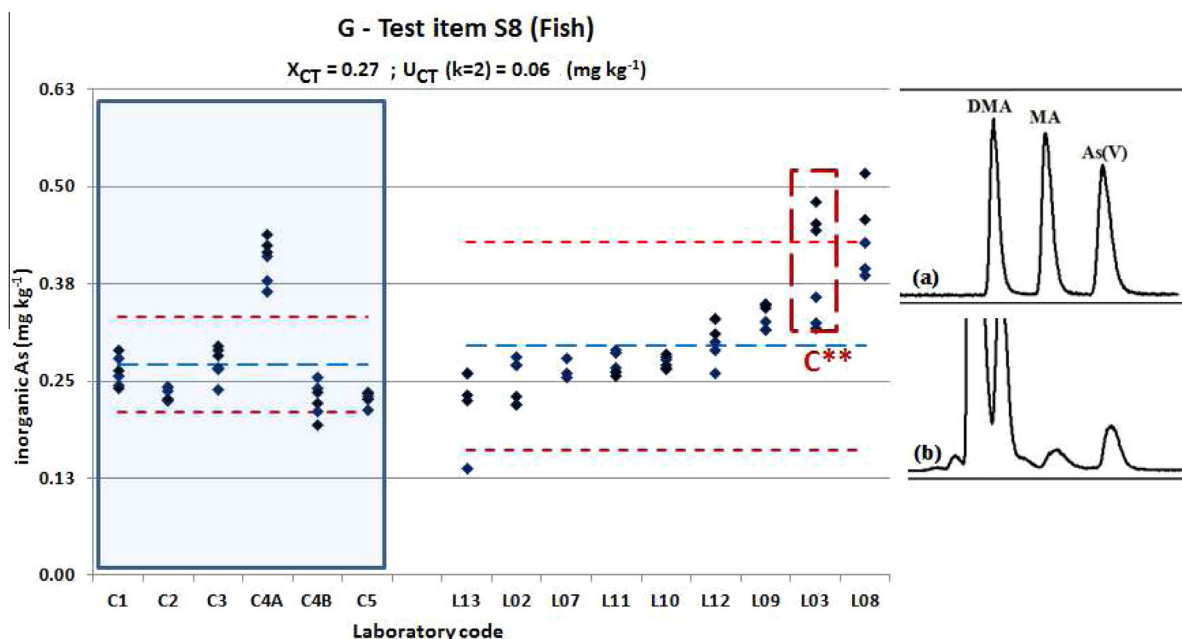


Fig. 1 (continued)

Table 3
 Method performance characteristics from the collaborative study (following ISO 5725-2:1994 [22]).

	Units	S1 – Rice (IMEP-107)	S2 – Wheat (IMEP-112)	S4 – Mussels (ERM-CE278k)	S5 – Cabbage (IAEA-359)	S6 – Mushroom (IMEP-116)	S7 – Seaweed (CRM 7405a)	S8 – Fish (DORM-4)
N° Laboratories (after outlier rejection)		8	9	6	8	7	9	8
N° Outlier Lab (test used)		1 (C)	–	1 (C), 1 (G)	1 (C)	2 (C)	–	1 (C)
N° Replicates excluded		6	–	11	6	12	–	6
Assigned value $X_{CT} \pm U_{CT} (k=2)$	mg kg ⁻¹	0.108 ± 0.011	0.165 ± 0.021	0.086 ± 0.008	0.091 ± 0.016	0.321 ± 0.026	10.1 ± 0.5	0.27 ± 0.06
Overall mean $X_{obs} \pm 2S_R$	mg kg ⁻¹	0.096 ± 0.030	0.146 ± 0.032	0.133 ± 0.048	0.074 ± 0.033	0.275 ± 0.034	7.548 ± 2.301	0.295 ± 0.134
S_r	mg kg ⁻¹	0.007	0.015	0.011	0.007	0.011	0.357	0.030
r	mg kg ⁻¹	0.021	0.041	0.032	0.020	0.031	1.001	0.085
RSD_r	%	7.8	10.1	8.6	9.6	4.1	4.7	10.3
S_R	mg kg ⁻¹	0.015	0.016	0.024	0.016	0.017	1.151	0.067
R	mg kg ⁻¹	0.042	0.045	0.068	0.046	0.047	3.222	0.188
RSD_R	%	15.6	10.9	18.2	22.1	6.1	15.2	22.8
Hor_{Rat}	%	0.71	0.52	0.83	1.02	0.32	1.29	1.18
Recovery $Rec \pm 2U_{Rec} (\sim 95\%)$	%	88.9 ± 29.4	88.7 ± 22.5	153.7 ± 57.6	81.6 ± 38.7	85.8 ± 12.6	74.7 ± 23.1	108.8 ± 55.4

C = Cochran test, G = Grubbs test (applied to laboratory means), GI = Grubbs internal test (applied to replicates within a laboratory).

