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Determination of inorganic As, DMA and MMA in marine and terrestrial tissue samples: a consensus extraction approach

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Environmental context. Arsenic can be found in all environmental compartments in a large number of chemical forms of varying toxicity. We performed an inter-laboratory comparison study focusing on quantitation of some of the most toxic arsenic forms in seven different biological materials and found very good agreement among the submitted results. Certification of the studied materials will provide suitable quality control samples for environmentally relevant concentrations of arsenic in food products and biota.

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ABSTRACT

Rationale. Arsenic (As) speciation analysis in biological matrices has been performed for several decades; however, there are very few matrix certified reference materials available for the validation of analytical methods. The literature data on the mass fractions of As species in the existing certified reference materials are inconsistent and suggest method extraction dependency. **Methodology.** In the present study, an international round-robin study was organised to identify the possible sources of discrepancies in quantitation of several As species in different matrices of biological reference materials: one plant tissue, three marine and three terrestrial biological tissues. Each participating laboratory was provided with a set of identical calibration standard solutions, and analysed the samples by following a common group extraction method as well as using an in-house protocol. **Results.** The results showed that significant biases can be introduced by insufficient verification of the analyte's mass fractions in the calibration standard solutions. The choice of extraction method seems to have very little impact on the quantitation of As species in the studied plant and terrestrial biological tissues. However, following a prescribed extraction method led to significant reduction of uncertainties in more complex samples such as marine animal tissues. **Discussion.** Some differences in the mass fractions of As species extracted from marine animal tissues in water (with and without H_2O_2) were observed and should be further investigated. Despite the variety of extraction methods used, very good agreement between reported mass fractions was achieved and the combined consensus values will be published as certified reference values of As species in the studied materials.

Keywords: Arsenic speciation, certified reference material, extraction method, high performance liquid chromatography, hydride generation, inorganic arsenic, marine tissue, mass spectrometry.

Introduction

The toxicity of As depends on its oxidation state but also whether it is present in its organic or inorganic chemical forms. In general, trivalent arsenic compounds are more toxic than pentavalent forms, and inorganic species are more toxic than organic species (Hughes 2002). However, a few studies have observed that trivalent monomethylarsonous acid (MMA^{III}) exhibits higher cytotoxicity than trivalent inorganic As (iAs) (Petrick *et al.* 2000; Styblo *et al.* 2000). Within the group of organoarsenic species, the toxicity generally decreases with increasing degree of methylation (Leermakers *et al.* 2006).

For instance, although still potentially carcinogenic, studies have suggested the toxicity of pentavalent dimethylarsinic acid (DMA^V) is lower than pentavalent monomethylarsonic acid (MMA^V) (Irvine *et al.* 2006). Organic compounds such as arsenobetaine (AsBet), arsenocholine (AsChol) and As containing sugars (arsenosugars) are considered non-toxic (Kaise *et al.* 1985; Francesconi 2010; Luvonga *et al.* 2020); however, some arseno-lipids (As-lipids), e.g. arseno-hydrocarbons, have shown considerable cytotoxicity and an ability to cross the blood–brain barrier in some studies (Meyer *et al.* 2014*a*, 2014*b*; Müller *et al.* 2018).

The current regulations of maximum levels of As in food are trying to address this complexity by targeting specific As species. For instance, the maximum allowable level of As in rice was changed from total As to inorganic arsenic (iAs (sum of As^{III} and As^V)) levels (EU 2015; USFDA 2016*b*; Health Canada 2021), which have been shown to vary greatly among rice samples surveyed in the literature (Bralatei *et al.* 2015; USFDA 2016*a*; Menon *et al.* 2020). However, in order for regulatory maximum levels to be established for foods, laboratories must have the capability to measure the contaminant accurately. For this purpose, several proficiency test and round-robin studies for iAs in rice were organised (de la Calle *et al.* 2012; Pétursdóttir *et al.* 2014*a*; Cordeiro *et al.* 2016). Depending on the specific design of the study, the general purpose of a round-robin study is to determine the reproducibility of a tested method and/or verify a new analytical method through an interlaboratory comparison. The consistency in the reported values of iAs in rice studies led to the conclusion that quantitation of iAs in rice is achievable, independent of the analytical methods evaluated, thus regulation of iAs is feasible (de la Calle *et al.* 2011). However, the quantitation of iAs in other foodstuffs, such as seafood, is proving to be more challenging (Baer *et al.* 2011; Fiamegkos *et al.* 2016). While As in rice is usually distributed between DMA and iAs, marine animal tissues and algae have been shown to contain larger numbers of organic As species.

Taking into consideration the differences in the toxic properties of organic and inorganic As species, method extraction efficiencies, chromatographic recoveries and species interconversion may complicate the quantitation of iAs in biological matrices. Matrix certified reference materials (CRMs) can serve as powerful quality control tools and are commonly used for accuracy evaluation of analytical methods. Although a large number of CRMs are available on the market for total As content, very few are certified for As species other than AsBet. The limited choices consist primarily of plant tissue matrices such as rice and seaweed. There are no available CRMs with certified iAs in animal tissue, whether it is of a terrestrial or marine origin. In such instances, speciation data on CRMs published in the peer-reviewed literature are often used as information values. However, the published data of major As species (MMA, DMA and iAs) showed very little agreement between the values in dogfish muscle (DORM-2), lobster hepatopancreas (TORT-2) and fish protein (DOLT-4) CRMs from the National Research Council Canada (NRC). For example, in DORM-2, the reported values ($mg\,kg^{-1}$) of iAs ranged between 0.004 ± 0.001 (Wang *et al.* 2007) and 0.64 ± 0.49 (Hirata *et al.* 2006), DMA between 0.16 ± 0.03 (Karthikeyan and Hirata 2004) and 0.66 ± 0.04 (Pizarro *et al.* 2003) and MMA between 0.015 ± 0.004 (Wahlen *et al.* 2004) and 0.31 ± 0.12 (Karthikeyan and Hirata 2004). Similar inconsistencies have been reported for iAs in TORT-2 (ranging between 0.186 ± 0.03 and $4.46 \pm 0.03\,mg\,kg^{-1}$) (Pétursdóttir *et al.* 2014*b*) and DOLT-4 (ranging between 0.011 ± 0.002 and $0.253 \pm 0.019\,mg\,kg^{-1}$) (Llorente-Mirandes *et al.* 2017). Clearly, there is a large inconsistency in the published values of As species in the selected CRMs and they should not be used as indicators of trueness of the results without rigorous verification.

The aim of the present study was to identify possible sources of discrepancies in measured mass fractions of iAs, DMA and MMA in different matrix CRMs (one plant tissue, three marine and three terrestrial biological tissues) from the NRC and define a simple and reproducible extraction protocol for several As species. The CRMs were certified for total As but not for As species. For this purpose, a round-robin study was designed with an international team of researchers in As speciation analysis from eight laboratories.

