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Establishment and validation of analytical methods for the determination of arsenic species in foodstuffs

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ESTABLISHMENT AND VALIDATION OF ANALYTICAL METHODS FOR THE DETERMINATION OF ARSENIC SPECIES IN FOODSTUFFS

Memoria presentada por Antoni Llorente Mirandes para optar al grado de **Doctor por la Universidad de Barcelona**

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HACEN CONSTAR

Que el presente trabajo de investigación ha sido realizado por el Sr. Antoni Llorente Mirandes en el Departament de Química Analítica de la Universitat de Barcelona bajo su dirección:

ESTABLISHMENT AND VALIDATION OF ANALYTICAL METHODS FOR THE DETERMINATION OF ARSENIC SPECIES IN FOODSTUFFS

Barcelona, octubre de 2015

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All things are poisons, for there is nothing without poisonous qualities.
It is only the dose which makes a thing poison

(Paracelsus)

AGRAÏMENTS

En primer lloc m'agradaria donar les gràcies a la Roser i el Fermín, que sempre m'han donat suport, ànims, bons consells i crítiques constructives.

També m'agradaria donar les gràcies a tots els companys/es del grup "Qüestram-M", que han passat pel grup durant aquests anys.

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Gràcies als meus pares i al meu germà que sempre han estat al meu costat i per totes les coses que han fet per mi.

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CONTENTS

ABBREVIATIONS AND ACRONYMS

A

AAS Atomic absorption spectroscopy
AB Arsenobetaine
AC Arsenocholine
AEC Anion exchange chromatography
AES Atomic emission spectrometry
AFS Atomic fluorescence spectroscopy
AOAC AOAC International (The association of the analytical communities)
AP Anion pairing
As(III) Arsenite
As(V) Arsenate
ASE Accelerated solvent extraction

B

BAc Bioaccessibility
BAv Bioavailability
BCR Bureau Community of Reference

C

CEC Cation exchange chromatography
cps Counts per seconds
CRM Certified reference material
Capillary Electrophoresis

D

DMA(III) Dimethylarsinous acid
DMA(V) Dimethylarsinic acid
DMAA Dimethylarsinoyl acetic acid
DMAE Dimethylarsinoyl ethanol

E

EC Electrochemical
EC European Commission
EPA Environmental Protection Agency
ESI Electrospray ionisation
ETAAS Electrothermal atomic absorption spectrometry
EU European Union

F

FAAS Flame atomic absorption spectroscopy
FDA Food and drug administration
FI Flow injection
FSA Food Standard Agency

G

GC Gas chromatography
GFAAS Graphite furnace atomic absorption spectroscopy
Gly-sug Glycerol arsenosugar
GI Gastrointestinal
GPX Glutathione peroxidase

H

HG Hydride generation
HG-AAS Hydride generation atomic absorption spectroscopy
HPLC High performance liquid chromatography

I

iAs Inorganic arsenic
IC Ion pairing chromatography
ICPMS Inductively coupled plasma mass spectrometry
ICPOES Inductively coupled plasma optical emission spectrometry
i.d. Internal diameter
INAA Instrumental neutron activation analysis
IRMM Institute for Reference Materials and Measurements
IS Internal standard
IUPAC International Union of Pure and Applied Chemistry

L

LC Liquid chromatography
LC50 Lethal concentration 50
LD50 Lethal dosis 50
LOD Limit of detection
LOQ Limit of quantification

M

Min minutes

MMA(III) Monomethylarsonous acid

MMA(V) Monomethylarsonic acid

MS Mass spectrometry

MS/MS Tandem mass spectrometry

m/v mass/volume

MW Microwave

m/z mass/charge

N

ND Not detected

NIES National institute for environmental studies

NRCC National Research Council Canada

NRCCR National Research Center for Certified Reference Materials

O

OES Optical emission spectroscopy

P

PBET Physiologically Based Extraction Test

PEEK Polyether ether ketone

PIPES Piperazine-NN-bis (2-ethane-sulfonic acid) disodium salt

PLE Pressurized liquid extraction

PO_4-sug Phosphate arsenosugar

PTFE Polytetrafluoroethylene

PTWI Provisional tolerable weekly intake

Q

Q Quadrupole

QQQ Triple Quadrupole

R

RP Reversed phase chromatography

rpm Revolutions per minute

S

SBET Simple bioaccessibility extraction test

SD Standard deviation

SEC Size exclusion chromatography

SHIME Simulator of the human intestinal microbial ecosystem

SO_3-sug Sulfonate arsenosugar

SO_4-sug Sulfate arsenosugar

SRM Selected reaction monitoring

T

T^a Temperature

tAs Total arsenic

TBAH Tetrabutylammonium hydroxide

TETRA Tetramethylarsonium ion

TMA+ Trimethylarsine

TMAO Trimethylarsine oxide

TOF Time of flight

U

USA United States of America

US-EPA United States environmental protection agency

UV Ultraviolet radiation

V

v/v Volume/volume

W

WHO World health organization

w/v weight/volume

STRUCTURE OF THE THESIS

The present doctoral thesis is structured in five parts. **Part I** deals with the introduction of the thesis. An introduction summarizing general information relative to arsenic properties, arsenic compounds, toxicity, analytical methods, arsenic dietary exposure, European Legislation, and quality assurance is presented in **Chapter 1**. The information reported in previous studies related to methods, measurement techniques and quality assurance assessment for inorganic arsenic determination in food is shown in **Chapter 2**. All this information is summarised and published in a review article. The main aim of this thesis as well as the specific objectives are summarised in **Chapter 3.**

The results obtained in this thesis are presented in **Part III** which is divided into two chapters. The first one, **Chapter 4**, is based on results obtained regarding development and validation of methods for the determination of arsenic species in foodstuffs. **In Chapter 5**, results related to the occurrence of arsenic species in foodstuffs are presented.

An overall discussion of the obtained results in this thesis is presented in **Part IV.** The discussion is divided into two chapters: **Chapter 6** related to development and validation of methods and **Chapter 7** to occurrence of arsenic species in foodstuffs.

Finally, the conclusions of the thesis are summarised in **Part V (Chapter 8)**.

Furthermore, a summary of the thesis in Spanish is shown in **Annex I** and other scientific contributions are presented in **Annex II** .

PART I: INTRODUCTION

Chapter 1

Arsenic: properties, species and occurrence

1.1. Properties and chemistry of arsenic

Arsenic is a metalloid with a complex chemistry demonstrating the properties of both metals and non-metals. Elemental arsenic has the atomic number of 33 and the atomic weight of 74.92 g mol^{-1}. Arsenic occurs in group 15 in the Periodic table, the same group as nitrogen and phosphorous, consequently the chemistry of arsenic is similar in many respects to these elements. Due to these chemical similarities, arsenic can often substitute for phosphorous in biological systems [1] being one of the reasons for the occurrence of arsenic at high levels in many marine organisms, and thus in many seafood [2]. Arsenic exhibits several known allotropic forms where the most stable allotrope of arsenic is the gray form, similar to rhombohedral form of phosphorous [3]. Arsenic is commonly found in sulfide-rich mineral the most abundant is arsenopyrite (FeAsS).

The only natural arsenic isotope is ^{75}As. Arsenic is widely distributed in the earth's crust and can exist in four oxidizing states; –3, 0, +3, +5 and in a variety of inorganic and organic forms [4]. The majority of the known arsenic species in organisms and food contain arsenic in oxidation states +5 and +3 [5]. Both of these inorganic arsenicals are toxic and can interconvert with changes in redox conditions and pH [6]. Furthermore, the existence of the -3 state in the environment has been questioned [6]. Arsenic may also be found in organoarsenic compounds, defined as those containing arsenic-carbon bonds. In addition to these compounds, arsenic can form lipid-based compounds. The affinity of arsenic for sulfur means that compounds with As-O components can also exist with As-S bonds; for example, As(III) can bond with sulfhydryl groups of proteins [7]. It is currently estimated that there are over 50 arsenic compounds found in the environment [5].

Arsenic is mobilized into the aqueous and atmospheric environment naturally through the weathering of rocks and minerals, volcanic activities and biological processes [8]. Arsenic can enter into the environment via anthropogenic processes including mining, smelting, combustion, the production and the use of pesticides, herbicides, insecticides and natural processes as weathering, volcanism, and dissolution of soils and sediments rich in arsenic. Arsenic is used mainly in agriculture (pesticides, wood preservation agents) and as the feed additive (Roxarsone) to improve growth of poultry although arsenic use in these applications have been reduced in recent years because of health concerns. Further, arsenic trioxide is used in medicine for treatment of certain type of leukemia [6].

1.2 Chemical speciation

Speciation is a word of importance of the work presented in this thesis. Even though the term speciation has been used for a long time it was not until the year 2000 that the international community agreed upon nomenclature and definition, and the IUPAC introduced a guideline for terms related to fractionation of elements and chemical speciation [9]. Modern applications of speciation analysis are powerful within e.g. the fields of food chemistry, environmental chemistry, health and hygiene as well as geology. Speciation is furthermore an important tool when investigating the toxicity and bioavailability of elements where the information of the total element concentration may be insufficient.
IUPAC definitions [9]:

I. *Chemical species*. Chemical elements: Specific form of an element defined as to isotopic composition, electronic or oxidation state, and/or complex or molecular structure.
II. *Speciation analysis*. Analytical chemistry: Analytical activities of identifying and/or measuring the quantities of one or more individual chemical species in a sample.
III. *Speciation of an element; speciation*. Distribution of an element amongst defined chemical species in a system.
IV. *Fractionation*. Process of classification of an analyte or a group of analytes from a certain sample according to physical (e.g. size, solubility) or chemical (e.g. bonding, reactivity) properties.

1.3 Arsenic species

More than 50 different naturally occurring As-containing compounds have been identified, comprising both organic and inorganic forms [5]. The following sections present the compounds relevant to this thesis and are of food relevance as well.

The structures of the most relevant arsenic species studied in this thesis are presented (**Figure 1**).Table 1 shows species name, abbreviations, formula, molecular weight and CAS for the main arsenic species studied in this thesis that are important in foodstuffs. Herein the nomenclature proposed in the Review articles of Maher [10] and Francesconi and Kuehnelt [11] is followed throughout the thesis. In these Reviews, the authors proposed the adoption of suitable names as well as abbreviations of arsenic compounds, considering that there is no agreement on this in the vast literature related to this issue. The proposal of the authors is clearly reasoned and undoubtedly contributes to reduce the confusion in the literature.

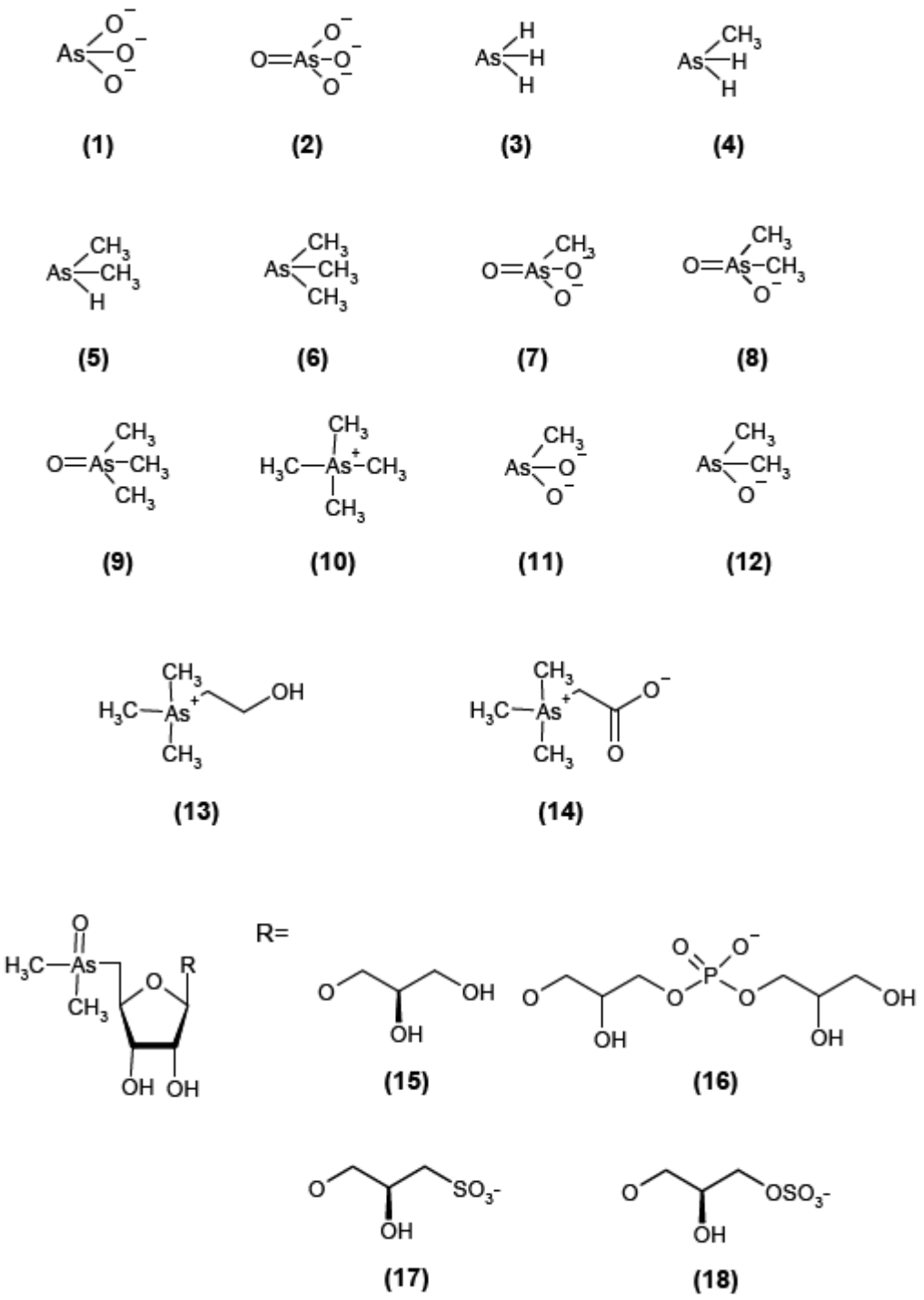


Figure 1. Structures of the main arsenic species studied in this thesis

Table 1. Common name, abbreviations, formula, molecular weight, and CAS for the main arsenic species in foodstuffs.