- iii) Check for laboratory outliers within the series of independent replicates applying the Grubbs-internal test (repeatability). This test is of particular relevance for laboratories being flagged as stragglers by the Cochran test;
- iv) Check for outliers in the laboratory mean applying the Grubbs test. This test checks for laboratory means deviating significantly from the overall mean (X_{obs}) calculated from data reported by all participants.

3. Results and evaluation

3.1. Method performance assessment

Trueness and precision of the method were estimated after identification and rejection (when applicable) of outliers. Table 4 provides an overview of the identified outliers for all test items.

According to ISO 5725 outlying results should be investigated and rejected only when an explanation is found for their anomaly.

Table 4

List of identified outliers for the different matrices. **Notation in the figures.

Sample	Laboratory (number of outlying results)	Outlier type**
S1 – Rice (IMEP-107)	L13 (6)	Cochran
S4 – Mussels (ERM-CE278 k)	C4A (6)	Grubbs
	C4B (6)	Grubbs
	L08 (5)	Cochran
	L03 (6)	Grubbs
S5 – Cabbage (IAEA-359)	L13 (6)	Cochran
S6 – Mushroom (IMEP-116)	L08 (6)	Cochran
	L03 (6)	Cochran
S8 – Fish (DORM-4)	L03 (6)	Cochran

Results should not be discarded only on the basis of statistical analysis.

Only the results reported by L03 for S4 (mussels) were flagged as Grubbs outliers; all the others were Cochran outliers. The

comments made by the laboratories in the questionnaire were scrutinised to understand the discrepancies of the results reported for some of the test items.

- L01 mentioned at the time of its registration that the instrument to be used for the analysis was old. It was not equipped with a flow injection system and it needed to be operated in the batch mode. The laboratory was not sure about the quality of the results that could be obtained with this instrument. The very large scatter of reported results for all matrices confirmed the laboratory's concern. Hence, the results of L01 were not included in the statistical evaluation.
- The results reported by L07 for S4 (mussels) were identified as Cochran outliers despite having an internal repeatability variance comparable to that of other sets of data. This mathematical artefact was due to the fact that the laboratory analysed only one bottle on one single day, thus having less degrees of freedom. It was therefore decided to retain these results for further statistical evaluation.
- L03 did not filter the chloroform phase after the first extraction. Filtering the chloroform phase is a crucial clean-up step necessary to avoid any traces of the HCl initially used to extract all As species from the matrix (cf. Point 9.3 of the SOP) (Fiamegkos et al., 2015). Residues of the concentrated HCl in the chloroform phase may introduce a high contamination with organic arsenic species. Such a contamination would be particularly important in samples in which iAs represents a small fraction of the total As, as it is frequently the case in samples of marine origin. This could explain the high values reported by L03 for S4 (mussels) as well as the high dispersion of data for S8 (fish) and S6 (mushroom) (in this sample about half of the total As mass fraction corresponds to organic compounds (Cordeiro et al., 2013) because the contamination is not necessarily constant in the different replicates. L12, which did not filter the chloroform phase was not flagged as outlier for any of the test items, proving that sound results can still be obtained when the organic phase is carefully sampled. Therefore, L03 was excluded from the statistical evaluation only when the results were flagged as outliers.

L08 reported having many problems with S4 (mussels) and S8 (fish), while L13 had problems with S5 (cabbage) and S8 due to the formation of emulsion during the back extraction from chloroform into 1 mol L⁻¹ HCl (point 9.4 of the SOP). Laboratory L02 did not apply the final filtration step 9.5.7 of the SOP which did not have a significant influence on the reported results.