Experimental

Round-robin campaign design

NRC Canada supplied each laboratory with existing NRC CRMs, i.e. three marine biological tissues [DORM-5 (fish protein) (Grinberg *et al.* 2021), DOLT-5 (dogfish liver) (Yang *et al.* 2014) and TORT-3 (lobster hepatopancreas) (Willie *et al.* 2013)], one plant material [CAME-1 (canola meal – *Brassica napus*) (Grinberg *et al.* 2023*a*)] and three terrestrial biological tissues [KRIK-1 (cricket powder – *Gryllodes sigillatus*) (LeBlanc *et al.* 2023*a*), BFLY-1 (de-fatted black soldier fly larvae, *Hermetia illucens*) (Grinberg *et al.* 2023*b*) and VORM-1 (mealworm meal – *Tenebrio molitor*) (LeBlanc *et al.* 2023*b*)]. Besides the CRMs, NRC also provided, to each laboratory, standard solutions of MMA, DMA and As^V of an accurate determined concentration, as described later. Samples were extracted using a common extraction method selected by the participating researchers, called the group extraction method (GEM), and each laboratory was also asked to use their in-house extraction method. Each laboratory used the analytical instrument of their choice for the As species measurement.

Dry weight determination

Accurately weighed 0.5 g aliquots of each CRM were placed in a vacuum oven in triplicate, and the oven was subsequently evacuated and maintained below 25 mm Hg to dry

the sample to a constant mass. Samples were left in the vacuum until a stable final dry weight was reached. Once dried, the mass loss was determined for the individual CRMs.

Sample extraction methods

The conditions for the GEM were agreed by the participating laboratories with an aim to reflect the current knowledge of As species integrity under various extraction conditions and simplicity, so the method could be applied by a large and diverse number of laboratories. Accurately weighed 0.25 g samples were extracted in 10 mL of 1% v/v H_2O_2 (prepared by diluting 16.6 mL of 30% H_2O_2 to a final volume of 500 mL) in deionised water which was pre-heated. The extraction temperature was set to 95°C in the extraction solution and a 60 min timer was started when the monitored samples placed in the hot block reached 95°C. Following the extraction, samples were centrifuged at $3000 \times g$ and the supernatant was analysed for As species by the instrumentation of choice (Table 1). In addition, six participating laboratories carried out another preparation of the CRMs using their preferred, in-house extraction method (Table 1).

Considering that the analysed samples were measured by high performance liquid chromatorgaphy (HPLC) where significant dilution occurs, the differences between sample and calibration standard matrices became negligible and it was not pertinent to prescribe the calibration method. Regardless, Lab 1 carried out quantitation using both external calibration and standard addition methods. The details of the chromatographic conditions are listed in Supplementary Table S1 and a description of chemicals used by participating laboratories can be found in the Supplementary material.

Calibration standards preparation

Solutions of MMA and DMA, respectively, were prepared by dissolving salts of disodium monomethyl arsenic acid (purity 99%, Sigma Aldrich) and cacodylic acid (purity 99%, Sigma Aldrich) in water to an approximate As mass fraction of 20 mg kg^{-1}. Similarly, the As^V standard solution was prepared by dilution of a 1000 mg kg^{-1} stock solution (Inorganic Ventures) to approximately 20 mg kg^{-1}. The exact mass fraction of As in each stock solution was determined against NRC HIAS-1 high purity As CRM (Methven *et al.* 2020). For this purpose, 1 mL of the MMA and DMA standard solutions were digested in 6 mL of concentrated HNO_3 in five replicates using a Multiwave 7000 digestion system, with a 15 min ramp to 200°C and held at 200°C for 15 min. The digested solution of MMA and DMA, together with the solution of As^V, were measured as unknown samples using an Agilent 5110 ICP-OES (Agilent Technologies, Canada). Each participant was provided with 10 mL of each standard solution.

Total As determination

Accurately weighed 0.25 g samples were digested in 7 mL of concentrated HNO_3 and 0.5 mL of 30% H_2O_2 using a Multiwave 3000 microwave system (Anton Paar) set to 1400 W with a 15 min ramp and a 30 min holding time.

Table 1. Summary of in-house extraction methods and analytical instruments used.

Lab	In-house extraction method	Analytical instrumentation	Calibration method
Lab 1	0.25 g + 10 mL of 1% H_2O_2, heated in water bath at 95°C for 60 min; centrifuged after extraction	HPLC-ICP-MS/MS	SA/EC
Lab 2	0.25 g of sample + 15 mL of water, heated at 90°C for a total of 75 min; centrifuged and filtered using PDVF 2-stage filters after extraction	HPLC-ICP-MS/MS	EC
Lab 3	0.25 g sample + 10 mL of water, heated in hot block at 90°C for 30 min with a ramp time of 45 min; centrifuged and filtered through a 0.45 µm pore size PVDF filter after extraction	HPLC-ICP-MS	EC
Lab 4	0.5 g sample + 10 mL 0.02 M TFA + 1% H_2O_2, heated in water bath at 95°C, 60 min (shaking); centrifuged after extraction	HPLC-ICP-MS/MS	EC
Lab 5	0.2 g + 10 mL of 2% HNO_3 and 3% H_2O_2, heated in quartz digestion tubes using an Ultrawave digestion system (10 min ramp to 90°C and 50 bar, held for 30 min); centrifuged after extraction	HPLC-ICP-MS	EC
Lab 6	Same as group extraction method[A]	HG-CT-ICP-MS/MS[B]	EC
Lab 7	Same as group extraction method[A]	HPLC-HG-ICP-MS/MS[C]	EC
Lab 8	0.5 g + 10 mL of 0.4% HNO_3 and 3% H_2O_2, heated and stirred at 90°C for 60 min; filtered via 0.45 µM PES filters after extraction	HPLC-ICP-MS	EC

[A]Labs 6 and 7 shared the same extract.
[B]For details see Matoušek *et al.* (2017).
[C]For details see Marschner *et al.* (2016, 2019).
PVDF, polyvinylidene difluoride membrane; TFA, trifluoroacetic acid; HG, hydride generation; CT, cryo-trapping; EC, external calibration; SA, standard addition; PES, polyethersulfone membrane; HPLC, high performance liquid chromatography; ICP-MS, inductively coupled plasma–mass spectromtery.

Two procedural blanks and CRM were included in each digestion cycle. After cooling, the digests were transferred into Teflon tubes and evaporated to approximately 1 mL. The final residues were quantitatively transferred to 25 mL polyethylene bottles and diluted to 25 g with water. All samples were analysed using two inductively coupled plasma–mass spectrometry (ICP-MS) instruments, an Element XR (ThermoFisher Scientific Inc., Bremen, Germany) and an Agilent 8900 (Agilent Technologies, Santa Clara, CA, USA). On the Element XR, As was measured at mass 75 using high resolution, while on the Agilent 8900, O_2 was used as a reactive gas to mass shift As from m/z 75 to 91. Quantitation was performed by one-point standard addition.