Compound	Common name	Abbreviation	Formula	Molecular weight	CAS number	Comment
1	Arsenite	As(III)	$As(OH)_3$	125.94	13464-58-9	Trace to low levels in most foods; highly toxic
2	Arsenate	As(V)	$AsO(OH)_3$	141.94	7778-39-4	Trace to low levels in most foods; a major form in water; highly toxic
3	Arsine	-	AsH_3	77.94	7784-42-1	Detected in thermal hot srings
4	Methylarsine	-	(CH_3AsH_2)			Detected at trace level in prawn and lobster, also in sewage sludge from wastewater treatment
5	Dimethylarsine	-	$((CH_3)_2AsH)$			
6	Trimethylarsine	-	$(CH_3)_3As)$			
7	Methylarsenate	MA	CH_5AsO_3	139.97	124-58-3	Trace arsenic species of some seafood and terrestrial foods; a significant human urine metabolite of iAs.
8	Dimethylarsenate	DMA	$C_2H_7AsO_2$	138	75-60-5	Minor arsenic species in seafood and some terrestrial foods; the major human urine metabolite of iAs, arsenosugars and arsenolipids
9	Trimethylarsine oxide	TMAO	C_3H_9AsO	136.02	4964-14-1	Minor arsenic species common in seafood.
10	Tetramethyl arsonium	TETRA	$C_4H_{12}As$	135.06	27742-38-7	Minor arsenic species common in seafood.
11	Methylarsenite	MA(III)	CH_5AsO_2	123.97	25400-23-1	Not usually detected in foods; detected in some human urine samples as a metabolite of iAs; a toxic species thought to be important for mode of toxic action.
12	Dimethylarsenite	DMA(III)	C_2H_7AsO	122	55094-22-9	Not detected in foods; detected in some human urine samples as a metabolite of iAs; a very unstable species that is very difficult to measure; highly toxic species considered by some researchers
13	Arsenocholine	AC	$C_5H_{14}AsO$	165.09	39895-81-3	Trace arsenic species found in seafood; is readily oxidised to arsenobetaine in biological systems
14	Arsenobetaine	AB	$C_5H_{11}AsO_2$	178.06	64436-13-1	Major arsenic species in most seafood; non-toxic
15	Glycerol arsenosugar	Gly-sug	$C_{10}H_{21}AsO_7$	328.19	1227057-97-7	Major (edible algae) or significant (molluscs) arsenic species in many seafood
16	Phosphate arsenosugar	PO_4-sug	$C_{13}H_{28}AsO_{12}P$	482.25	88216-76-6	
17	Sulfonate arsenosugar	SO_3-sug	$C_{10}H_{20}AsO_9S$	391.25	123288-10-8	
18	Sulfate arsenosugar	SO_4-sug	$C_{10}H_{20}AsO_{10}S$	407.25	123257-94-3	

Inorganic arsenic species

Inorganic arsenic (iAs) is widely distributed in the environment and found mainly in the +3 or +5 oxidation state, either bound in thio-complexes or as the two oxyanions As(III) and As(V). The reported data is usually arsenite and arsenate even though the inorganic arsenic is likely to be bound to thio-groups in peptides or proteins in food [5]. Under normal environmental oxygen levels, As(V) is thermodynamically favored. They are however easily interconverted and often found together. In seawater and freshwater, As(V) is the major arsenic species and essentially all arsenic in drinking water is arsenate. Concentrations of arsenic in natural waters are typically below 10 μg As L^{-1}, frequently below 1 μg As L^{-1} and can reach up to 5000 μg As L^{-1}. This large range occurs under natural conditions [12, 13]. In the rare cases where high concentrations of arsenic are found, particularly in groundwater, the effects are severe where the drinking water of millions of people are highly contaminated, e.g. in the Bengal Basin [13].

Food products of terrestrial origin are generally low in concentration of total arsenic (tAs) and subsequently also low in iAs content, usually below 0.05 mg As kg^{-1} [2]. Exception to this is rice which contains significant amounts of iAs often between 0.05 to 0.4 mg As kg^{-1} [14, 15] and at times considerably higher, up to 1.9 mg As kg^{-1} in rice bran solubles [16]. Fish and other seafood are on the other hand high in tAs concentration where most samples fall within a range of 5 to100 mg As kg^{-1} [2, 17], but with much lower levels of iAs, typically <0.2 mg As kg^{-1} [18–20]. No general relationship between the tAs concentration and the level of iAs in seafood has been shown [20]. Most seafood has only trace quantities of iAs, and seafoods high in iAs are the exceptions. For instance, the edible seaweed Hijiki has high levels of iAs: 66 mg As kg^{-1} or more [21–29]. Furthermore, unusually high levels of iAs in mussels have also been reported where iAs was reported up to 5.8 mg As kg^{-1} [30].

Methylated arsenic species

The arsenic species that belong to this group are methylarsonate (MA), dimethylarsinate (DMA), trimethylarsine oxide (TMAO) and tetramethylarsionium ion (TETRA). DMA and MA species occur jointly is in accordance with the pathway proposed by Challanger for arsenate biotransformation involving reduction and methylation of As(V) [31]. These methylated compounds are formed as a result of biomethylation, where biomethylation refers to an enzymatic transfer of a methyl group from a donor atom to an acceptor atom within a living organism [32]. The biomethylation of arsenic compounds is described by the pathway previously suggested [31], shown in **Figure 2**, which involves a series of alternating reduction and oxidative methylation reactions mediated by arsenic methyltransferase enzymes and S-adenosylmethionine (SAM), a near universal methyl donor in biological systems [33]. Within this model As(V) is first reduced to As(III) before being methylated and oxidized to form MA [31]. The reduction and oxidative methylation steps are repeated producing the trivalent and pentavalent forms of MA and DMA and finally trimethylarsine [33].

Both MA and DMA are generally detected at low levels (<0.5 mg As kg^{-1}) in living organisms [34], and are also common minor arsenic metabolites and are often found together. TMAO is generally only found at low or trace levels in terrestrial and marine organisms [11]. Despite is a metabolite in the biotransformation process, TMAO is usually detected at trace levels especially in the marine environment [35], and in some cases found as major compound [36]. In the terrestrial environment it was detected at trace levels in samples of plants and lichens [37]. TETRA is usually a minor species in the marine environment but can be found as major species in some molluscs [6]. Furthermore, TETRA has been reported in frogs, mushrooms and some plant species [7].

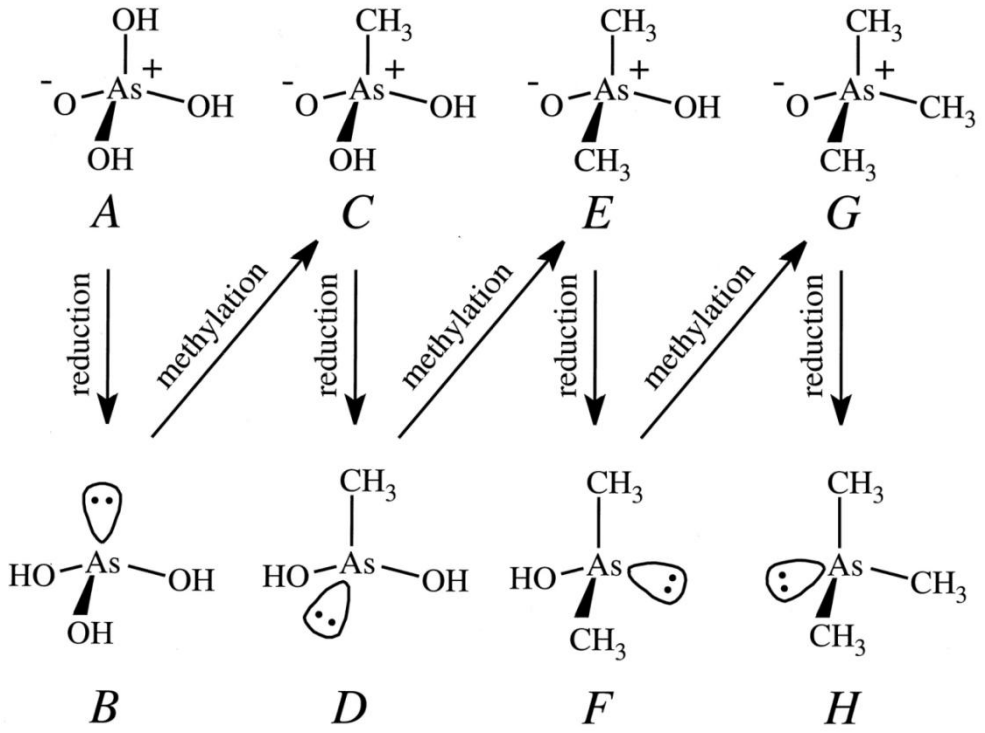


Figure 2. A scheme of the pathway proposed by Challenguer for the conversion of arsenate to trimethylarsine (adapted from [31]). (A) Arsenate; (B) arsenite; (C) methylarsenate; (D) methylarsenite; (E) dimethylarsenate; (F) dimethylarsenite; (G) trimethylarsine oxide; (H) trimethylarsine. The top line of structures shows the As(V) intermediates. The vertical arrows indicate the reduction reactions to the As(III) intermediates (bottom line), and the diagonal arrows indicate the methylation steps by SAM.

Arsenocholine (AC)

Arsenocholine (AC) is commonly found at trace levels in marine organisms typically <0.2 mg As kg^{-1}. It is a metabolic precursor of AB [38, 39] and is rapidly converted into this compound [38, 40, 41]. In the terrestrial environment it was first detected in samples of fungi growing in arsenic contaminated area [40, 42]; and has been detected at trace levels in some samples of terrestrial plants [43].

Trimethylarsoniopropionate (TMAP)

Trimethylarsoniopropionate (TMAP), a compound similar to arsenobetaine, was first identified in 2000 in a fish species [44], and is now known to be a common minor constituent of marine organisms (typically at concentrations of 0.2-2 mg As kg^{-1}; [45, 46].

Arsenobetaine (AB)

Arsenobetaine (AB) was first identified by Edmonds and Francesconi in 1977 [47]. Currently, the main hypothesis for AB formation is that it is formed from the degradation products of dimethylated arsenosugars (**Figure 3, pathway #2)**. Arsenosugars are thought to be precursors for the formation of AB because the dietary sources for marine organisms, such as phytoplankton and marine kelp, contain elevated levels of arsenosugars [36]. However other routes have also been mentioned in terrestrial or deep-sea environments, in biotic, and in abiotic environments. The three pathways of arsenobetaine formation often mentioned in the literature are shown in **Figure 3**. Pathways #1 and #2 involve the degradation of arsenosugars, and Pathway #3 involves DMA(III) as precursor.

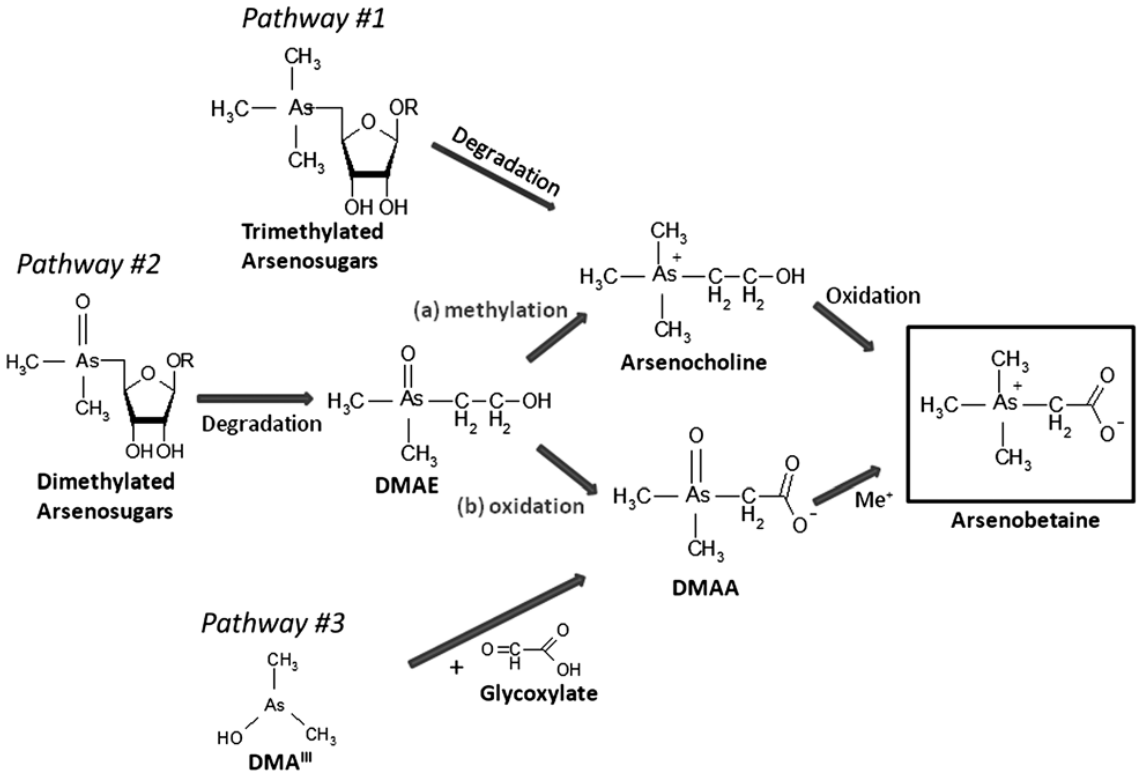


Figure 3. Proposed pathways of AB formation (adapted from [48]. Biotransformation of arsenobetaine from trimethylathed arsenosugars (pathway #1) and from dimethylathed arsenosugars (pathway #2). Pathway #3: biotransformation of arsenobetaine by amino acid synthesis by simple methylated compounds.