Regarding the results reported by the expert laboratories it is interesting to mention that the results obtained by HG-ICP-MS (C4A) for S4 (mussels), S5 (cabbage) and S8 (fish) were not in agreement with the results reported by the experts using HPLC-ICP-MS within their respective expanded measurement uncertainties (corresponding to a 95% confidence level). Nevertheless, only the results reported by C4 (for both methods) for S4 were flagged as Grubbs outliers. In addition the expert laboratory reported that when analysing S4 and S8 the extracts had to be diluted to avoid formation of foam during hydride generation. Foam generation during HG-ICP-MS was most likely resulting from the high protein content of these samples. Extraction of samples using 2% (v/v) nitric acid and 3% hydrogen peroxide does not destroy the protein matrix and since no further purification of the solutions took place solubilised proteins can react with sodium borohydride leading to strong foam formation.

All the remaining measurement results were used to evaluate the trueness and precision of the method under validation. Table 3 provides for each sample:

- the number of laboratories used to assess the performance characteristics of the method (after outlier exclusion);
- the number of outlier laboratories and replicates;
- the assigned values and associated expanded measurement uncertainties (X_{CT} , U_{CT});
- the overall observed mean (after the outlier rejection, X_{obs}) and their respective expanded uncertainty, expressed as the reproducibility standard deviation (S_R) multiplied by a coverage factor of 2, to approximate a 95% confidence interval;
- the repeatability standard deviation (S_r) the repeatability limit r (computed as 2.8 S_r) and the repeatability relative standard deviation, or within-laboratory variability, (RSD_r);
- the reproducibility standard deviation (S_R), the reproducibility limit R (computed as 2.8 S_R) and the RSD_R ;
- the Horwitz ratio (HorRat) expressed as the ratio between the observed RSD_R value divided by the predicted reproducibility relative standard deviation ($PRSD_R$) value calculated from the Horwitz equation (Thompson, 2000); and
- the overall analytical recovery R , is calculated as:

$$R = 100 \frac{X_{obs}}{X_{CT}} \quad (4)$$

while the associated uncertainty (u_R) is estimated as [29]:

$$u_R = R \sqrt{\left(\frac{u_{obs}}{X_{obs}}\right)^2 + \left(\frac{u_{CT}}{X_{CT}}\right)^2} \quad (5)$$

Where: u_{obs} is the estimated standard deviation under reproducibility conditions (S_R),

u_{CT} is the standard uncertainty associated to the X_{CT} .

No statistically significant difference could be identified between the overall observed mean and the assigned values for all test items when taking into account the estimated expanded uncertainty of the analytical recovery ($2u_R$, to approximate the 95% confidence interval). Therefore, no significant bias could be identified for the matrices investigated.

Consequently, the method is considered fit for its intended purpose, since the HorRat ratios are below 2 in all cases.

No significant difference was observed for the two identical samples (S1 and S3, both rice), where the following ranges (expressed as $X_{obs} \pm 2 S_R$) were obtained: 0.096 ± 0.030 mg kg⁻¹ for S1 and 0.089 ± 0.022 mg kg⁻¹ for S3.

3.2. Degree of difficulty in the determination of iAs mass fraction in different types of matrices

An evaluation of the results and of the comments reported by the participants in IMEP-41 on the method under validation, and by the expert laboratories using the method of their choice, made it possible to extract some conclusions about the inherent difficulty of iAs determination in different types of matrices. Two major clusters could be identified: 1) matrices of marine origin, and 2) matrices of non-marine origin.

3.2.1. Matrices of marine origin

The selective determination of iAs seems to be particularly challenging in food of marine origin: mussels (S4), seaweed (S7) and fish (S8). In those samples iAs represents only a small fraction of the total As mass fraction (Fig. 1C, F and G). Samples of marine origin contain often a very large number of different As-species, some of which may form also volatile hydrides which can interfere with the determination of iAs by HG-AAS or HG-ICP-MS, and for HPLC-ICP-MS there is always the risk of co-eluting species. This is particularly true in the case of S4 (mussels) and S8 (fish), where the iAs

mass fractions ($0.086 \pm 0.008 \text{ mg kg}^{-1}$ and $0.27 \pm 0.06 \text{ mg kg}^{-1}$, respectively), represent 1 and 4% of the total As mass fraction ($6.7 \pm 0.4 \text{ mg kg}^{-1}$ in S4 and $6.80 \pm 0.64 \text{ mg kg}^{-1}$ in S8). Seaweeds (S7) typically also contain high levels of several organic arsenic species, but in this sample the iAs mass fraction ($10.1 \pm 0.5 \text{ mg kg}^{-1}$) represents 28% of the total As mass fraction ($35.8 \pm 0.9 \text{ mg kg}^{-1}$).