Consensus value assignment

Although the ISO document 'Guide to the expression of uncertainty in measurement' (JCGM 1995) states that the uncertainty associated with a measured value should reflect the contributions of all sources of uncertainty; the uncertainty provided by laboratories is often underestimated.

Consensus values were obtained using the NIST Consensus Builder and Hierarchical Bayes (Laplace) as the method of analysis (Koepke *et al.* 2020). The NIST Consensus Builder combines measurement results provided by participating laboratories into a consensus estimate. The consensus estimate calculates the measurement uncertainty comprised of stated uncertainties associated with the individual measured values and any additional component of uncertainty that manifests itself only when these measured values are inter-compared. It uses a random effects model and is capable of detecting, evaluating and propagating an uncertainty component that accounts for the mutual inconsistency of the measurement results, called dark uncertainty (Thompson and Ellison 2011).

For detailed chromatograms, chromatographic conditions, summary of measured mass fraction and databases containing mass fractions from each participating laboratory please see the Supplementary material.

Results and discussion

Extraction method

The reviewed publications (Pizarro *et al.* 2003; Karthikeyan and Hirata 2004; Wahlen *et al.* 2004; Hirata *et al.* 2006; Wang *et al.* 2007; Pétursdóttir *et al.* 2014*b*; Llorente-Mirandes *et al.* 2017) of As species mass fractions in the CRMs suggested that quantitation of As species in biological tissues is likely extraction method dependent. In a previous study, the results from proficiency tests of iAs quantitation in rice (de la Calle *et al.* 2011) showed that if equal extraction efficiency is achieved among labs then, regardless of the analytical instrumentation used for the detection, agreement among values can be found. Therefore, when designing the present study, one GEM was established which was followed by each participant. A large number of extraction methods have been described in the literature and some rigorous testing has been performed on CRMs and biological tissues (Pétursdóttir *et al.* 2014*b*; Wolle and Conklin 2018*b*). Some methods are able to extract a larger proportion of total As; however, it may be at the expense of As species integrity (Wolle and Conklin 2018*a*). Our preliminary experiments using marine animal tissue (data not shown) showed a linear relationship between the degradation of phosphate arsenoribose (arsenosugar-482) and extraction time when performed under acidic conditions. Similarly, with increasing extraction time, an increase in DMA mass fraction was recorded; most likely the result of arsenosugars degradation (data not shown). An extensive study of extraction methods on a large number of biological tissues showed that acidic (HNO_3, HCl) and alkali (TMAH) solutions have a higher extraction recovery for total As than water under the same heating conditions (Wolle and Conklin 2018*a*). However, further investigation of As species stability under acidic and alkali extraction conditions showed degradation of the arsenosugars, which was also observed in other studies (Gamble *et al.* 2003; Foster *et al.* 2007; Narukawa *et al.* 2012; Wolle and Conklin 2018*a*). Additionally, degradation of non-polar As species into DMA, AsChol and other unknown compounds was also observed when the lipid fraction was extracted in acidic or alkali solution (Wolle and Conklin 2018*b*). Importantly, no degradation of arsenosugars or lipids was observed when extractions were performed in water (Wolle and Conklin 2018*a*). Therefore, the GEM extraction was carried out in water. To eliminate binding of As^{III} to thiols, which may hinder the extraction process, as well as to facilitate oxidation of As^{III} to As^{V}, the extraction water contained 1% H_2O_2.

Our preliminary experiments also have shown a higher extraction yield when the extraction was carried out in vials of larger diameter, i.e. 100 mL versus 18 mL microwave vessels (data not shown). At the set extraction temperature (95°C), the samples are not boiling, hence there is no continuous agitation of the extraction mixture. Therefore, the GEM used by all participants employed 50 mL vials which have a distinctly larger diameter than the 15 mL alternative. The GEM did not prescribe filtration of the supernatants; however, some participants filtered the extracts. As can be seen in

Table 2. Mass fractions (mg kg^{-1}) of iAs measured in unfiltered and filtered CRM DOLT-5 extracts.

Condition tested	*n*	Range
0.45 µm PES	5	0.0160–0.020
0.45 µm PVDF	2	0.0176–0.0179
0.2 µm Nylon	2	0.0181–0.0183
Unfiltered	2	0.0178–0.0181

PES, polyethersulfone membrane; PVDF, polyvinylidene difluoride membrane.

Table 2, no considerable difference between the mass fraction of filtered and unfiltered samples was observed.

For the in-house extraction methods, each participating lab selected a method routinely used for analysis of similar samples. Lab 1 used 1% H_2O_2 in water, Lab 2 and 3 used water without H_2O_2, Lab 4 used 20 mM TFA with 1% H_2O_2 and Lab 5 used 2% HNO_3 with 3% H_2O_2. Lab 8 closely followed an established CEN method for the determination of iAs in foodstuffs (European Committee for Standardization 2016) which also prescribed a mixture of HNO_3 and H_2O_2 but with a lower HNO_3 concentration (0.4%) (Table 1). The extraction apparatus used were water bath (Lab 1 and 4), hot block (Lab 2, 3 and 8) and microwave using 18 mL vessels (Lab 5).

Quantitation method

The choice of quantitation method and quality of calibration standards can have significant impact on the accuracy of the results. Lab 1 carried out quantitation using both external calibration and standard addition methods and reported no significant differences between the calculated values. However, the commercially available salts of As species for preparation of calibration standard solutions can be of varying purity and their exact concentration and purity should be verified using National Metrology Institute (NMI) traceable standards. To determine an exact concentration, the calibration standards were digested in closed vessels to achieve complete oxidation of As species to As^V. It was previously observed, although not widely discussed in the literature, that the ICP-MS response differs among individual As species (Narukawa *et al.* 2006, 2007). Therefore, to avoid any systematic errors caused by calibration standards, each participant was provided with identical calibration standard solutions of DMA, MMA and As^V prepared in water. One participant reported values quantified by NRC provided calibration standards as well as by their own calibration standards. Both results, from group and in-house extraction methods, were higher when quantified using NRC provided calibration stock standards (data not published). The systematic underestimation can be explained by an incorrect As mass fraction in the in-house DMA stock solution used for quantitation, which reiterates the importance of cross-calibrating calibration standards with NMI traceable stock solutions.