Pathway #1 starts with trimethylated arsenosugars degrading into arsenocholine and then being converted into arsenobetaine. Pathway #1 is supported by the fact that trimethylated sugars have been identified in abalone (*Haliotis rubra*) as 28% of tAs in intestinal tissues [49] and in gastropods from mangrove ecosystems [50] as 6–8% of tAs. However, trimethylated arsenosugars are usually present at very low concentrations in marine organisms, which is unlikely to be the source of arsenobetaine at a high concentration in marine animals [51]. At the same time, rapid uptake or transformation may deplete concentrations of intermediate compounds, and controlled studies with trimethylated arsenosugars have not yet been carried out to assess these possibilities.

Pathway #2 is the most widely described in marine environments, as dimethylated arsenosugars are widely spread and available at the base of aquatic food chains in phytoplankton, algae and microbial mats. Pathway #2 involves the degradation of dimethylated arsenosugars. Dimethylarsinoylethanol (DMAE) is the first product of degradation, and then either (a) AC or (b) dimethylarsinoylacetate (DMAA) act as intermediates (**Figure 3)**. The biotransformation starts with dimethylathed arsenosugars which degradate to give DMAE, followed by an oxidation and subsequent methylation via the proposed intermediate DMAA to the end product arsenobetaine **(Figure 3, pathway #2b)**. The formation via the arsenosugars was supported by a study showing that DMAE was formed after anaerobic decomposition of a brown alga; *Ecklonia radiata* [52]. Besides, Duncan et al. [53] showed the presence of the arsenobetaine precursor DMAE in phytoplankton. DMAA has been demonstrated to be a major degradation product of arsenosugars, second to DMA(V), in sheep [54]. DMAA has also been shown to be a precursor in the formation of AB in laboratory studies involving lysed bacteria extracts [55]. Furthermore, it has been proposed that the formation of AB may occur from the degradation of dimethylated arsenosugars to DMAE intermediate and then to arsenocholine (AC) which is then converted to AB (**Figure 3, pathway #2a**). The conversion of AC to AB has been demonstrated with many laboratory studies with various bacteria, mice, rats and rabbits [38, 56]. However the degradation of arsenosugars to AC has not been as well demonstrated. Studies with shrimp showed that arsenosugars remained unchanged or transformed to trace amounts of DMA(V) suggesting the formation pathway from arsenosugars to AB does not involve AC as an intermediate [56].

Another proposed pathway (**Figure 3, pathway #3**) is based on the amino acid synthesis by simple methylated compounds involving dimethylarsinous acid (DMA(III)) and 2-oxo acids, glyoxylate and pyruvate, to form DMAA and then AB [57, 58].

Arsenobetaine is the major form of arsenic in marine fish and most other seafood [5, 59–61] . Arsenobetaine has also been found in some terrestrial foods, in particular in some mushroom species, although generally as a minor compound [62]. More recently, it was shown that arsenobetaine also occurs in marine algae at low concentrations [63]. Arsenobetaine has not yet been detected in seawater although it is likely present at trace levels. The concentrations of arsenobetaine in freshwater organisms are generally much lower than in marine organisms, often below 0.1 mg As kg^{-1} [64, 65]. However, farmed freshwater fish (aquaculture products) can contain arsenobetaine at higher concentrations because they are provided with feed containing marine ingredients [66].

Arsenosugars

Arsenosugars comprise a dimethylarsinoyl or a trimethylarsonium derivative bound to a ribofuranoside sugar. More than 20 naturally occurring arsenosugars have been identified, most of which are dimethylarsinoylribosides and trimethylarsonoribosides are usually minor constituents [67]. The main commonly found arsenosugars in algae are shown in **Figure 1.** The first arsenosugars were isolated from brown algae *Ecklonia radiata* in 1981 and were sulfonate and glycerol arsenosugars [68]. Sulfate arsenosugars was isolated from the kidney of the giant clam, *Tridacna maxima* in 1982 [52]. The last common arsenosugar, phosphate arsenosugar was identified in 1983 and was also isolated from *Ecklonia radiata* [69].

Arsenic uptake from water by aquatic organisms initially occurs because of its similar chemical properties to the essential macronutrient phosphorus. It is assumed that algae absorb As(V) from seawater and accumulate it as arsenosugars. Algae have a membrane transport system to take up the essential phosphate from seawater, but this cannot distinguish between phosphate and arsenate. This hypothesis is supported where the addition of phosphate in a phytoplankton growth medium decreased the uptake of arsenic in the phytoplankton cells, indicating competition between arsenate and phosphate for cellular uptake [70]. Therefore, to eliminate the toxic arsenate, algae have developed a process of converting it to arsenosugars. This finding has been supported by the study of the detoxification process for the brown alga *Fucus serratus* [71]. The study shows that at low arsenate concentration (20 μg As L^{-1}) the alga takes up arsenate readily and converts it efficiently to arsenosugars while at the high exposure (100 μg As L^{-1}) the detoxification process was overloaded, the toxic arsenic species (presented mainly as arsenite and methylarsonate) accumulated to levels fatal to the alga and arsenosugars were not significantly produced [71].

Arsenosugars appear to be the key intermediates in the biochemical cycling of arsenic. They may serve as precursors to arsenobetaine (**Figure 4**), the major form of arsenic in marine animals. Available evidence indicates that these compounds are formed from arsenate, taken up by algae from seawater, in a process that involves S-adenosylmethionine as both the donor of the methyl groups and of the ribosyl (sugar) group [61, 72, 73]; (**Figure 4**). The biosynthetic pathway proposed, is based on the methylation pathway of arsenic by microorganisms [31]. In this pathway the third methylation step is replaced by an adenosylation step followed by glycosidation [73] (**Figure 4**). This scheme was supported by the identification of the key intermediate arsenosugar-nucleoside in the kidney of the giant clam *Tridacna maxima* [74].

Arsenosugars are usually the major arsenical constituents of marine algae (typically 2-50 mg As kg), and they also are found at significant concentrations in animals feeding on algae (e.g. mussels and oysters; typically 0.5-5 mg As kg) [62]. In terrestrial organisms, arsenosugars occur generally at trace levels only, although interesting exceptions have been reported [75].

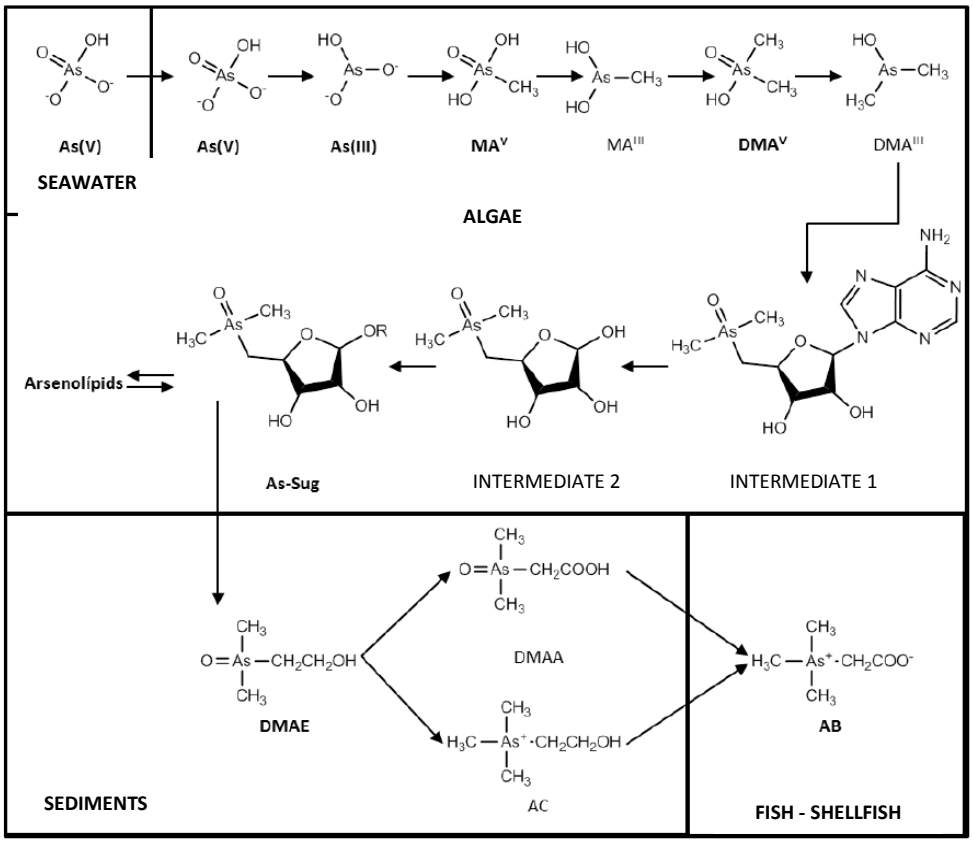


Figure 4. Proposed biosynthetic pathway of metabolic for arsenosugars from arsenate in algae (adapted from [51, 73]

Thio-arsenosugars

Some arsenosugars can exist in two different forms, oxo- and thio-arsenosugars. These compounds are the sulfur analogues of oxo-arsenicals, where the arsinoyl (As=O) group is substituted by an arsinothioyl group (As=S). The structures of the main thio-arsenosugars are presented in **Figure 5 and Table 2**. The first identified thio-arsenical was (thio-dimethylarsinoyl) acetate DMAAS in 2004 in the urine of sheep feed on algae [76] . Several other thio-arsenic species were identified in molluscs, algae, and human urine [77].

Table 2. Species name, abbreviations, formula, molecular weight and CAS for main thio-arsenosugars species.

Compound	Common name	Abbreviation	Formula	Molecular weight	CAS number
19	Thio-arsenosugar glycerol	Thio-OH	$C_{10}H_{21}AsO_6S$	344.26	761458-55-3
20	Thio-arsenosugar phosphate	Thio-PO_4	$C_{13}H_{28}AsO_{11}PS$	498.32	761458-56-4
21	Thio-arsenosugar sulfonate	Thio-SO_3	$C_{10}H_{20}AsO_8S_2$	407.31	1227407-67-1
22	Thio-arsenosugar sulfate	Thio-SO_4	$C_{10}H_{20}AsO_9S_2$	423.31	1227407-68-2

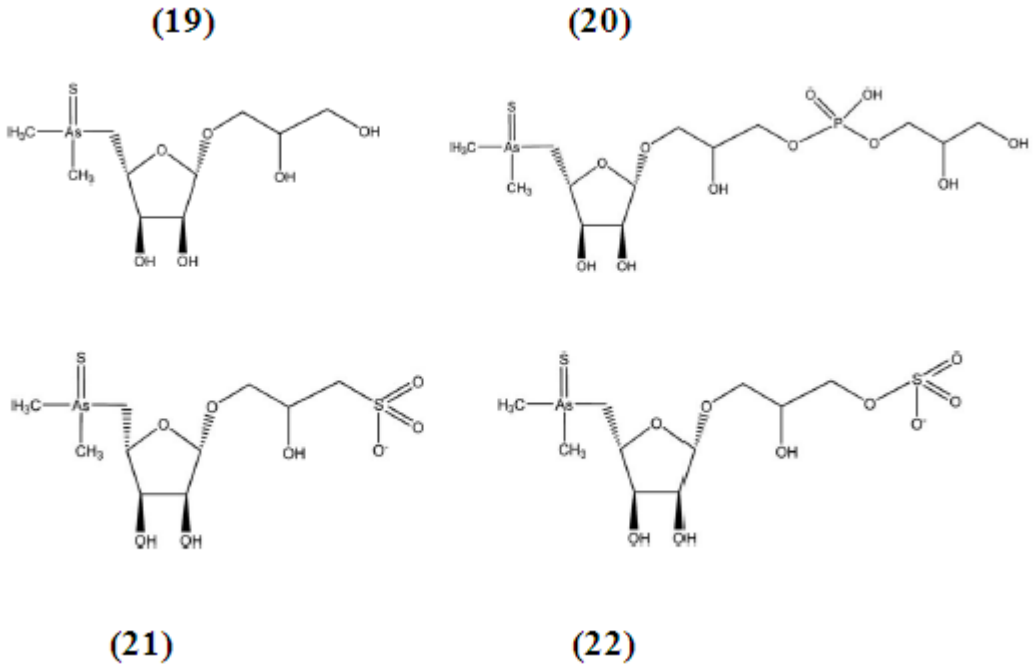


Figure 5. Structures of the main thio-arsenosugars species: thio-arsenosugar glycerol (19); thio-arsenosugar phosphate (20); thio-arsenosugar sulfonate (21); thio-arsenosugar sulfate (22)

Arsenolipids

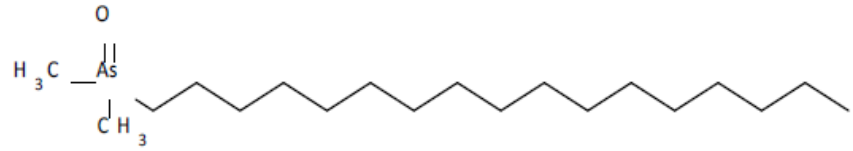
Arsenolipids is a broad term for all fat-soluble naturally occurring compounds that contain arsenic (**Table 3 and Figure 6**). Arsenolipids have been much less investigated compared to the water-soluble arsenicals and are present in marine oils, such as fish oils and oils extracted from algae. However, the distribution of these compounds in various marine organisms is not well studied. The arsenolipids compounds have shown to vary from organism to organism [78].