As discussed before, several laboratories (L03, L08 and L13) had problems with the analysis of S4 (mussels) and/or S8 (fish), and reported results which were either biased or characterised by a large dispersion. The same difficulties were observed in the population of expert laboratories although only the results reported by expert C4 (for both methods: HG-ICP-MS and HPLC-ICP-MS) for S4, were flagged as Grubbs outliers. This expert reported that “S4 contains an organic As-compound eluting very near to As(V), which may co-elute with As(V) depending on column conditions used”. If that compound could also generate the hydride, it would explain the results obtained by this laboratory for S4, using the two different techniques, for which the results are in good agreement.

The results reported for S4 (mussels) by the participants in IMEP-41 using the method under validation are systematically higher than the assigned value, although still overlapping with it within their respective expanded uncertainties. Bivalves are known to contain MA (Schoof & Yager, 2007). In the samples analysed by Muñoz et al. (2000), MA did not exceed 0.4% of the total As mass fraction in any sample. The content of MA in S4 (data provided by expert C2) is $0.183 \pm 0.003 \text{ mg kg}^{-1}$ (expressed as As), corresponding to 3% of total As. The MA mass fraction in this test item is therefore high and could explain the results reported for iAs in IMEP-41, because as indicated before, MA can interfere in the determination of iAs in the method being validated. The high content of MA in S4 could also be a feasible explanation for the high result reported by C4 using HG-ICP-MS but not the result obtained using HPLC-ICP-MS because this expert laboratory confirmed that co-elution of MA and As(V) did not occur under the conditions used. The high value obtained by C4 for S4 must be due to the interference of some unknown As species.

However, since: 1) the method under validation does not perform differently than other methods, 2) the reference value, calculated out of the values reported by experts, is included in the range covered by the results obtained by the participants in the collaborative trial (95% confidence interval) and 3) the HorRat value for this matrix (0.83) falls in the accepted range 0.5–2, we consider that the method is fit for the purpose of determining iAs in bivalves. In addition the validated method does not imply interpretation of the complex chromatograms obtained by HPLC-ICP-MS where the inorganic arsenic peak could be hidden/enhanced if proper separation is not achieved.

As mentioned before, some laboratories encountered also some problems in the determination of iAs in S8 (fish), due to the formation of an emulsion. Also expert C4 reported that “S8 produced highly divergent results between HPLC and HG-ICP. The samples have been done several times with the same results; the reason for this is not clear”. Indeed, the results obtained by C4 using HG-ICP-MS (C4A) were twice as high as the result obtained by the same laboratory using HPLC-ICP-MS (C4B), and were not in agreement with any of the results reported using HPLC-ICP-MS, within their respective uncertainties. Nevertheless, C4A was not flagged as an outlier for S8. Although the mean of the results reported for S8 by the participants in IMEP-41 is in good agreement with the assigned value, it has to be mentioned that the standard uncertainties associated to the assigned value and to the mean of the participant's results for S8 are the largest among all the matrices included in the validation (Table 3) showing the difficulties experienced with the analysis of this specific sample.

The results reported for S7 (seaweed, *Sargassum fusiforme*, syn. *hizikia-fusiforme*) by the two populations, experts and participants in the collaborative trial, deserve some in-depth discussion. There is quite a good agreement within each of the two populations, being the standard uncertainties associated to the assigned value and to the mean of results reported by the participants 2.5 and 15%, respectively. However, the recovery obtained for S7 with the method under validation is the lowest among all the test items: 75%. Several arguments can be provided to try to explain this fact:

- Due to the high iAs mass fraction in S7 (seaweed), about two orders of magnitude higher than those in the other test items, laboratories had to dilute the final extract (1:4 to 1:25 dilution factors were applied) to be able to use the calibration curve constructed following the SOP, introducing in this way an additional error in the final calculation. The dilution bias might be caused by a systematic dilution error, by a change in the matrix effect in the diluted extract and/or by subtraction of a reagent contribution to the blank without taking into consideration the dilution factor.
- Arsenosugars are the major arsenic compounds in marine algae (Schmeisser et al., 2004). Hijiki contains about 50% arsenosugars which can be changed or completely destroyed by heating or acid treatment (Narukawa et al., 2012). S7 (seaweed) is a certified reference material in which the As(V) mass fraction has been certified on the basis of results obtained with HPLC-ICP-MS and ion chromatography (IC)-ICP-MS, using two different extraction methods and with water as extractant: ultrasonication (for 1 h) and microwave assistance (for 30 min), in both cases at 60 °C. Under those conditions and according to the CRM producers (Narukawa et al., 2012), arsenosugars would not be changed or destroyed, what would have resulted in an overestimation of iAs. In the method being validated in IMEP-41, the extraction is based on: 1) solubilisation of the protein matrix with 6 mol L^{-1} HCl at room temperature shaking for 15 min with a mechanical shaker and leaving then the mixture to rest for 12–15 h, 2) subsequent extraction with chloroform of the iAs present in the acid medium, shaking for 5 min with a mechanical shaker.
- In the method being validated only extracted species capable of generating hydrides would be detected using atomic absorption spectrometry, contrary to what would happen when using ICP-MS. The high temperatures reached in the ICP torch would atomise and ionise any arsenic species (including the organic species, such as arsenosugars), which under certain chromatographic conditions could co-elute with As(V). In the same paper the authors succeeded to generate volatile arsenic hydride from arsenosugars, although the mechanism of reaction could not be clarified.

The chance that the results obtained by all expert laboratories working under different extraction conditions would have been affected by the same interference or by inter-conversion of species with the same extent is rather low. For this reason the explanation provided in the first bullet point (dilution necessary) seems more plausible.

3.2.2. Matrices of non-marine origin

Four test items of non-marine origin were included in this collaborative trial, namely plants/funghi: Rice (S1), wheat (S2), cabbage (S5) and mushrooms (S6).

The simplest matrix regarding distribution pattern of arsenic species, was wheat (S2) where only iAs was detected. In the rice test item (S1) the major arsenic species was iAs, followed by DMA and some traces of MA. The pattern was slightly more

complex in mushrooms (S6) where not only iAs, DMA and MA were present but also some other non-identified As compounds.

The more challenging matrix in the group of non-marine test items was cabbage (S5): in the chromatogram obtained by expert C2 for this test item (Fig. 1.D) two peaks can be observed, one corresponding to iAs and a second non-identified compound. The dispersion of results reported for S5 was the second largest after fish (S8) for both populations, expert laboratories and participants in IMEP-41, with 17.6% and 44.6% expanded standard uncertainties associated to the assigned values and to the overall mean of the collaborative trial, respectively. Very likely the non-identified arsenic species is able to generate a volatile hydride to some extent, which would explain the results obtained by expert C4 using HG-ICP-MS. Although not flagged as outlier, those results (C4A) do not overlap with any of the results obtained by the expert labs when using HPLC-ICP-MS. Also L13 reported problems with S5 due to the formation of an emulsion during the back extraction from chloroform into diluted HCl, which would explain the large dispersion of results reported by L13.

The determination of iAs in cereals seems to be more straightforward than in other food of plant origin and for these sample types good agreement between the results obtained by expert laboratories and participants was obtained.

4. Conclusions

The trueness and precision of a method for the determination of iAs in a broad range of food commodities has been assessed by means of a collaborative trial. The method does not imply the use of sophisticated/expensive instrumentation and can be implemented, even in challenging matrices. Marine matrices were particularly challenging although the requirements of international guidelines for validation of methods were met. For instance problems in the determination of iAs in bivalves were encountered by participants when using the method under validation and by one of the experts applying two different methods: direct hydride generation –ICP-MS and by HPLC-ICP-MS. In this sense it could be questioned if it is possible to determine iAs in bivalves using any analytical approach because unknown arsenic species may interfere depending on the conditions and method of analysis used.

The proposed method can be used to monitor iAs in food and helps in providing more data on the fraction of As with the highest toxicity in the human diet. Such data are strongly needed for refining risk assessment of human dietary exposure to iAs.

The main drawback of the method is that it implies the use of such an organic solvent as chloroform.