Poor chromatographic separation can lead to a significant quantitation bias. The majority of the studied samples contained only a few As species resulting in good baseline separation. However, more complex samples, such as marine biological tissues, presented several challenges. AsBet elutes in the void volume of anion exchange columns on which As^{III} is also poorly retained, and therefore can elute very close to AsBet. Since AsBet is present at a several orders of magnitude higher mass fraction than As^{III}, in marine tissue samples, As^{III} may be unidentified due to a broad elution peak of AsBet (Supplementary Fig. S1). This of course, can be eliminated by the addition of H_2O_2 either prior or post extraction to convert As^{III} into As^V. DOLT-5 contained an unknown compound eluting close to As^V under the chromatographic conditions used by Lab 1 (Fig. 1*a*). However, the chromatographic conditions selected by Lab 4 did not provide sufficient separation between the unknown compound and As^V (Fig. 1*b*) and the initially reported mass fraction of As^V by Lab 4 was the sum of both species. The unknown compound co-eluted with As^V, as shown in Fig. 1*b*, and although the peak of As^V is relatively broad, there is no visible shoulder which would indicate insufficient separation.

As mass fraction determination

Plant tissue matrix

The plant tissue used in this study was CAME-1, canola meal, the high protein residue left over after extraction of oil. The

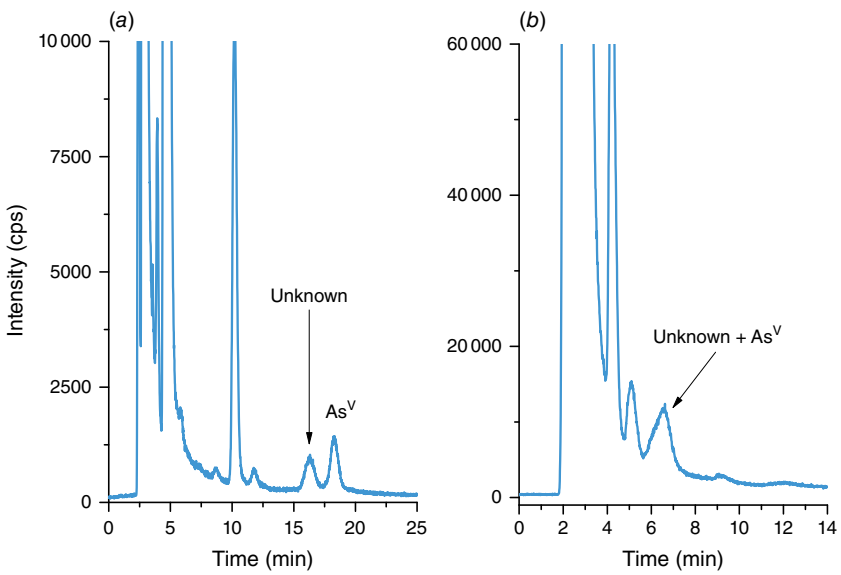

Fig. 1. Chromatograms of DOLT-5 extract showing separation between unknown As species and As^V (*a*) using a PRP-X100 anion exchange column, 40 mM ammonium carbonate mobile phase with 5% methanol at 1 mL min^{-1} flow rate and co-elution of unknown compound and As^V (*b*) using a PRP-X100 anion exchange column, 60 mM ammonium carbonate mobile phase at 1 mL min^{-1} flow rate. For more details on chromatographic conditions see Supplementary Table S1.

residual material was a coarse powder with large sized particles, and is used in animal feed formulations. Several participants reported the need to filter the supernatant due to difficulties in sedimentation of the plant tissue. Most of the participants reported iAs (Fig. 2) as the only species present in the material, with a consensus value (Koepke *et al.* 2020) of $0.013 \pm 0.001\ mg\ kg^{-1}$ when GEM was used and $0.014 \pm 0.001\ mg\ kg^{-1}$ obtained by in-house methods (Table 3). The uncertainty associated with the consensus values was reduced from 7 to 5% by following the GEM.

Lab 6 reported both DMA and MMA at a low mass fraction (0.00028 ± 0.00002 and $0.00008 \pm 0.00001\ mg\ kg^{-1}$, respectively, Supplementary Tables S3, S4) as well as traces of some other unknown species. Lab 8 also reported the presence of DMA; however, the mass fraction was below the limit of quantitation of their methods ($LOQ > 0.0008\ mg\ kg^{-1}$). The mass fraction of iAs accounted for 40% of total As. A low overall extraction efficiency of As species from plant materials is common (Kuehnelt *et al.* 2000; Mattusch *et al.* 2000; Geiszinger *et al.* 2002; Mir *et al.* 2007). There are several factors that may contribute to this observation. Arsenic may be present in a number of chemical forms with concentrations below the LOD of the analytical methods. Besides the iAs identified in this study, plants may accumulate other As species: DMA, MMA, tetramethylarsonium ion (TETRA), AsBet, AsChol and trimethylarsine oxide (TMAO) as reported by other publications (Kuehnelt *et al.* 2000; Geiszinger *et al.* 2002). Owing to lower detection limits, one of the participants, Lab 6, reported several unknown compounds at trace levels which may be a result of either their partial extractability from the tissue or low natural concentrations.

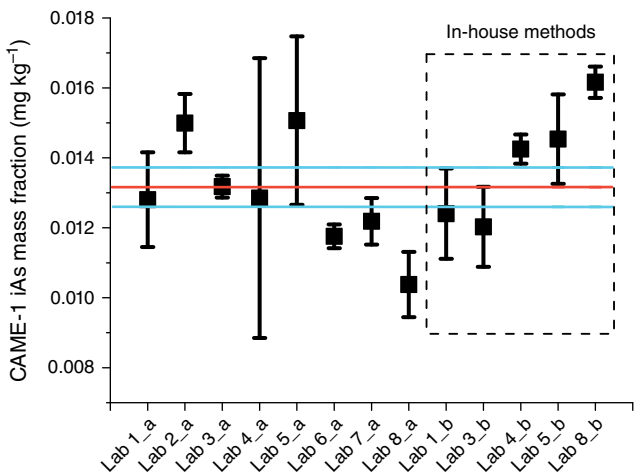

Fig. 2. Mean mass fractions of iAs ($mg\ kg^{-1}$) in NRC CRM CAME-1, canola meal. Mass fractions were measured by the GEM (*a*) and in-house methods (*b*). Error bars indicate standard deviation ($n \geq 3$), the red line marks the consensus value and the blue lines mark the standard uncertainty of the consensus value ($k = 1$) (Koepke *et al.* 2020).

Terrestrial animal tissue matrix

There is a very good agreement between the levels of iAs extracted with the group and in-house extraction methods from terrestrial biological tissues (BFLY-1, VORM-1, KRIK-1). It can be seen in Table 3 that the uncertainty associated with the consensus value for iAs in BFLY-1 was significantly lower when GEM was applied and no significant difference ($P > 0.05$) was observed in the uncertainties for iAs in VORM-1 and KRIK-1.