Table 3. Common name, abbreviations, formula, molecular weight and CAS number for arsenolipids: arsenic hydrocarbons, arsenic fatty acids and arsenic phospholipids.

Compound	Common name	Abbreviation	Formula	Molecular weight	CAS number
23	**Arsenic hydrocarbons**	AsHC	$C_{19}H_{39}AsO$	358.44	1456610-45-9
			$C_{19}H_{41}AsO$	360.46	1083077-43-3
			$C_{20}H_{43}AsO$	374.48	1423745-42-9
			$C_{21}H_{45}AsO$	388.51	1393357-63-5
			$C_{22}H_{47}AsO$	402.54	1423745-43-0
			$C_{24}H_{39}AsO$	418.5	1456610-47-1
24	**Arsenic fatty acids**	AsFA	$C_{17}H_{35}AsO_3$	362.39	1032052-02-0
			$C_{22}H_{35}AsO_3$	422.44	1423745-44-1
			$C_{22}H_{37}AsO_3$	424.46	1423745-45-2
			$C_{23}H_{37}AsO_3$	436.47	1032052-10-0
			$C_{24}H_{37}AsO_3$	448.48	1296225-43-8
			$C_{43}H_{84}AsO_{14}P$	931.03	1423745-30-5
			$C_{43}H_{84}AsO_{14}P$	931.03	1423745-46-3
			$C_{44}H_{86}AsO_{14}P$	945.05	1423745-31-6
			$C_{45}H_{88}AsO_{14}P$	959.08	115921-38-5
			$C_{45}H_{86}AsO_{14}P$	957.07	1423745-40-7
25	**Arsenic phospholipids**	AsPL	$C_{45}H_{84}AsO_{14}P$	955.05	1393357-60-2
			$C_{46}H_{90}AsO_{14}P$	973.11	1423745-32-7
			$C_{47}H_{86}AsO_{14}P$	981.09	1423745-39-4
			$C_{47}H_{92}AsO_{14}P$	987.14	1423745-34-9
			$C_{47}H_{86}AsO_{14}P$	981.09	1423745-47-4
			$C_{47}H_{90}AsO_{14}P$	985.12	1423745-37-2
			$C_{47}H_{88}AsO_{14}P$	983.1	1423745-38-3
			$C_{48}H_{94}AsO_{14}P$	1001.16	1423745-35-0
			$C_{49}H_{96}AsO_{14}P$	1015.19	1393357-61-3
			$C_{51}H_{100}AsO_{14}P$	1043.24	1631038-74-8
			$C_{53}H_{104}AsO_{14}P$	1071.3	1393357-62-4

The presence of fat-soluble arsenic compounds were first reported in fish in the late 1960s [79]. Several years later, in 2008 some structures of the first reported lipid-soluble arsenic compounds were finally elucidated and six fatty acids containing arsenic were identified in cod liver oil [80] and three arsenic-containing hydrocarbons in capelin oil [81]. Several other arsenolipids were present in the oils whose structures are still unknown. Arsenolipids were recently found in tuna, which is the first identification of arsenolipids in commonly consumed seafood [82] and can occur in a wide range of biological samples and can reach concentrations over 90% of the tAs [78]. Even though arsenolipids appear to be common, especially in fatty fish, quantitative data is scarce. In the fish oils examined so far, the arsenolipid content varied between about 4-12 mg arsenic/kg of oil [78, 81]. Although the first investigations on the lipid-

soluble arsenic in marine oils were reported more than 40 years ago, knowledge regarding their biosynthesis, chemical structures, levels and toxicity is still limited [83].



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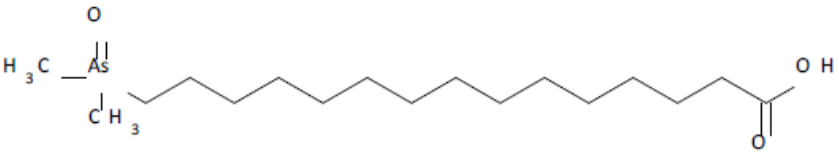

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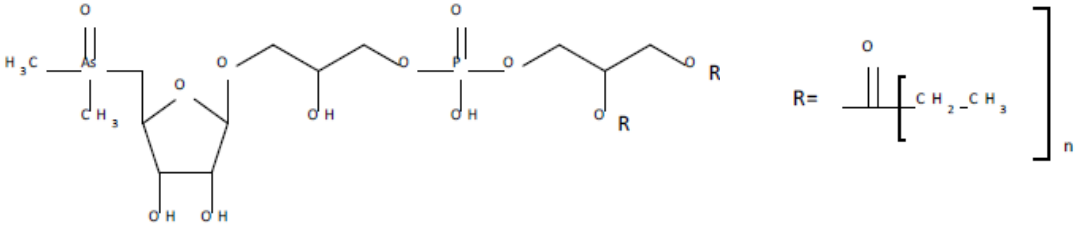

(25)

Figure 6. Structures of the arsenic hydrocarbons (23), Arsenic fatty acids (24) and arsenic phospholipids (25). Structures are generalised and do not show degree of saturation for fatty acids, hydrocarbons or phospholipids.

1.4 Toxicity of arsenic species

The toxicity of arsenic is dependent on its chemical form and the oxidation state (Irvin 1995) and also on how they are metabolized in the body [54]. The oxidation state and chemical form of arsenic are important factors that affect toxicity and they may be changed by biological processes. Therefore the metabolic pathway of arsenic species needs to be considered.

In toxicology, the most common manner to evaluate the acute toxicity are LD_{50} and LC_{50}. The median lethal dose, LD_{50} (abbreviation for "lethal dose, 50%") or LC_{50} (“lethal concentration, 50%”) is a measure of the lethal dose of a toxin, radiation, or pathogen. The value of LD_{50} for a substance is the dose required to kill half the members of a tested population after specified test duration. LD_{50} figures are frequently used as a general indicator of a substance acute toxicity. A lower LD_{50} is indicative of increased toxicity. Two types of arsenic toxicity have been described:

· *Acute*: caused by ingestion of high amounts of inorganic forms of arsenic where this has shown to have effect on almost all physiological systems of the body and can be lethal. Acute exposure to some arsenic compounds can cause death. As commented, a common parameter to evaluate acute toxicity is the LD_{50} and values of several arsenic species are displayed in **Table 4**.

· *Chronic*: humans can be chronically exposed to iAs mainly through drinking water. Effects of prolonged exposure to arsenic can lead to cancer of the skin, bladder, lung as well as other cancers [84, 85], and to skin lesions such as skin hyperpigmentation and keratosis. Chronic exposure can further include effects on the peripheral nervous system, the central nervous system and has been associated with cardiovascular diseases [86] . The effects of chronic exposure of organic arsenic are not fully known [5].

Table 4. Acute toxicity (LC_{50} and LD_{50} values) of some arsenic species.

As species	**LC_{50}** [a] (μmol L^{-1})	**LD_{50}** [b] (mg kg^{-1})	**References LD_{50}**
DMA (III)	2.16	-	
As(III)	5.49	14.0-42.9	[87]
As(V)	571	20-800	[88]
DMA (V)	843	1.200-2.600	[89]
MA (V)	-	700-1.800	[90]
MA (III)		3.5	[87]
AC		6.500	[91]
AB	-	>10.000	[92]
TETRA	-	890	[90]
TMAO		10600 [c]	[89]

[a] LC_{50} values for human cells [93]. [b] LD_{50} for mice (oral ingestion)

Arsenic speciation studies have revealed the dependence of toxicity according to the species of arsenic [94]. The different toxicities of the As species reinforce the importance of its chemical speciation, as the total amount of As does not provide enough information about the toxicity of the analysed sample. In general, it can consider the following gradation in toxic character of arsenic compounds [95].

:

R_3As (R = H, Me, Cl) > As_2O_3 (As(III)) > As_2O_5 (As(V)) > $RnAsO(OH)_3$-n (n = 1,2) > $R_4\ As^+$

Inorganic arsenic (arsenite or As(III) and arsenate or As(V)) is considered the most dangerous form due to its biological availability, as well as physiological and toxicological effects (iAs is classified as a non-threshold, class 1 human carcinogen) [96]. Inorganic arsenic compounds are generally more toxic than the organic arsenic compounds and trivalent arsenic is considered more toxic than pentavalent arsenic [12]. It has been proposed that the acute toxicity

of trivalent arsenicals is because of their binding to thiol groups of biologically active proteins thus inhibiting the function of various metabolic enzymes [97]. The acute toxicity generally decreases with increasing degree of methylation (**Table 4**), with the exception of TETRA, whose acute LD_{50} value is lower than for the other methylated compounds (MA, DMA, TMAO) [98]. The chronic toxicity of most organic arsenicals, such as MA and DMA, has not been decisively established [5]. AB is considered non-toxic and can be consumed without concern [34], and AC essentially nontoxic [99]. Arsenosugars are not acute toxic but there is a possibility that they might have chronically toxic effects as their toxicity and metabolism have only been sparsely studied [100]. Thus, not much is known about the toxicity of arsenosugars, which are commonly found in algae and could be considered as potentially toxic [100] since they are biotransformed by humans into toxic organoarsenicals [101]. In addition, lipid-soluble As compounds (arsenolipids) have been reported as major compounds of arsenic in fatty fish and their toxicity is not yet known [2, 100].

1.5 Arsenic dietary exposure and risk assessment

In 1989, the Joint Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) Expert Committee on Food Additives (JECFA) established a provisional tolerable weekly intake (PTWI) of 15 μg kg^{-1} body weight (bw) for iAs (equivalent to 2.1 μg kg^{-1} bw per day) [102]. In 2009, the EFSA Panel on Contaminants in the Food Chain (CONTAM Panel) assessed the risks to human health related to the presence of arsenic in food in European population [5]. More than 100,000 occurrence data on arsenic in food were considered but approximately 98 % reported as total arsenic. The highest total arsenic levels were measured in the following food commodities: fish and seafood, food products or supplements based on algae, especially hijiki, and cereal and cereal products, with particularly high concentrations in rice grains and rice-based products, and bran and germ.

The inorganic arsenic exposure from food and water across 19 European countries has been estimated to range from 0.13 to 0.56 μg kg^{-1} body weight (b.w.) per day for average consumers and from 0.37 to 1.22 μg kg^{-1} b.w. per day for 95th percentile consumers. The food subclasses of cereal grains and cereal based products, followed by food for special dietary uses, bottled water, coffee and beer, rice grains and rice based products, fish and vegetables were identified as largely contributing to the inorganic arsenic daily exposure in the general European population. A simplified overview of arsenic species in some food commodities is shown in **Figure 7**. Feldmann and Krupp [100] proposed a strategy for such a routine analytical approach to classify the arsenic compounds into three fractions according its toxicity: (i) the toxic iAs; (ii) AB as established non-toxic arsenical; (iii) the leftover organoarsenical fraction, which may contain arsenosugars and other organoarsenicals, including non-water extractable, fat-soluble or lipophilic arsenic compounds; those would have to be reported as the sum of potentially toxic arsenicals. The expected proportions of this arsenic fractions in fish, seaweed, molluscs and rice are illustrated **Figure 7**. As can be noted, high tAs content is found in marine food commodities compared to rice. The speciation pattern reveals that iAs is predominant in rice and is expected to be low than 5% in marine food commodities. Meanwhile the non-toxic arsenobetaine is the

major compounds in fish and mollusks. Furthermore, it is illustrated the importance of the potentially toxic fraction of which arsenosugars in seaweed and molluscs are the important species.

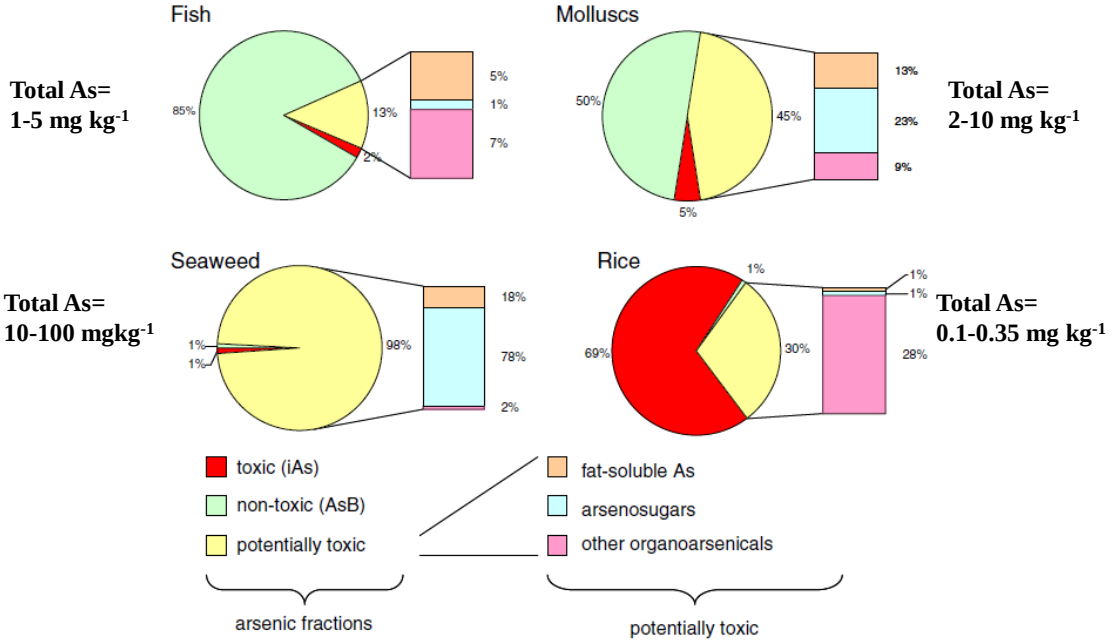

Figure 7. Expected proportions of the three different arsenic fractions in fish, seaweed, molluscs and rice (adapted from [100]).