Moreover, this exercise, including the results reported by the participants of the collaborative trial and by expert laboratories using HPLC-ICP-MS based methods, reveals the difficulty of determining iAs in food of marine origin and that any method to be used for that purpose needs to be properly validated and/or implemented by the control laboratories.

References

- BS EN-16278 (2012). *Animal feeding stuffs – Determination of inorganic arsenic by hydride generation atomic absorption spectrometry (HG-AAS) after microwave extraction and separation by solid phase extraction (SPE)*. European Committee for Standardization.
- Commission Regulation (2006). (EC) 1881/2006 setting maximum levels for certain contaminants in foodstuffs, issued by the European Commission. Official Journal of the European Union, L364/5.
- Cordeiro, F., Robouch, P., Emteborg, H., Seghers, J., Fiampegkos, I., Cizek-Stroh, A., & de la Calle, B. (2013). IMEP-116: Determination of total cadmium, lead, arsenic, mercury and inorganic arsenic in mushrooms. JRC Scientific and Policy Reports. EUR 26214.
- D'Amato, M., Turrini, A., Aureli, F., Moracci, G., Raggi, A., Chiaravalle, E., Mangiacotti, M., ... Cubadda, F. (2013). Dietary exposure to trace elements and radionuclides: The methodology of the Italian Total Diet Study 2012–2014. *Annali dell Istituto Superiore di Sanita*, 49(3), 272–278.
- de la Calle, M. B., Baer, I., Robouch, P., Cordeiro, F., Emteborg, H., Baxter, M. J., Brereton, N., ... Cubadda, F. (2012). Is it possible to agree on a value for inorganic arsenic in food? The outcome of IMEP-112. *Analytical and Bioanalytical Chemistry*, 404, 2475–2488.
- EFSA (2014). Dietary exposure to inorganic arsenic in the European population. *EFSA Journal*, 12(3), 3597–3665.
- EN 15517 (2008). *Determination of trace elements – Determination of inorganic As in seaweed by hydride generation atomic absorption spectrometry (HG-AAS) after acid digestion*.
- EPA (2010). Toxicological review of inorganic arsenic in support of summary information on the integrated risk information system (IRIS) (Draft) EPA/635/R-10/001 <http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=219111:575>. Accessed at: .
- FAO JECFA MONOGRAPHS 8 (2011). *Safety evaluation of certain contaminants in food. WHO – Food Additives Series: 63*. Geneva: World Health Organization.
- FDA (2004). *Total diet study statistics on element results, revision 2, market baskets 1991-3 through 2002-4*.
- Fiampegkos, I., Cordeiro, F., Devesa, V., Vélez, D., Robouch, P., Emteborg, H., Leys, H., Cizek-Stroh, A., & de la Calle, B. (2015). IMEP-41: Determination of inorganic As in food. Collaborative Trial Report. JRC Technical Reports. JRC94325.
- Francesconi, K. A., & Kuehnelt, D. (2004). Determination of arsenic species: A critical review of methods and applications, 2000–2003. *Analyst*, 129(5), 373–395.
- GB/T5009.11 (2003). *Determination of total arsenic and abio-arsenic in foods*.
- Hajeb, P., Sloth, J. J., Shakibazadeh, S., Mahyudin, N. A., & Afsah-Hejri, L. (2014). Toxic elements in food: Occurrence, binding, and reduction approaches. *Comprehensive Reviews in Food Science and Food Safety*, 13(4), 457–472. <<https://ec.europa.eu/jrc/en/interlaboratory-comparisons>> (Accessed on 31-10-2015).
- Hughes, M. F., Beck, B. D., Chen, Y., Lewis, A. S., & Thomas, D. J. (2011). Arsenic exposure and toxicology: a historical perspective. *Toxicological Sciences*, 123(2), 305–332.
- ISO 5725-2 (1994). *Accuracy (trueness and precision) of measurement methods and results – Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*. Geneva: ISO.
- ISO Guide 35 (2006). *Reference Materials – General and statistical principles for certification, issued*. Geneva (CH): ISO.
- Lynch, H. N., Greenberg, G. I., Pollock, M. C., & Lewis, A. S. (2014). A comprehensive evaluation of inorganic arsenic in food and considerations for dietary intake analyses. *Science of the Total Environment*, 496, 299–313.