In VORM-1, the consensus value following the GEM for iAs was $0.113 \pm 0.002\ mg\ kg^{-1}$, and following in-house extraction methods was $0.104 \pm 0.005\ mg\ kg^{-1}$. The combined consensus value of iAs ($0.107 \pm 0.003\ mg\ kg^{-1}$) represented 83% of total As, with iAs being the only As species detected by the majority of participants. Lab 6 was the only lab that reported low levels of DMA ($0.00027 \pm 0.00001\ mg\ kg^{-1}$, Supplementary Table S3) and MMA ($0.00032 \pm 0.00002\ mg\ kg^{-1}$, Supplementary Table S4). The use of a defined GEM did not reduce the associated uncertainty, which was relatively low, 4% for both extraction methods.

The group extraction method provided good agreement among the iAs values in BFLY-1 with an associated uncertainty of 4%, however, the results from in-house extraction methods are skewed by a single lab result (Fig. 3; red line and arrow) which increased the associated uncertainty to 10% (Table 3). Lab 3 reported a significantly lower iAs mass fraction ($0.035 \pm 0.001\ mg\ kg^{-1}$, Supplementary Table S2) using their in-house extraction method (water with no added H_2O_2) in comparison with other labs. The Lab's result using the GEM was $0.050 \pm 0.003\ mg\ kg^{-1}$ (Supplementary Table S2). The combined consensus mass fraction of iAs with the GEM was $0.052 \pm 0.002\ mg\ kg^{-1}$ (Table 3), which was equivalent to $0.0013\ mg\ kg^{-1}$ of iAs in the extraction solution. If As^{III} was present in this sample, its mass fraction would be below the LOD in the extraction solution and without oxidation of As^{III} it would be undetected, and this is what could account for the lower mass fraction of iAs reported by Lab 3. In fact, Lab 3 performing in-house extraction without H_2O_2 did not detect any As^{III} in the chromatograms. Furthermore, As^{III} binds strongly to peptides such as glutathione through cysteinyl sulfhydryl (-SH) groups (Zhao *et al.* 2009). These complexes are stable in water without H_2O_2. Other As species detected in BFLY-1 were DMA and MMA with consensus mass fractions of $0.007 \pm 0.0003\ mg\ kg^{-1}$ and $0.003 \pm 0.0002\ mg\ kg^{-1}$, respectively, determined by GEM and $0.007 \pm 0.001\ mg\ kg^{-1}$ and $0.003 \pm 0.0004\ mg\ kg^{-1}$, determined by an in-house method (Table 3). The percentage uncertainty associated with the mass fractions of both MMA and DMA increase when in-house methods were applied. The sum of As species calculated on combined mass fractions from both group and in-house methods accounted for 59% of the total As. The low recovery could be due to some As bound to chitins in the exoskeleton, or the presence of As in lipid soluble

Table 3. iAs, DMA and MMA consensus values (mg kg^{-1}) and their associated uncertainties (u, k = 1) in the studied CRMs, N^A = 8 for GEM and $N^A \geq 5$ for in-house method, n = number of replicates.

		iAs			DMA			MMA		
CRM (AsT, mg kg^{-1})	Extraction method	n	Consensus value (mg kg^{-1})	u%	n	Consensus value (mg kg^{-1})	u%	n	Consensus value (mg kg^{-1})	u%
CAME-1 (0.03 ± 0.003)	GEM	58	0.013 ± 0.001	5	–	NQ	–	–	NQ	–
	In-house	28	0.014 ± 0.001	7	–	NQ	–	–	NQ	–
	Combined	86	0.013 ± 0.001	4	–	NQ	–	–	NQ	–
BFLY-1 (0.104 ± 0.005)	GEM	61	0.052 ± 0.002	4	55	0.007 ± 0.0003	4	61	0.003 ± 0.0002	7
	In-house	28	0.054 ± 0.006	10	22	0.007 ± 0.001	12	28	0.003 ± 0.0004	11
	Combined	89	0.052 ± 0.002	4	77	0.007 ± 0.0003	4	89	0.003 ± 0.0002	5
VORM-1 (0.129 ± 0.004)	GEM	58	0.113 ± 0.005	4	–	NQ	–	–	NQ	–
	In-house	28	0.104 ± 0.005	4	–	NQ	–	–	NQ	–
	Combined	86	0.110 ± 0.003	3	–	NQ	–	–	NQ	–
KRIK-1 (0.129 ± 0.004)	GEM	46	0.006 ± 0.001	14	52	0.021 ± 0.001	4	–	NQ	–
	In-house	18	0.006 ± 0.001	11	25	0.022 ± 0.001	6	–	NQ	–
	Combined	64	0.006 ± 0.001	8	77	0.021 ± 0.001	4	–	NQ	–
TORT-3 (59.5 ± 3.8)	GEM	57	0.624 ± 0.023	4	54	3.79 ± 0.352	9	56	0.360 ± 0.032	9
	In-house	36	0.510 ± 0.076	15	18	2.41 ± 0.837	35	33	0.208 ± 0.036	17
	Combined	93	0.596 ± 0.029	5	72	3.44 ± 0.412	12	89	0.307 ± 0.034	11
DOLT-5 (34.6 ± 2.4)	GEM	55	0.033 ± 0.003	9	51	3.31 ± 0.222	7	55	0.249 ± 0.034	13
	In-house	36	0.037 ± 0.005	14	30	2.55 ± 0.536	21	33	0.157 ± 0.033	21
	Combined	91	0.034 ± 0.002	7	81	3.09 ± 0.188	6	88	0.201 ± 0.024	12
DORM-5 (13.3 ± 0.7)	GEM	58	0.015 ± 0.001	4	60	0.281 ± 0.030	11	54	0.014 ± 0.002	13
	In-house	36	0.019 ± 0.002	12	30	0.280 ± 0.060	21	28	0.011 ± 0.003	24
	Combined	94	0.016 ± 0.001	7	90	0.288 ± 0.027	9	82	0.013 ± 0.001	11

[A]N, the number of laboratories; AsT, total arsenic; GEM, group extraction method; NQ, not quantified.

form; both cannot be extracted using the applied mild extraction conditions. To the authors' best knowledge, in the only study reporting As species in the black soldier fly larvae, (Biancarosa *et al.* 2019) the major mass fraction of As was an unidentified organic As species, accounting for 61% of total As, followed by DMA, AsBet and iAs. In the current study, the distribution of As species within black soldier fly larvae (BFLY-1) is very different from the results published by Biancarosa *et al.* (2019). This most probably results from the diet the insects were fed. The black soldier fly larvae in Biancarosa *et al.* (2019) had been fed on a substrate containing 60% seaweed, which is known to be rich in arsenosugars and As-lipids, hence high fractions of organic As species. Unfortunately, the feed of the black soldier fly larvae studied in the present work was not known.