The EFSA CONTAM Panel concluded that dietary exposure to inorganic arsenic for children under three years of age is in general estimated to be from 2 to 3-fold that of adults [5]. It was concluded that the provisional tolerable weekly intake (PTWI) established by JECFA was no longer appropriate as data had shown that inorganic arsenic causes cancer of the lung and urinary bladder in addition to skin, and that a range of adverse effects had been reported at exposures lower than those reviewed by the JECFA. The CONTAM Panel modelled the dose-response data from key epidemiological studies and selected a benchmark response of 1 % extra risk. A range of benchmark dose lower confidence limit ($BMDL_{01}$) values between 0.3 and 8 µg kg^{-1} b.w. per day was identified for cancers of the lung, skin and bladder, as well as skin lesions. Besides, the estimated dietary exposures to inorganic arsenic for average and high level consumers in Europe were within the range of the $BMDL_{01}$ values identified, and therefore they concluded that there is little or no margin of exposure and the possibility of a risk to some consumers cannot be excluded.

Four recommendations among the eight proposed by EFSA concerned analytical methodology:

- Dietary exposure to inorganic arsenic should be reduced.
- In order to refine risk assessment of inorganic arsenic, there is a need to produce speciation data for different food commodities to support dietary exposure assessment and dose-response data for the possible health effects.

- Although several arsenic speciation methods have been reported, their suitability for a range of food samples and/or arsenic species needs to be established.
- There is a need for robust validated analytical methods for determining inorganic arsenic in a range of food items.
- Certified reference materials especially for inorganic arsenic in products such as water, rice and seafood are required. The production of such a material should be a priority to facilitate future surveys of the inorganic arsenic content of foods.
- Future epidemiological studies should incorporate better characterization of exposure to inorganic arsenic including food sources.
- There is a need for more information on critical age periods of arsenic exposure, in particular in early life. Studies should include effects later in life of early life arsenic exposure.
- There is a need for improved understanding of the human metabolism of organoarsenicals in foods (arsenosugars, arsenolipids etc.) and the human health implications.

Also the FAO/WHO Joint Expert Committee on Food Additives (JECFA) [103] has evaluated dietary exposure to iAs. Similar conclusions as EFSA report were published, highlighting that: there is a need for validated methods for selective extraction and determination of iAs in food matrices and for CRMs for iAs. There is a need for improved data on occurrence of different species of arsenic in, and their bioavailability from, different foods as consumed in order to improve the estimates of dietary and systemic exposure. Further information on the toxicity of arsenic species found in food is also required. It was recommended that future epidemiological studies of the health impacts of arsenic should incorporate appropriate measures of total exposure to iAs, including from food and from water used in cooking and processing of food. Finally, the Committee further recommended that epidemiological studies not only focus on relative risks, but also analyse and report the data such that they are suitable for estimating exposure levels associated with additional (lifetime) risks, so as to make their results usable for quantitative risk assessment. Besides, the inorganic arsenic lower limit on the benchmark dose for a 0.5% increased incidence of lung cancer ($BMDL_{0.5}$) was determined from epidemiological studies to be 3.0 μg/kg bw per day (2–7 μg/kg bw per day based on the range of estimated total dietary exposure) using a range of assumptions to estimate total dietary exposure to inorganic arsenic from drinking-water and food. The Committee noted that the provisional tolerable weekly intake (PTWI) of 15 μg/kg bw is in the region of the $BMDL_{0.5}$ and therefore was no longer appropriate. The Committee withdrew the previous PTWI.

Recently, in 2014 EFSA evaluate the dietary exposure to inorganic arsenic in the European population and provided information on the levels of arsenic (tAs and iAs) found in a range of foods on the European market [104]. A dataset comprised of 103,773 food samples (including drinking water) collected in 21 European countries was used to calculate the dietary exposure to iAs. Of these, 101,020 were based on tAs and 2,753 on iAs. Among the reported results on tAs, 66.1 % were below the limit of detection or quantification; meanwhile for the

reported data on iAs the percentage of data was 41.9 %. Most of the data (92.5 %) reported as tAs were converted to iAs using different approaches (in general a conversion factor of 70 % was used) before calculating dietary exposure to iAs. Dietary exposure estimated among the adult population was considerably lower compared to those in the 2009 EFSA opinion (EFSA, 2009), ranged from 0.09 to 0.38 µg kg^{-1} b.w. per day, and 95th percentile dietary exposure estimates ranged from 0.14 to 0.64 µg kg^{-1} b.w. per day. Among the reported conclusions, the main contributor to dietary exposure to iAs was the food group 'Grain-based processed products (non rice-based)' (**Figure 8**), in particular, wheat bread and rolls, for all the age classes except infants and toddlers. Other food groups that were important contributors to iAs exposure were rice, milk and dairy products (main contributor in infants and toddlers), and drinking water.

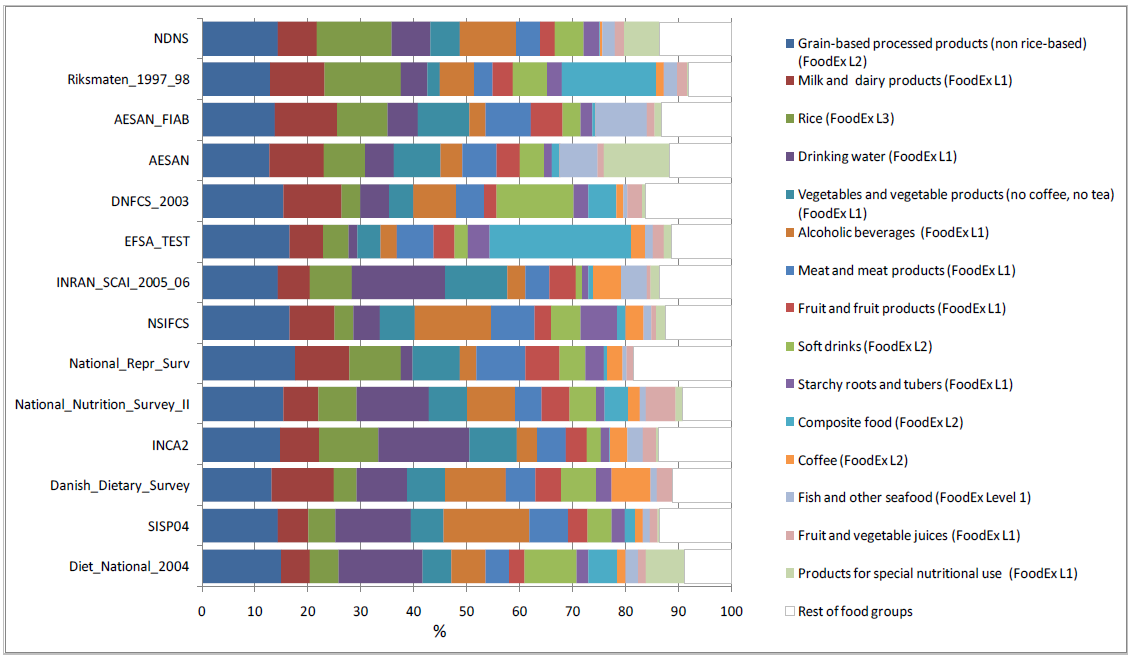

Figure 8. Main food groups contributing (%) to the mean chronic dietary exposure to iAs for the age class '18 to 65 years old' (Adapted from [104]).

Besides, the highest dietary exposure was estimated in the younger population (infants and toddlers) and the mean dietary exposure ranged from 0.20 to 1.37 µg/kg b.w. per day, while the 95th percentile dietary exposure estimates ranged from 0.36 to 2.09 µg/kg b.w. per day. The main contributors were 'Milk and dairy products' followed by 'Drinking water', 'Grain-based processed products (non rice-based)' and 'Food for infants and young children' (**Figure 9**). Consumption of three portions (90 grams/day) of rice-based infant food could represent an important source of iAs (1.59- 1.96 µg/kg b.w. per day). Finally it was stated that, the most important sources of uncertainty in the present assessment are related to the heterogeneity of the food consumption data, the conversion of tAs into iAs and to the treatment of the left-censored data. Furthermore, as a recommendation more analytical data on iAs would be needed, in particular in fish and seafood, and in food groups that provide a significant contribution to the

dietary exposure to iAs (e.g. rice and wheat-based products) in order to reduce the uncertainty of the exposure assessments to iAs.

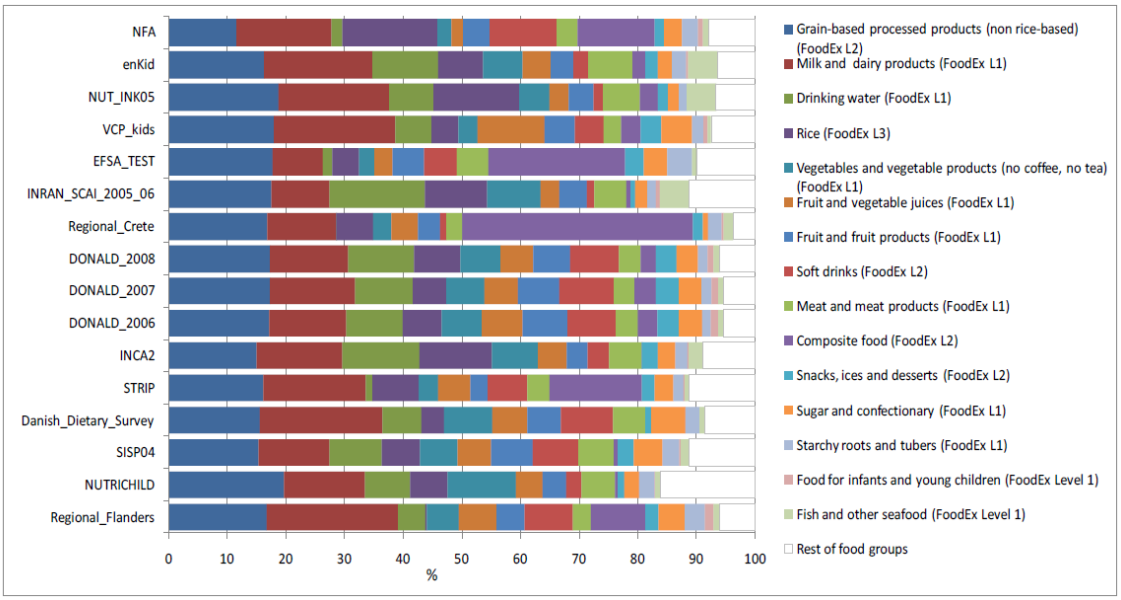

Figure 9. Main food groups contributing (%) to the mean chronic dietary exposure to iAs for the age class '3 to 10 years old' (adapted from [104]).

1.6. European Legislation

The EU has set a maximum limit of 10 μg As L^{-1} for arsenic in water intended for human consumption based on the recommendation of WHO [105]. The directive 2002/32/EC on undesirable substances in animal feed sets maximum contents for total arsenic in a number of feed commodities (refer to a feedingstuff with a moisture content of 12 %) [106]. The directive states that contents of iAs below 2 mg kg^{-1} are recommended in feed commodities, especially those based on the seaweed species *Hizikia fusiforme* Very recently, the European Union published Regulation (EU) 2015/1006 [107]amending Annex to Regulation (EC) No 1881/2006 [108] regarding the maximum levels of iAs in rice and rice-based products. The new MLs of iAs range from 0.10 to 0.3 mg As kg^{-1} depending of the rice product. Furthermore, a new recommendation has recently published by European Union [109]. Member States should perform a monitoring on the presence of arsenic in food during the years 2016, 2017 and 2018. The monitoring should include a wide variety of foodstuffs reflecting consumption habits including food such as cereal grains, cereal based products (including bran and germ), fruit and vegetable juices, drinking water (including bottled water), coffee, dry tea leaves, beer, fish and sea food, vegetables, algae products (including hijiki), milk, dairy products, food intended for infants and young children, food for special medical purposes and food supplements in order to enable an accurate estimation of exposure. Member States should carry out the analysis of arsenic, preferably by determining the content of iAs and tAs and, if possible, other relevant

arsenic species by making use of a method of analysis that has been proven to generate reliable results.

1.7 Analytical techniques for the determination of total arsenic, arsenic species and bioaccessible arsenic

An exhaustive revision of analytical methods and measurement techniques for inorganic arsenic determination are reviewed and shown in **Article I.** Thus, in this section a general overview of analytical techniques is presented.

1.7.1 Total arsenic determination

Recent reviews indicate that the main techniques used for the determination of arsenic in biological samples are graphite furnace atomic absorption spectrometry (GFAAS), inductively coupled plasma optical emission spectrometry (ICPOES), inductively coupled plasma mass spectrometry (ICPMS) and hydride generation atomic absorption spectrometry (HG-AAS) [110, 111]. Appropriate selection of the sample preparation procedure in trace analysis is essential due to the integrity of chemical information that strongly depends on the initial steps. The most frequently used methods in the preparation of food samples are dry ashing and microwave-assisted acid digestion [112]. The presence of arsenobetaine (AB) in fish and marine species could be a problem in the determination of tAs by GFAAS, HG-AFS or HG-AFS. This species is considered a stable metabolic species and its chemical decomposition is very difficult [51, 113]. The conversion of all organic arsenic species into iAs is usually required or the determination of tAs by atomic spectrometry. Consequently, the high stability of AB becomes unfavorable for the determination of the tAs content [114]. Wet digestions using strong oxidizing agents combined with strong acids and high temperatures (280ºC), are required for complete degradation of AB [115]. In some cases, even with the use of these reagents at higher temperatures, AB is not degraded completely and tAs content can be easily underestimated [116, 117]. Thus, it is necessary to be aware of this fact in order to select the most suitable digestion procedure to overcome this problem.