- Maher, W., Krikowa, F., Ellwood, M., Foster, S., Jagtap, R., & Raber, G. (2012). Overview of hyphenated techniques using an ICP-MS detector with an emphasis on extraction techniques for measurement of metalloids by HPLC-ICPMS. *Microchemical Journal*, 105, 15–31.
- Muñoz, O., Devesa, V., Suárez, M. A., Vélez, D., Montoro, R., Urieta, I., Macho, M. L., & Jalón, M. (2000). Total and inorganic arsenic in fresh and processed fish products. *Journal of Agriculture and Food Chemistry*, 48, 4369–4376.
- Muñoz, O., Vélez, D., & Montoro, R. (1999). Optimization of the solubilization, extraction and determination of inorganic arsenic [As(III) + As(V)] in seafood products by acid digestion, solvent extraction and hydride generation atomic absorption spectrometry. *Analyst*, 124(4), 601–607.
- Musil, S., Pétursdóttir, A. H., Raab, A., Gunnlaugsdóttir, H., Krupp, E., & Feldmann, J. (2014). Speciation without chromatography using selective hydride generation: Inorganic arsenic in rice and samples of marine origin. *Analytical Chemistry*, 86(2), 993–999.
- Narukawa, T., Inagaki, K., Zhu, Y., Kuroiwa, T., Narushima, I., Chiba, K., & Hioki, A. (2012). Preparation and certification of hijiki reference material, NMIJ CRM 7405-a, from the edible marine algae hijiki (Hizikia fusiforme). *Analytical and Bioanalytical Chemistry*, 402, 1713–1722.
- National Research Council (1999). *Arsenic in Drinking Water Sub-committee on Arsenic in Drinking Water*. National Academy Press National Research Council (NRC). 310.
- National Research Council (2013). *Critical aspects of EPA's IRIS assessment of inorganic arsenic: interim report*. Division on Earth and Life Studies, Board on Environmental Studies and Toxicology Committee on Inorganic Arsenic. National Academies Press.
- Pétursdóttir, Á. H., Gunnlaugsdóttir, H., Krupp, E. M., & Feldmann, J. (2014). Inorganic arsenic in seafood: Does the extraction method matter? *Food Chemistry*, 150, 353–359.
- Rasmussen, R. R., Hedegaard, R. S. V., Larsen, E. H., & Sloth, J. J. (2012). Development and validation of an SPE HG-AAS method for determination of inorganic arsenic in samples of marine origin. *Analytical and Bioanalytical Chemistry*, 403(10), 2825–2834.
- Rasmussen, R. R., Qian, Y., & Sloth, J. J. (2013). SPE HG-AAS method for the determination of inorganic arsenic in rice – Results from method validation studies and a survey on rice products. *Analytical and Bioanalytical Chemistry*, 405(24), 7851–7857.
- Schmeisser, E., Goessler, W., Kienzl, N., & Francesconi, K. (2004). Volatile analytes formed from arsenosugars: determination by HPLC-HG-ICPMS and implications for arsenic speciation analyses. *Analytical Chemistry*, 76, 418–423.
- Schoof, R. A., & Yager, J. W. (2007). Variation of total and speciated arsenic in commonly consumed fish and seafood. *Human and Ecological Risk Assessment: An International Journal*, 13, 946–965.
- Sloth, J. J., Cordeiro, F., Rasmussen, R. R., Hedegaard, R. V., Emteborg, H., Verbist, I., Danier, J., & de la Calle, M. B. (2011). *Determination of inorganic arsenic in animal feed of marine origin*. JRC Scientific and Technical Reports. JRC 66416.

- Thompson, M. (2000). Recent trends in inter-laboratory precision at ppb and sub-ppb concentrations in relation to fitness for purpose criteria in proficiency testing. *Analyst*, 125, 385–386.
- U.S. Department of Health and human services, Public Health Service, Agency for Toxic Substances and Disease Registry (2007). *Toxicological Profile for Arsenic (Final)*, 559.
- Vieira, M. A., Grinberg, P., Bobeda, C. R. R., Reyes, M. N. M., Campos, R. C., Dedina, J., & Tsalev, D. L. (2009). Non-chromatographic atomic spectrometric methods in speciation analysis: A review. *Spectrochimica Acta – Part B Atomic Spectroscopy*, 64(6), 459–476.
- WHO (2011). *Evaluation of certain contaminants in food. Seventy-second report of the joint FAO/WHO expert committee on food additives.*