The percentage uncertainty in mass fractions of iAs in KRIK-1 extracted by GEM was slightly higher (14%) when compared with in-house methods (11%), and the mass fractions of iAs in this sample was the lowest from all the studied samples: 0.006 ± 0.001 mg kg^{-1} (GEM, Table 3) and 0.006 ± 0.001 mg kg^{-1} (in-house methods, Table 3). Only Lab 6 reported trace levels of MMA. However, the mass fraction of DMA, present at almost one order of magnitude higher than iAs and MMA, had a relatively low associated uncertainty for both extraction methods: 0.021 ± 0.001 mg kg^{-1} (4%) for GEM and 0.022 ± 0.001 mg kg^{-1} (6%) for in-house methods (Table 3). Thus, it is very likely that the higher uncertainties associated with iAs are driven by instrumental precision due to the low mass fraction of these species rather than extraction inconsistencies. It should be noted that the extracts from this matrix were cloudy solutions, most probably due to solubilisation of fat components. Neither additional centrifugation nor filtration improved the quality of the extracts. Although, outside of the scope of this study, the sample was analysed for organic As species using HPLC-ICP-MS (cation exchange chromatography) and LC-orbitrap-MS by Lab 1, where the largest fraction of As was found to be AsBet at 0.210 ± 0.015 mg kg^{-1}. The sum of the As species (including AsBet) accounted for 81% of the total As for both consensus values, following group and in-house extraction methods.

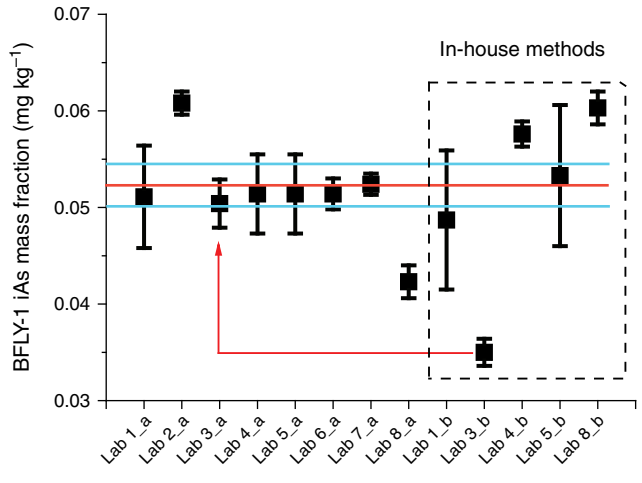

Fig. 3. Average mass fractions of iAs (mg kg^{-1}) in NRC CRM BFLY-1. Mass fractions were measured by the GEM (*a*) and in-house methods (*b*). Error bars indicate standard deviation ($n \geq 3$), red line marks the consensus value and blue lines mark the standard uncertainty of the consensus value ($k = 1$) (Koepke *et al.* 2020). Red line with arrow indicates GEM and in-house results from a single lab with low in-house results.

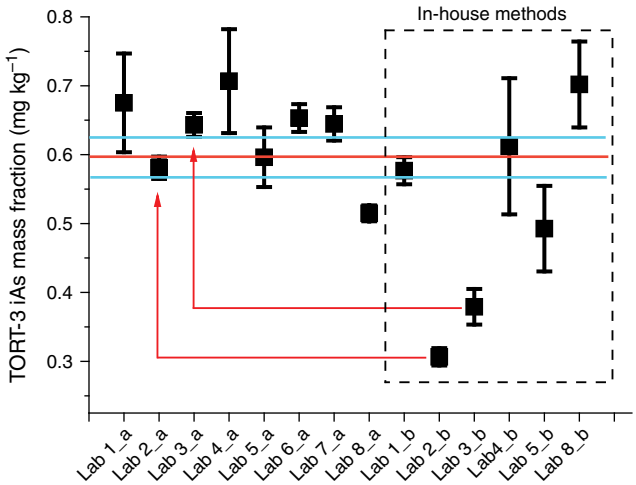

Fig. 4. Average mass fractions of iAs (mg kg^{-1}) in TORT-3 certified reference material. Mass fractions were measured by the GEM (*a*) and in-house methods (*b*). Error bars indicate standard deviation ($n \geq 3$), the red line marks the consensus value and blue lines mark the standard uncertainty of the consensus value ($k = 1$) (Koepke *et al.* 2020). Red lines with arrows indicate GEM and in-house results from Labs 2 and 3 with very low in-house results.

So far, there are no published studies on As species in edible cricket products; however, the total As concentration in the studied samples falls within the range reported in the recent survey of edible insects available on the Canadian market (0.035–0.34 mg kg^{-1}) (Kolakowski *et al.* 2021).

Marine animal tissue matrix

Arsenic speciation analysis in marine animal tissue has been performed for several decades, but no suitable CRM is available for iAs to validate the measurements. In this study, existing CRMs (TORT-3, DORM-5 and DOLT-5) were used to provide a consensus value for iAs, DMA and MMA. The consensus mass fraction of iAs in TORT-3 (0.624 ± 0.023 mg kg^{-1}, Table 3), following the GEM, had a low associated uncertainty of 4%, which nonetheless increased to 15% using the in-house extraction methods (consensus value of 0.510 ± 0.076 mg kg^{-1}, Table 3). The percentage uncertainty from the in-house extraction methods was mainly driven by results reported by Labs 2 and 3 (Fig. 4; red lines and arrows).

It should be noted that Labs 2 and 3 were not using H_2O_2 in their in-house extraction methods (water and heat only). A similar value of iAs (0.341 ± 0.030 mg kg^{-1}) was reported by Wolle and Conklin (Wolle and Conklin 2018*b*) using the same extraction method as Lab 2. In a recently published study, Tibon *et al.* (2021) used water/methanol (1:1) as an extraction medium without H_2O_2 and reported a mass fraction of As^{III} of 0.361 ± 0.012 mg kg^{-1} and As^{V} of 0.270 ± 0.024 mg kg^{-1}, for which the sum is comparable with the mean value reported in the present study. To the best of our knowledge, there are no other published results of iAs in TORT-3 extracted without H_2O_2 and thus several investigations were carried out in the present study in an attempt to explain the lower mass fractions of iAs reported by Labs 2 and 3. Generally, H_2O_2 is used to oxidise As^{III} to As^{V} which aids the quantitation of iAs in the presence of excess AsBet, which can co-elute with As^{III} from anion exchange columns. Additionally, small mass fractions of As^{III} or As^{V} may fall below the LOD if they are not analysed as the sum of both species after treatment with H_2O_2.