1.7.2 Arsenic speciation

General considerations of arsenic speciation

In analytical element speciation the key parameter is to ensure that there are no alterations of these arsenic species across the overall analytical process, including sampling. Speciation of arsenic is still challenging, particularly in analysis of food samples with a complex organic matrix. The goal of speciation is the quantitative extraction of all arsenic species without changing their original characteristics. The main steps in speciation analysis are: (1) extraction, (2) separation and (3) measurement and quantification of species. As an example, the steps in arsenic speciation analysis by HPLC-ICPMS are shown in **Table 5**. The steps need proper optimization and evaluation to obtain a quantitative extraction and guarantee minimal changes to the original species, especially in complex matrices, such as different foodstuffs. For

instance, the selection of extractants and the apparatus used are crucial in step 1; mobile phases are carefully considered to achieve an adequate chromatographic separation (2); and finally the selection of the most suitable ICPMS conditions is the paramount importance in the step 3.

Table 5. Steps in arsenic speciation by HPLC-ICPMS.

Step	Evaluation
Extraction	Selection of extractants
	Maintaining integrity of species
	Quantitative extraction
Separation (HPLC)	Selection of mobile phases
	Interaction of species with column
	Availability of standards
	Elution mode
Measurement and quantification (ICPMS)	Nebulization
	Monitored masses
	Interferences

Extraction of arsenic species

According to Maher and colleagues [118], metalloid species could be classified as: "Easy to extract species," stable species existing as discrete molecules or relatively weakly bound to cellular constituents, and "Hard to extract species," unstable species that dissociate on extraction and species incorporated within cellular constituents such as proteins [118]. In case of arsenic, aqueous soluble arsenic species and lipid soluble arsenic species were classified in the first group meanwhile arsenic phytochelatins (PC) in plants in the second. Therefore, the selection of a suitable extractant solvent is the paramount importance in arsenic speciation analysis. In general, the extraction conditions can vary greatly depending on the extractant:sample ratio, the extracting approach used and the ranges of time and temperature. Extraction conditions influence not only the extraction efficiency but also the integrity of the native arsenic species during extraction. The extraction protocol should be optimized to obtain reliable results on the basis of the extraction efficiency. Finally, it is crucial to pay special attention to the stability of arsenic species in the extracts. Another crucial point is to ensure stability of As species at the stages of sample storage and pre-treatment by using appropriately selected sample conservation, since several factors may promote inter-conversion of As species (e.g., microbial activity, temperature, and light) [10, 119, 120].

A wide variety of extraction solvents have been used in foodstuffs: mixtures of MeOH:water, water, acids, bases, sequential extraction and enzymatic are the most used supported by mechanical extraction, hot plate extraction, ultra-sonic extraction, heating in a water bath, pressure extraction or microwave-assisted extraction [10, 119, 120]. Crucial methodological parameters that could affect extraction efficiency and species stability are: the type of solvent, the sample particle size, the solid/liquid ratio, the extraction time and

temperature; and, the extraction technique. Methanol, water and methanol–water mixtures are commonly used to extract water-soluble As species from marine algae and animals, but are inefficient in extracting iAs species from, terrestrial animals. For these samples the use of a dilute acid is recommended. Sequential extraction procedures, for instance: methanol-water extraction followed by dilute acid extraction, will increase the extraction efficiencies of hard to extract As species. Microwave-assisted extraction is now widely used and has been shown to give better recoveries relative to mechanical mixing and sonication [10]. Some As species such as the arsenosugars and AB are relatively stable but As(III), As(V), MA(III), DMA(III), As-GSH and As–PC species are not. Considerable care should be taken to ensure that the As species determined are not artifacts of the preservation or extraction procedures. Speciation of As in food samples requires extraction under mild conditions in order to maintain integrity of all As species. Problems associated with low recovery of As species and oxidation or reduction between As(III) and As(V) species, and conversion of organic species to iAs species have been reported [119].

Techniques

The speciation analysis usually involves many steps, including extraction, separation and detection. Several methods have been employed to perform arsenic speciation analysis. However, the appearance of coupled-techniques has allowed the development of very powerful analytical methods for speciation purposes. The most common separation techniques used for this purpose, are gas chromatography (GC), high-performance liquid chromatography (HPLC), including ion chromatography (IC), capillary electrophoresis (CE) and field-flow fractionation (FFF), among others. Among the detectors coupled to a separation technique, HG-AAS, HG-AFS, ICPOES and especially ICPMS, with HG and without HG, are those most used among the element specific detectors [11, 121]. The selection of the separation technique will be determined by the properties of the arsenic species of interest, such as volatility, charge and polarity, whereas the detection technique is determined by the expected concentration level in the assayed sample. The most common analysis used for arsenic speciation is HPLC-ICPMS (**Figure 5**). Furthermore, there are additional analytical methods suitable for obtaining data to complement the information on arsenic speciation obtained when applying the aforementioned method, for instance: X-ray absorption spectroscopy (XAS) and electrospray mass spectrometry (ESI-MS) [122]. Analytical methods and measurement techniques for inorganic arsenic determination are reviewed and shown in **Article I.**

1.7.3 Determination of arsenic bioaccessible content

Food provides nutrients, but also non-nutritional components and contaminants. The consumption frequencies are needed to evaluate the risks and benefits associated to the intake of a given food. Furthermore, such evaluation must take into account that foods are typically subjected to further culinary treatment before ingestion. Cooking affect total arsenic content and also arsenic species distribution. For a better knowledge of the risks and benefits associated to food consumption, the assessment of arsenic bioavailability, total content and arsenic species, is fundamental for complete food safety assessment. Bioavailability refers to the fraction of the

substance that reaches the systemic circulation (blood) from the gastrointestinal (GI) tract (bioavailable fraction) and which is available to promote its action in the exposed organism [123]. A first step in bioavailability assessment is the study of bioaccessibility, which indicates the maximum fraction of a trace element or other substance in food that is theoretically released from its matrix in the GI tract (bioaccessible fraction), and thus becomes available for intestinal absorption (i.e. enters the blood stream) [124].

Both *in vitro* and *in vivo* methods for evaluating arsenic bioavailability in food have been proposed [125–127]. Each approach has its own strengths and weaknesses for the assessment of bioaccessibility (Fernandez-Garcia 2009). The *in vivo* is mainly advantageous due to its closeness to reality, particularly so, if individuals chosen as experiment subjects belong to the target population but is very time demanding, requires carefully planning and specific resources for an adequate experimental control, and has some ethical constraints. On the other hand, the *in vitro* methods provide an effective approximation to *in vivo* situations and offer the advantages of good reproducibility, simplicity, rapidity, ease of control, low cost and high precision, as it is possible to control conditions better than with *in vivo* tests [126]. Experimental conditions are controlled to a much higher degree and validation and standardization with reference materials is possible, which enables reproducibility and reduces uncertainty [125]. Appropriate temperature, shaking, pH, type of enzyme and chemical composition should be selected to simulate the gastric and/or gastrointestinal conditions. For the *in vitro* methodological approaches, most studies only address the availability for intestinal absorption. For this purpose, there is a division between static and dynamic digestive modeling. In the static methodologies, the biochemical reactivity found in the human GI tract (oral cavity, gastric environment, and intestinal lumen) is sequentially simulated. Dynamic methodologies are intended to be more realistic, encompassing various phenomena that occur in vivo, such as, shear, mixing, hydration, or peristalsis. Moreover, these methodologies attempt to simulate how conditions change over time during each main digestive stage (mouth, stomach, and intestine).

Various *in vitro* approaches to evaluate the bioaccessibility have been reported in the last years [124–127]. The most applied *in-vitro* approaches to measurement bioaccessibility are: (1) the maximum soluble concentration of the target compound in the simulated GI solution (bioaccessible fraction); (2) the soluble fraction of the compound (BA fraction) achieved by using human GI microbiota (Simulator of the Human Intestinal Microbial Ecosystem, SHIME); (3) the dialyzable fraction of the compound, which can dialyze through a semi-permeable membrane with a specified pore size (dialysate or bioavailable fraction) at equilibrium or non-equilibrium conditions; and,(4) the fraction of the compound capable of being retained or transported through a solid or micro porous supports (bioavailable fraction) in which human Caco-2 cells grown are incorporated (intestinal epithelial model) [126].

A limited number of arsenic bioaccessibility studies has been conducted, mostly concerning conventional food items; fish and shellfish [128, 129], edible seaweeds [24, 128, 130, 131], rice [132], vegetables [133, 134] and country foods (food obtained by hunting and gathering) from contaminated sites in Canada [135]. There is thus a lack of data on the bioaccessibility of arsenic species in foodstuffs considering the effect of cooking. In order to improve the risk assessment process, the need to perform such studies seems to be evident.

1.8 Quality assurance in speciation analysis

The determination of arsenic species is still not a routine procedure, and hence clear quality criteria are not yet established. The reliability of speciation data depends on the accuracy of the speciation method. For this, the need to minimise errors that can occur during sampling, sample preparation, separation and detection is of paramount importance. A common way to verify analytical procedure and to evaluate the accuracy of the method is to check it with certified reference materials (CRMs). These should be as similar as possible to real sample and should be treated in the same way as the other samples [136, 137]. CRMs offer an excellent way to ensure that the employed method provides acceptable results. Several CRMs are available for tAs content in several matrices. However, only a few CRMs for arsenic speciation are commercially available. Furthermore, as external QC, proficiency tests or interlaboratory comparisons are a valuable tool to test the reliability of a method by comparing results with an assigned reference value. A summary of CRMs as well as PTs for arsenic speciation analysis is fully discussed and described in **Article I.**

Chapter 2

Recent developments and quality assessment of inorganic arsenic determination in food: a review

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Recent developments and quality assessment of inorganic arsenic determination in food: a review

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ABSTRACT

Here we review recent developments in analytical proposals for the assessment of the inorganic arsenic (iAs) content of food products. Interest in the determination of iAs in food is fueled by the wide recognition of its toxic effects on humans, even at low concentrations. Currently, the need for robust and reliable analytical methods is recognized by various international safety and health agencies, and by organizations in charge of establishing acceptable tolerance levels of iAs in food. This review summarizes the state of the art of analytical methods while highlighting tools for the assessment of quality assurance of the results, such as the production and evaluation of certified reference materials (CRMs) and the availability of specific proficiency testing (PT) programs.

Since the number of studies dedicated to the subject of this review has increased considerably over recent years, the sources consulted and cited here are limited to those from 2010 up to the present (May 2015).

Keywords: Inorganic arsenic; Food analysis; Analytical techniques; Quality assurance; Proficiency testing; Certified reference materials.

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1. INTRODUCTION

The determination of inorganic arsenic (iAs) in food is considered a subject of paramount importance. Of the great number of known arsenic species that have been identified in different types of food, arsenic health concerns are derived primarily from the inorganic forms of this element. Moreover, food is the main contributor to human arsenic intake (excluding occupational exposure and drinking contaminated water). This interest is supported by a huge number of publications in the literature over many years [1]. The most recent reviews highlight new research concerning both the toxic and carcinogenic character of iAs (although the mechanisms of arsenic carcinogenicity remain unclear) [2–5]. Two reviews use the meta-analysis of toxicity data [6,7] to obtain information concerning the assessment of iAs exposure risk or the possible dose–response relationship, among other approaches. Mechanisms involved in the pathogenesis of arsenic-induced toxicity have been reviewed [8]. Among the studies of the toxicity of iAs, vulnerable groups are especially considered, such as children [9–11] and pregnant women [12].

The toxic effects of inorganic arsenic forms led the Joint Commission FAO/WHO in 1989 to set a provisional tolerable weekly intake (PTWI) for inorganic arsenic of 15 μg kg^{-1} of body weight (equivalent to 2.1 μg kg^{-1} bw per day) [13]. Recently, the European Food Safety Authority (EFSA) [14] and the JECFA (Joint FAO/WHO Expert Committee on Food Additives) [15] evaluated dietary exposure to iAs. Both concluded that the PTWI parameter is no longer appropriate and should no longer be used and it is thus withdrawn. The EFSA and JECFA evaluations provided estimates of toxic intake limits for iAs as a benchmark dose level (BMDL): 0.3–8 μg kg^{-1} b.w. per day for cancers of lung, skin and bladder as well as for skin lesions (EFSA $BMDL_{01}$ [14]); and 3.0 μg kg^{-1} b.w. per day (2-7 μg kg^{-1} b.w. per day based on the estimated range of total dietary exposure) for lung cancer (JECFA $BMDL_{0,5}$ [15]). Also, both reports emphasized the need to produce speciation data, particularly iAs data, for different food products to estimate the health risk associated with dietary As exposure. EFSA and JECFA highlighted the need for a robust, validated analytical method for the determination of iAs in a range of food items; and the need for certified reference materials (CRMs) for iAs. In 2014, EFSA evaluated dietary exposure to iAs in the European population [16]. It concluded that for all ages except infants and toddlers, the main contributor to dietary exposure to iAs is the food group: "grain-based processed products (non-rice-based)". Other food groups that were important contributors to iAs exposure were rice, milk and dairy products (the main contributor in infants and toddlers), and drinking water. Furthermore, in order to reduce the uncertainty in the assessment of exposure to iAs, more analytical data on iAs are needed. This mainly refers to speciation data in fish and seafood, and for food groups that contribute substantially to dietary exposure to iAs (e.g., rice and wheat-based products).