To identify the possible sources of lower mass fractions of iAs as reported by the water extractions, TORT-3 was extracted with H_2O_2 (Fig. 5 blue line) and without H_2O_2 (Fig. 5 black line). Subsequently after the extraction (and removal of the residual matrix), a portion of the extract without H_2O_2 was spiked with H_2O_2 (Fig. 5 red line) and analysed, whereas another portion spiked with H_2O_2 was subjected to heating conditions as during the extraction step (Fig. 5 green line). It was observed that, when H_2O_2 is added into the extract post-extraction and analysed directly, in addition to the oxidation of As^{III} to As^{V} there is a slight increase in the mass fraction of a compound eluting in the shoulder of DMA, most probably DMAA (Supplementary Fig. S2), and low quantities of the unknown compound U2 could be detected (Fig. 5 red line). However, when H_2O_2 was used during extraction (Fig. 5 blue line) or after extraction and subjected to heating (Fig. 5 green line), a significantly larger number of changes in As species were observed. The peak area of iAs increased by 35%, equivalent to the 0.206 mg kg^{-1} of the consensus value from the group extraction method. After the mathematical correction, the results from Lab 3b would be in agreement with the rest of the labs; however, the results from Lab 2b would approach,

but remain slightly outside, the confidence interval of the consensus value.

Similarly, the DMA mass fraction and the compound eluting in the shoulder of the DMA peak increased, which could be a result of the breakdown of some arsenosugars, as previously reported (Ebisuda *et al.* 2003; Wolle and Conklin 2018*a*). Interestingly, the peak area of the compound eluting at the retention time of MMA also increased. There is no evidence in the literature of MMA being a degradation product of organic As compounds, therefore it is possible that the compound is not actually MMA. Changes in peak areas were also observed for unknown compounds U1 and U2, the peak areas of the former decreased, whereas the peak area of U2 significantly increased. The unknown compound U2 was not present in the chromatogram after extraction without H_2O_2 suggesting that this compound is either entirely formed by the degradation of larger organoarsenicals when H_2O_2 is being used or it is an oxidised analogue of thio-arsenical. Clearly, when it comes to complex matrices such as TORT-3, the choice of an extraction method is important. Presently, it is not clear whether extraction in water without H_2O_2 leads to underestimation of the As species mass fraction or if addition of H_2O_2 into the extractant causes species conversion and/or enables detection of thio-arsenicals.

Other reported values for iAs in TORT-3 were published in a study focusing on selective hydride generation (HG), where results from a reference detection technique (HPLC-HG-AFS) were compared with selective HG-ICP-MS (Marschner *et al.* 2019). The same HPLC-HG based method was used in the present study although it was coupled to a different detector (ICP-MS/MS); however, the results were significantly different. The published results for iAs determined by HPLC-HG-AFS were $0.428 \pm 0.006\,mg\,kg^{-1}$ in comparison with 0.645 ± 0.024 and $0.653 \pm 0.020\,mg\,kg^{-1}$ (Supplementary Table S2) in the present study for HPLC-HG-ICP-MS/MS and for HG-CT-ICP-MS/MS, respectively. The method in the Marschner *et al.* (2019) study employed a microwave digestion system for extraction. Interestingly, a similar in-house extraction method was used by Lab 5, with the exception of the hold time at 90°C for 10 min used by Marschner *et al.* (2019), instead of 30 min in the present study (Lab 5). The mass fraction reported by Lab 5 using their in-house extraction method was $0.493 \pm 0.062\,mg\,kg^{-1}$ (Supplementary Table S2), which is in good agreement with the published results (Marschner *et al.* 2019). Both microwave digestion systems used narrow 18 mL vessels in which the contact area between the sample and extractant is smaller compared to larger volume tubes, which could contribute to the lower extraction efficiencies.

Because of the degradation of arsenosugars into DMA, quantitation of DMA in the matrices containing arsenosugars is challenging. As it was already mentioned, the addition of H_2O_2 increased the peak area of DMA, and even if there was a good agreement between the reported values, the accuracy of the consensus value would be questionable. The results provided by labs that didn't use H_2O_2 in their in-house method, i.e. Lab 2 ($1.85 \pm 0.015\,mg\,kg^{-1}$, Supplementary Table S3) and Lab 3 ($1.60 \pm 0.039\,mg\,kg^{-1}$, Supplementary Table S3), are significantly lower than the results provided by other participants. Although they are in good agreement with previously published data ($1.181 \pm 0.030\,mg\,kg^{-1}$ (Tibon *et al.* 2021) and $1.303 \pm 0.067\,mg\,kg^{-1}$ (Wolle and Conklin 2018*b*)), the number of publications is very limited. Similarly, as it was in the case for iAs, values reported by HG-CT-ICP-MS/MS and HPLC-HG-ICP-MS/MS were significantly higher (Lab 6, $4.33 \pm 0.210\,mg\,kg^{-1}$ and Lab 7, $3.49 \pm 0.168\,mg\,kg^{-1}$, Supplementary Table S3) in the current study than those previously published ($1.617 \pm 0.072\,mg\,kg^{-1}$ (Marschner *et al.* 2019)) using the HPLC-HG-AFS method.

The analysis of DOLT-5 for iAs has been challenging due to its low mass fraction; about 10-fold lower than in TORT-3. Inorganic As accounts for $<$ 0.2% of total As with a consensus mass fraction of 0.033 ± 0.003 (GEM, Table 3) and $0.037 \pm 0.005\,mg\,kg^{-1}$ (in-house methods, Table 3). The relative standard uncertainty decreased from 14 to 9% when GEM was applied. It was observed that the results provided by Lab 3 using an in-house method that does not contain H_2O_2 resulted in a 36% higher mass fraction than when quantified by the GEM (Supplementary Table S2). This observation is rather unusual as the trend was reversed in CAME-1 and the terrestrial animal CRMs. There are two published studies reporting values for iAs in DOLT-5 and while one of them is within the confidence interval of the consensus value reported in this study, $0.029 \pm 0.007\,mg\,kg^{-1}$ (Marschner *et al.* 2019), the other is significantly higher, $0.218 \pm 0.009\,mg\,kg^{-1}$ (sum of 0.125 ± 0.008 as As^{III} and 0.093 ± 0.004 as As^{V}) (Tibon *et al.* 2021). The latter publication did not show a chromatogram for this CRM and hence interpretation of the observed difference can only be addressed by assumptions. Under the extraction conditions used in Tibon *et al.* (2021), As^{III} would elute close to the void volume with AsBet and other cations. The mass fraction of AsBet is nearly 200 times higher than the reported As^{III} which could potentially influence the quantitation of As^{III}. Additionally, under the applied chromatographic conditions of Labs 1, 2, 3 and 5 (Supplementary Table S1), an unknown As compound was eluting close to As^{V} (Fig. 1). It is not clear from the published results if these two compounds were chromatographically resolved or if they could contribute to the reported mass fraction of As^{V}.