Rice and rice-based products are the type of food in which iAs toxicity is of most concern in many countries [17–22]; especially in countries, such as those in Southeast Asia, where irrigation practices increasingly include flooding with water containing arsenic. This can lead to an increase of the arsenic contents of rice and so control of such practices is frequently called for [23]. The other type of food product that merits special interest regarding iAs toxicity is those

with a marine origin [24–27] and in lesser extent other food commodities such as apple juice [28] or mushrooms [29]. Furthermore, the assessment of iAs concentrations in food products aimed particularly at children deserves special interest [30–33]. Other studies also reveal that knowledge of iAs contents is important in the control of processes of biotransformation in marine organisms that constitute a food source, after exposure to iAs compounds [34]. Lynch et al. [35] considered four food groups, in accordance with their iAs contents, reporting estimated mean values as: *seaweed/algae/seafood*, 11,000 μg kg^{-1} for seaweed/algae and 130 μg kg^{-1} for seafood; *rice*, 130 μg kg^{-1} ; *apple juice*, 5.8 μg kg^{-1} ; and *infant food*, rice, other cereals and related products, 92 μg kg^{-1} and vegetables, 20 μg kg^{-1} .

Guidelines and directives regulating iAs the contents of food have been assessed and the establishment of maximum levels (MLs) is emphasized [36–44]. Meharg and Raab [45] discusses several proposals and relates them with detection capacities and the availability of measurement techniques, highlighting the assessment of iAs contents. Among the regulations proposing MLs of arsenic tolerated in food, few establish specific levels for iAs. The maximum tolerable level of total arsenic (tAs) in drinking water defined by the World Health Organization (WHO) is 10 μg L^{-1} [46]. Very recently, the European Union published Regulation (EU) 2015/1006 [47] amending Annex to Regulation (EC) No 1881/2006 [48] regarding the maximum levels of iAs in foodstuffs, especially rice and rice-based products. The new MLs of iAs range from 0.10 to 0.3 mg As kg^{-1} depending of the rice product. Furthermore, the EU established a maximum levels for iAs in animal feeds, contents of below 2 mg kg^{-1} are recommended, especially those based on the seaweed species *Hizikia fusiforme* [49]. The Ministry of Health of China established a maximum level of iAs in food products depending on type of food [50]. The CODEX Alimentarius Commission in a draft report on contaminants in food accepts a ML of 0.2 mg kg^{-1} of iAs for polished rice and analysis of total As as a screening method [51]; the same document states that no agreement was reached for a ML of iAs in husked rice, but a value of 0.4 mg kg^{-1} is ongoing discussed [51,52] and may be adopted at the next session of the Committee. The Australia New Zealand Food Standard Code (FSANZ) [53] established a limit of 1 mg kg^{-1} for seaweed and mollusks; while for crustacean and fish, iAs is not allowed to exceed 2 mg kg^{-1}. Meanwhile, the authorities in the UK have advised consumers to avoid consumption of hijiki seaweed [54] while the Canadian Food Inspection Agency (CFIA) advises consumers to avoid that seaweed [55]. Specific regulations for iAs in edible seaweed have been established in some countries: 3 mg kg^{-1} (dw) as the maximum permitted level in the USA [56] and France [57]. The content of iAs in apple juices is considered a matter of concern by the U.S Food Drug and Administration (FDA) [58] and by the FSANZ [53]. The FDA recommends 10 ppb (as in drinking water) as a ML for iAs adequate to protect public health. The Canadian government, thorough Health Canada, established 0.1 ppm as the maximum tolerated limit for arsenic in fruit juices, fruit nectar and ready-to-serve beverages [59]; furthermore, this organization is currently considering establishing a specific lower tolerance of 0.01 ppm for apple juice. Several national initiatives and authorities have advised against consumption of rice drinks for infants and toddlers because it can increase the intake of iAs. The UK Food Standards Agency [60] does not recommend substitution of breast milk, infant formula, or cows' milk by rice drinks for toddlers and young children up to 4.5 years, whereas the Swedish National Food Agency [61] recommends no rice-

based drinks for children younger than 6 years and, in Denmark [62], children are advised against consuming rice drinks and biscuits.

The analytical technology to be applied for the assessment of arsenic species, highlighting iAs, is continuously updated and reviewed [36,63–74]. Nearing et al. [75] reviewed additional analytical methods suitable for obtaining data to complement the information on arsenic speciation obtained when applying the methods commonly used. Among such complementary methods, those authors report X-ray absorption spectroscopy (XAS) and electrospray mass spectrometry (ESI-MS); although such techniques are most useful for identifying or complementing information on several arsenic compounds with more complex molecular structures than those corresponding to iAs species. Some other general reviews of element speciation provide broad information on arsenic speciation, including analytical methodology and types of food [67,76–80].

Efforts have also been made in recent years to establish a methodology for the specific determination of iAs in food products. The validation of such methods is mandatory to demonstrate their suitability for routine analysis in control laboratories. Future regulations will probably be established as the iAs analytical methods improve. For this, the European Committee for Standardization (CEN) (CEN TC 327/WG 4) standardized 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 iAs by solid phase extraction (SPE) [81]. Other two standards are published, such as: Chinese Standard Method GB/T 5009.11-2003 [82]; and EN 15517:2008 [83]. Currently, there is an ongoing proposal for CEN method to determine iAs in foodstuffs by HPLC coupled to inductively coupled plasma mass spectrometry (HPLC-ICPMS) (CEN TC275/WG10). The AOAC has proposed a reference method for quantitation of As species and total iAs in selected food and beverage matrices, based on measurement by HPLC-ICPMS, currently in its fourth draft version [84]. Furthermore, for future implementation of analytical methods for iAs determination in food control laboratories, the availability of validated methods as well as participation in proficiency testing (PT) and the analysis of CRMs is mandatory, according to the ISO/IEC 17025 standard [85]. Obviously, this is applicable to speciation of iAs in food; considering its toxicity and the need to develop methods that can be applied in routine analysis.

The present review summarizes recent analytical proposals, including the use of CRMs and the availability of specific PT for the determination of iAs in the most widely consumed food products, covering the period 2010-May 2015. Increasing interest in the iAs contents of food products has led to a large number of studies being published on subjects such as: the evaluation of toxicity, bioaccessibility and bioavailability studies; the estimation of dietary intake; and estimations of iAs consumed by populations in different geographical areas. Such studies and the data they generate are beyond the scope of the present review; thus they are not included in it.

1.1. Overview of the literature

A preliminary search of the Web of Science database of 50.2 million journal papers, provided us with more than 18,000 papers and reviews whose titles contain the term "arsen*" between 1985 and 2014. Refining the search and including the search terms "speci*" or "compo*" or "inorg*" in the titles, led to 3301 publications (Figure 1). The distinction between "species" and "compounds" is not entirely clear and several authors use the terms as though they were synonyms; so both terms could be found interchangeably in the titles, meaning the same. As shown in Figure 1, the rate of publication related to As speciation has increased; making interest in arsenic speciation within the scientific community over the last fifteen years evident. The blue plot in Figure 1 reveals a peak in interest in arsenic species over 2011-2014, which could be related to the increased focus on iAs in food by authorities and institutions [14,15]. It seems that this call could have encouraged researchers to produce more data on arsenic species in different food products and hence the number of publications has increased from 2010 to the present.

Refining the initial search and including "arsenite" or "arsenate" or "food", or food synonyms as well as types of food (rice, seaweeds, fish, etc.), in the title led to approximately 500 which are represented by the red plot in Figure 1. A tendency can be observed in the literature related to arsenic and dealing with several subjects such as speciation, compounds, inorganic or food; this is an increase of the publication rate over recent years (2009-2014).

Finally, the terms "speci*" and "compo*" were excluded from the last search and a more specific search was performed. Hence, we searched for papers and reviews including "arsen*" and either "inorg*", "arsenite" or "arsenate" in the title as well as including several terms in the title such as "food" or "nutrit*" and several types of food. This provided us with 250 approximately (Figure 1). The green plot in Figure 1 shows the same tendency: a rise in the numbers of publications dealing with iAs in food, surely due to the increasing emphasis on iAs in food by the authorities and institutions mentioned above.

Focusing on the period 2010-2015, 115 publications were found in the Web of Science database that deal with iAs in foodstuffs. These papers were sorted according to the research area of the publication and the Web of Science classification criteria (Figure 2a). A wide variety of fields was obtained and as can be seen, areas such as "chemistry", "environmental sciences ecology", "food science technology", and "toxicology" are the most cited in these publications related to iAs in food. From the data consulted, a detailed distribution of these publications, according to type of food analyzed, was elaborated and is represented in Figure 2b.

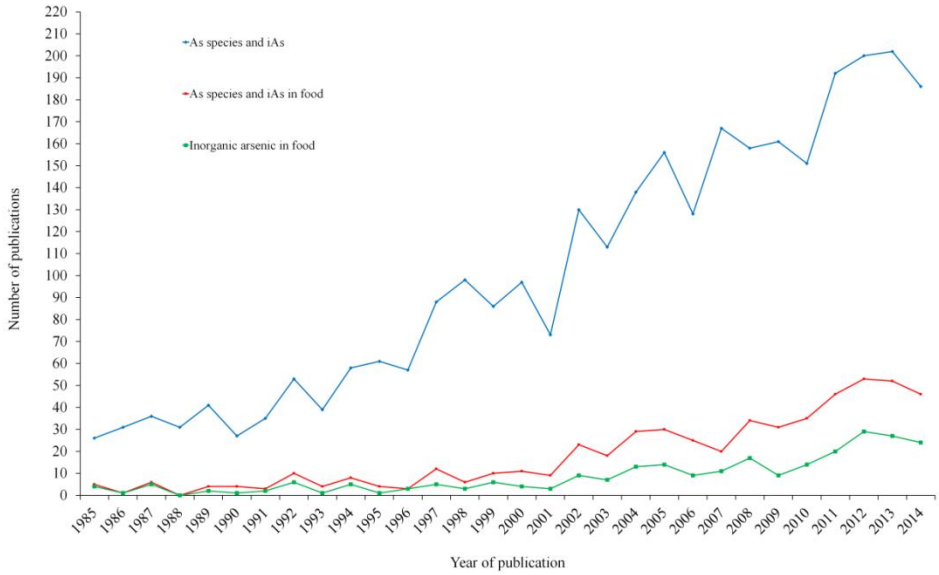

Figure 1. Blue plot is the number of papers published each year dealing with the As species either iAs as a function of time (1985-2014). Red plot refers to number of papers dealing with speciation of As species and iAs in the field of food and alimentation. Green plot shows the number of publications dealing only with iAs and relationship with food and alimentation.

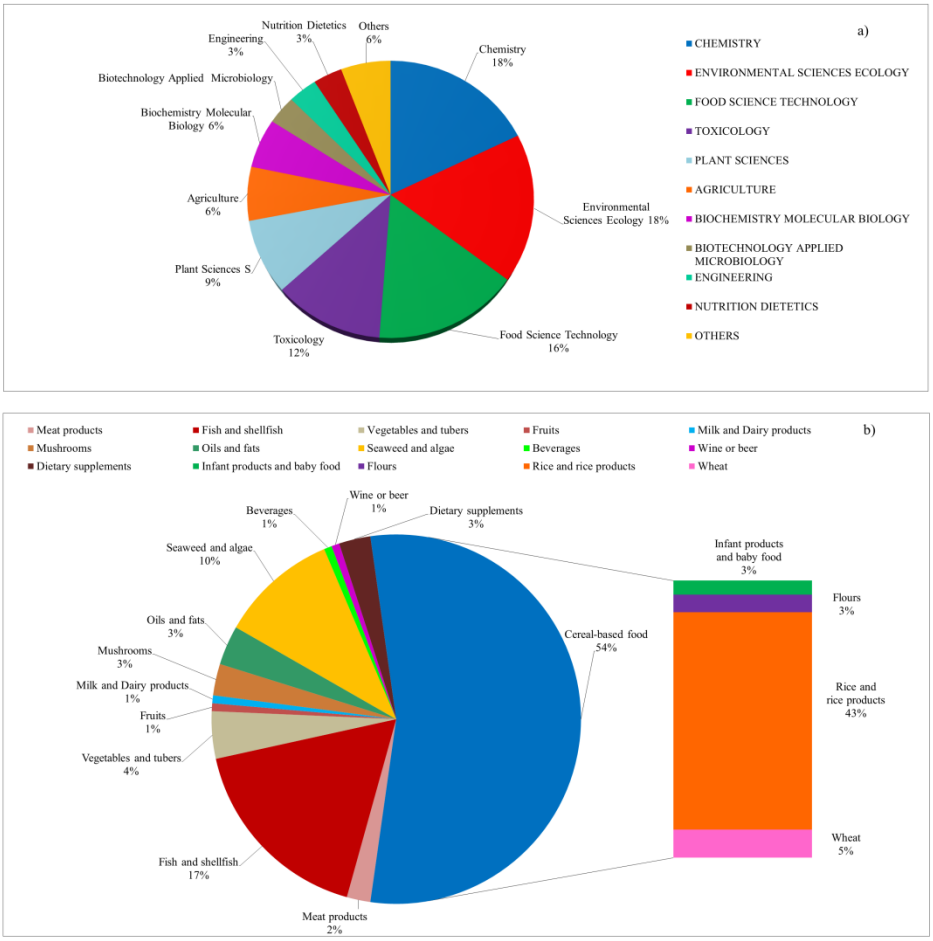

Figure 2. Distribution of publications (2010-2015) on the basis of research area of inorganic arsenic (a) and on the basis of types of analyzed foods of inorganic arsenic (b).