The presence of arsenosugars in DOLT-5 has been previously reported (Tibon *et al.* 2021), as well as observed in this study (Supplementary Fig. S3), and thus the quantitation using H_2O_2 will most probably overestimate the mass fraction of DMA in this material. Results reported from the in-house method without H_2O_2 were significantly lower than the consensus value from GEM ($3.31 \pm 0.222\,mg\,kg^{-1}$, Table 3),

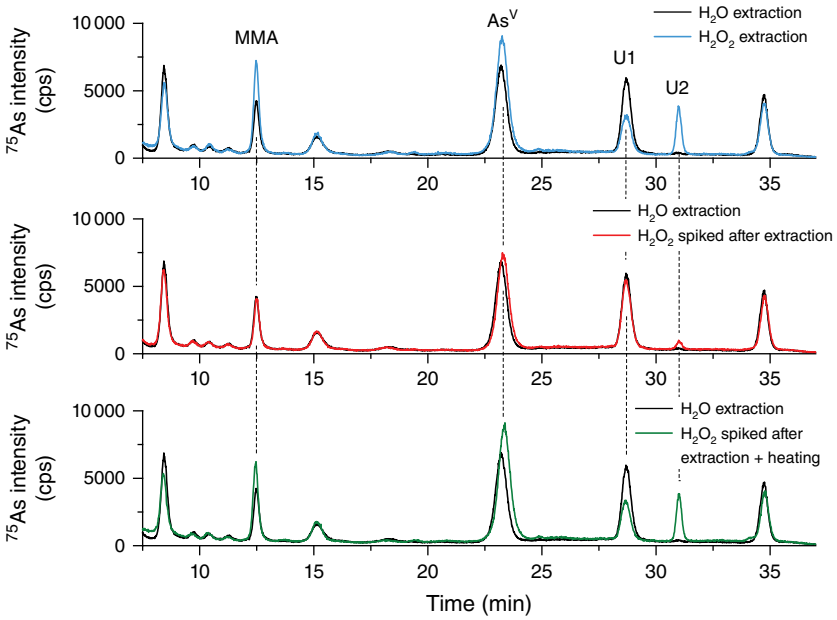

Fig. 5. Chromatograms of extracted TORT-3 in water (black line), in 1% H_2O_2 (blue line), in water and spiked with H_2O_2 after extraction (red line) and in water and spiked with H_2O_2 after extraction and subjected to thermal treatment (green line).

1.19 ± 0.126 mg kg^{-1} reported by Lab 2 and 1.64 ± 0.055 mg kg^{-1} by Lab 3 (Supplementary Table S3). A higher mass fraction was reported by others, 1.870 ± 0.120 mg kg^{-1} using water/methanol (1:1, v/v) as an extractant (Tibon *et al.* 2021) and 1.742 ± 0.045 mg kg^{-1} was reported by a HPLC-HG-AFS method following extraction in 2% HNO_3 with 3% H_2O_2 (Marschner *et al.* 2019).

Similarly, in DORM-5, the iAs mass fraction accounted for <0.2% of total As with a consensus mass fraction of 0.015 ± 0.001 mg kg^{-1} (Table 3) reported using the GEM. The associated relative standard uncertainty of 4% was driven by results provided by Lab 1, which reported significantly higher mass fractions than the rest of the participants but could not provide any technical explanation which would justify exclusion of these results (Supplementary Table S2). Application of in-house methods increased the relative standard uncertainty to 12% with a consensus value of 0.019 ± 0.002 mg kg^{-1} (Table 3). The iAs mass fraction in DORM-5 is one of the lowest among the studied CRMs. There was also an added challenge of insufficient sedimentation, even after prolonged centrifugation, as reported by several labs. DORM-5 has only recently been released and thus there are no published studies of As in this CRM. However, the low mass fraction of iAs is representative of typical iAs values found in a similar matrix – fish protein (Wolle and Conklin 2018*b*).

The consensus values for DMA from both the group (0.281 ± 0.030 mg kg^{-1}, Table 3) and in-house (0.280 ± 0.060 mg kg^{-1}, Table 3) extraction methods matched very well for DORM-5, although the associated uncertainty increased from 11 to 21% when in-house methods were applied. The results using in-house methods of Labs 2 and 3 were significantly lower than the rest of the reported mass fractions, as was in the case of TORT-3 and DOLT-5 (Supplementary Table S3). From the studied samples, as well as evidence provided in the literature (Wolle and Conklin 2018*b*), it became clear that if the matrix contains arsenosugars, the assignment of an accurate mass fraction to DMA is very challenging. Using the various in-house extraction conditions by the participating labs, which to a certain degree represent some of the most common extraction conditions reported in the literature (Ardini *et al.* 2020), arsenosugars undergo degradation which leads to formation of DMA and other compounds. Additionally, tissues high in fat may contain As-lipids, which can be hydrolysed during the extraction. As previously shown (Ebisuda *et al.* 2003), DMA is one of the As-lipid hydrolysis products, potentially providing a significant contribution to DMA mass fraction in some samples.

Conclusion

In summary, the presented round-robin study led to several important observations. By following the steps in the prescribed group method, several variables were eliminated which led to reduced variability of reported values. As seen in Table 3, the uncertainties associated with the consensus values generated by the in-house extraction methods are greater in comparison with GEM but even with these very complex matrices, such as seafood, they are below 14% for iAs. The increased uncertainties of the consensus values generated by the in-house extraction methods imply that some inconsistences can be expected when comparing published data produced by different extraction methods and applied to similar matrices. However, only a slight reduction in the uncertainties was observed in the plant and terrestrial animal tissues, which is indicative of an extraction method

independence for the tested methods in the presented study. Additionally, the low uncertainties in the consensus values generated by GEM suggest that if the extraction efficiencies between labs are comparable, the choice of analytical methods will have negligible impact on the quantitation. Nonetheless, it has to be stressed that the choice of appropriate chromatographic conditions that account for complexity and number of As species present is critical for obtaining accurate results. It was shown that the use of identical calibration standard solutions by the participating laboratories contributed to the reduction of the consensus uncertainty values. Therefore, greater attention should be paid to the verification of an accurate mass fraction in the calibration stock solutions. And lastly, the present results identified extractant dependent behaviour of As species in TORT-3 and possibly other similar matrices, where origin and implications are not clear at the moment and will require further investigation.

Supplementary material

Supplementary material is available online.

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Data availability. All data cited has been obtained from papers given in the reference section.

Conflicts of interest. Joerg Feldmann is an Editor for Environmental Chemistry and was blinded from the peer review process for this manuscript. Two of the study's authors (K. Kubachka and M. Wolle) are US Food and Drug Administration (FDA) employees, and it must be declared that the opinions expressed by the authors do not reflect any current or future opinion or position by the FDA regarding arsenic analysis or regulation, and that the mention of manufacturers or trade names is for clarity and does not constitute an endorsement.

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