It can be seen that more than 50% are related to "cereal-based food" and specifically "rice and rice products", which accounted to 43%. This means that research on iAs in the last five years focused on rice and its products; which is not surprisingly since rice is the main food of over half the world's population, owing to its nutritive properties and its relatively low cost. It is estimated that in many countries, rice may contribute as much as 50% of the daily intake of protein, and in Asian countries it is a staple food. Furthermore, it is estimated that the As content of rice is over 10 times greater than that found in other cereals [86,87]. As stated above, cereal-based food and especially rice and its products are among the foods that contribute most to iAs exposure in the European population. It seems quite clear that speciation research focused on cereals and rice, motivated by the recommendations of the EFSA [14] and JECFA [15] reports. The second and the third groups are "fish and shellfish" and "seaweed and algae" which represent 17% and 10%, respectively (Figure 2b). Marine foods usually have higher tAs (in the range of mg As kg^{-1}) than rice or cereals (in the range of µg As kg^{-1}); however, the proportion of iAs in such food is very low compared to that in terrestrial foodstuffs. The non-toxic arsenobetaine is the major compound in fish and shellfish; while it is the so-called "potentially toxic" arsenosugars in "seaweed and algae" [88]. Other minor groups (3%) are "vegetables and tubers", "mushrooms" and "dietary supplements".

2. ANALYTICAL METHODS AND MEASUREMENT TECHNIQUES

In analytical element speciation the best way to ensure there are no alterations of the species across the overall analytical process, including sampling, in general consists of the use of techniques capable of performing the measurements *in situ*. Nevertheless, very few techniques are selective and sensitive enough to determine individual elemental species at trace levels. In practice, analytical speciation involves two main steps: extraction and measurement. Figure 3 summarizes an overall scheme including the most important steps in element speciation, and highlights specific information for iAs determination in food products. The steps need proper optimization to guarantee minimal changes to the original species, especially in complex matrices, such as different foodstuffs. The challenge is greater when a single group of species has to be determined, as in the case of iAs, from among other arsenic species that are present in the samples. Some reviews focus on specific analytical aspects, such as sampling and sample pre-treatment [72,89–93]. From the large number of proposals for arsenic speciation within the field of food analysis, we summarize here those developed with the aim of determining iAs contents. Two groups of methods are reported here, based on either direct measurement techniques (2.1) or on the use of coupling systems between separation and detection (2.2). In both cases, preliminary steps of extraction or selective separation are also reported.

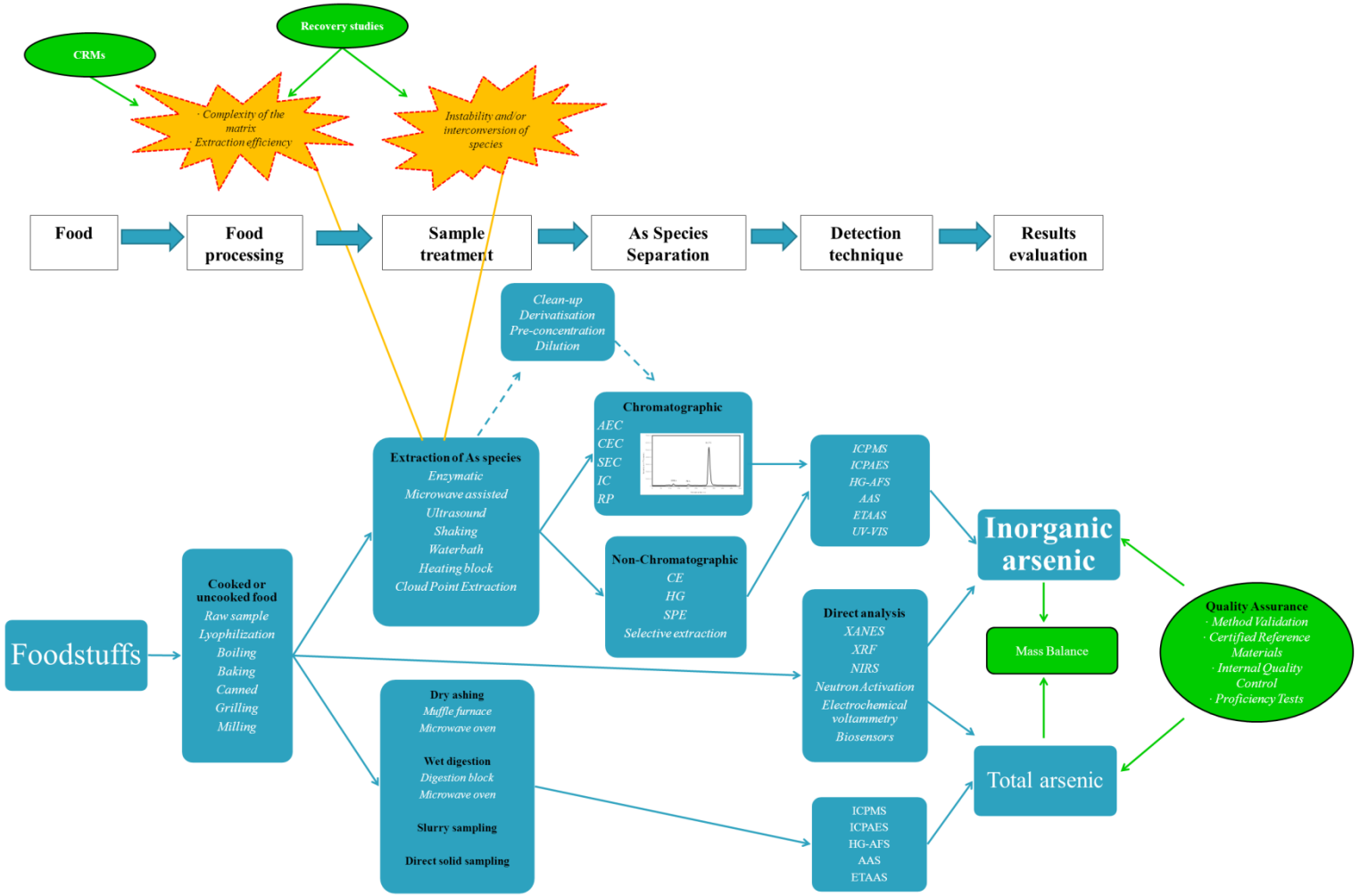

Figure 3. Scheme of the different steps required to perform total and inorganic arsenic determination in foodstuffs.

2.1 Methods involving non-coupled techniques

The vast majority of these methods are based on selective separation of arsenic species and spectroscopic detection; they are designed to determine only iAs species, the most toxic, and many of them are presented as alternatives to the use of ICPMS, which is more costly than other element detection techniques. Methods and applications based on such techniques are reported here by separately summarizing those that use direct measurement (A) and those that use HG, as a previous derivatization technique (B).

2.1.A Techniques involving direct measurement

Electrothermal atomic absorption spectrometry (ETAAS)

Electrothermal atomic absorption spectrometry (ETAAS) is considered to be one of the most sensitive techniques for trace element analysis, and several proposals have been made for As speciation in different matrices of interest, among them food samples. The determination of arsenic species can be considered a challenge when using ETAAS, since accurate optimization of the operational parameters, as well as the type of chemical modifiers, is required.

Lopez-Garcia et al. proposes arsenic speciation in fish-based baby foods using ETAAS [94]. According to those authors, iAs, MA, DMA and AB can be determined using sample suspensions in TMAH (tetramethyl ammonium hydroxide) and by means of several injections using three different chemically modified ETAAS atomizers: cerium (IV), palladium salts and a zirconium-coated tube. This approach is qualified by those authors as semi-quantitative due to the incomplete discrimination among arsenic species; but they claim it is suitable for food products where AB is the predominant compound, compared to methylated arsenic species. The same authors [95] applied dispersive liquid–liquid micro extraction to determine separately total As and the water soluble fraction of arsenic in edible oil; both fractions are analyzed later by ETAAS, assuming that the polar fraction obtained by a dispersive procedure assisted by ultrasound, contains the most toxic forms: As(III), As(V) and MA. Shah et al. [96] determines total As and iAs in samples of edible fish from the arsenic-contaminated Manchar Lake, Pakistan, and evaluated the estimated daily intake (EDI) of iAs. The method adopted allows the measurement of total As, after prior acidic digestion; whereas As(III) and As(V) are separated by two sequential steps with chloroform as the extracting agent and reducing As(V) to As(III). The corresponding extracts, as well as total As, are measured by ETAAS, using Mg $(NO_3)_2$ + Pd as a modifier. Pasias et al. [97] develops and fully validates a method to determine total As and iAs in rice. The method is then applied to determine total As and its inorganic forms in several varieties of rice and rice flour samples from local markets in Lamia (Greece). In it, total iAs is extracted by HNO_3 combined with EDTA; while As(III) is extracted using the same extraction system followed by complexation with APCD and further back extraction. For the ETAAS measurement, Pd is chosen, among other chemical modifiers, for the measurement of As in all the extracts obtained. In a study of As speciation in mono-varietal wines purchased in Mendoza (Argentina) Escudero et al. [98] determines total As and iAs in samples of Malbec and Sauvignon Blanc varieties using ionic liquid (IL) dispersive micro extraction as a pre-concentration

technique, coupled with ETAAS. This system is applied to each separate fraction previously obtained of As(III), total iAs and total As. Zmozinski et al. [99] proposes direct solid sample analysis with a graphite furnace (SS-ETAAS) as a screening method for iAs determination in fish and seafood. A method for the determination of arsenate and total iAs in rice samples is proposed by Dos Santos Costa et al. [100]; after whole extraction with HNO_3, arsenate is determined by cloud point extraction (CPE) of the complex formed with molybdate and As(V) in a sulfuric acid medium; while total iAs is extracted by the same CPE method, after previous oxidation of As(III) to As(V). In both cases, the final measurement is performed by ETAAS using Ir as the modifier.

Inductively coupled plasma mass spectrometry (ICPMS)

ICPMS has been widely used as a system for arsenic determination at very low levels and fundamental studies are frequently published.

D'Ilio et al. [101] reports and discusses the most common interferences found in As measurements, and proposals for correction. Rajakovic et al. [102] reports a study focused on estimating the limits of detection (LOD) for arsenic at trace levels, when using ICPMS. Those authors review current approaches and discuss them, supporting the conclusions with their experimental work. Bolea-Fernandez et al. [103] reports information concerning performance mechanisms, interferences and new proposals dealing with the use of such detection systems applied to arsenic determination.

Among the applications of ICPMS as a technique for iAs determination in food, differences arise in the pre-treatment of the sample and the extraction system applied. Kucuksezgin et al. [104], in a study on risk assessment based on the consumption of some edible marine organisms from Izmir Bay (eastern Aegean Sea), uses acidic digestion to determine total As; whereas separation of iAs is carried out in an alkaline medium with further oxidation of the arsenate. In both cases, final measurement of As is performed by ICPMS. Lewis et al. [105] develops a study of the stability of fish (megrim) samples over time, under different conditions, to ascertain whether some variability of arsenic species can occur. Within the study, iAs, obtained by applying the method using extraction with chloroform after acidification and further reduction, and final back-extraction, is measured by an HR-ICPMS detector with Ga as the internal standard.

2.1.B Techniques involving hydride generation (HG) as a derivatization step

The use of HG as a tool may improve selectivity and sensitivity in elemental analysis and different proposals are frequently reviewed [106–109]. Such system can easily be combined with spectroscopic and ICPMS detectors. Regarding arsenic, volatile arsines generated by reduction can be transported to the detector, avoiding chemical interference, thus achieving a very low LOD. The boiling points of the volatile arsines generated by reduction of inorganic and methylated forms of arsenic are sufficiently different to allow their separation. Nevertheless, HG is not suitable for arsenic compounds which cannot generate volatile hydrides by reduction; among such compounds arsenobetaine and arsenocholine, both usually present in fish-based food products, require transformation into iAs, capable of generating arsines by reduction.

Moreover, efficiency in the formation of volatile arsines strongly depends not only on the type of original arsenic compounds in the sample, but on the matrix composition. The mechanisms of arsine generation, the gas transport systems leading to the detector and detection conditions are frequently discussed. Tetrahydroborate, $NaBH_4$, in acidic media, which is probably the most commonly used reducing agent for the generation of volatile arsines, is required in substantial amounts; and some alternatives have been proposed. Several specific conditions have been proposed and reviewed.

Thus, Wu et al. [106] reviews applications of several reducing systems other than tetrahydroborate; while D'Ulivo et al. [110] discusses the mechanisms of hydrides forming from iAs and from methylated arsenic species, by using $NaBH_4$ and the formation of intermediate byproducts. Anawar [111] discusses the advantages and disadvantages of the combined HG-ETAAS system, in a review focused on this combined technique applied to arsenic speciation. Lehmann et al. [112] proposes the determination of iAs by controlling the medium of reduction and detection by FI-HG-MF-AAS (flow injection–HG–metal furnace–atomic absorption spectrometry) as the final measurement technique. Leal et al. [113] and Chaparro et al. [114] in studies using flow systems as on-line pre-concentration systems, propose a multi-commutation flow system coupled to HG atomic fluorescence spectrometry **(**AFS) for the analysis of As. The method is applied to arsenic speciation and the determination of DMA and iAs using multi-syringe flow injection analysis (MSFIA) coupled to an HG-AFS system. Yang et al. [115] uses a low-temperature plasma-assisted chemical vapor generation method to avoid the use of large amounts of sodium tetrahydroborate for the generation of volatile arsines, with detection by HG-AFS. Chen et al. [116] proposes a method for selective separation of As(III) from As(V) based on adsorption on multi-wall carbon nanotubes functionalized with branched cationic polyethyleneimine (BPEI-MWNTs) and measurement by HG-AFS. Matousek et al. [117] develops a method for arsenic speciation based on selective HG-cryotrapping-ICPMS, based on cryotrapping of arsines and desorption at their boiling points. Dados et al. [118] proposes a system to trap *in situ* arsenic hydrides previously generated using a nano-sized ceria-coated silica-iron oxide and final measurement of the slurry by ICPOES.

The recent applications of HG-spectroscopic detection, focused on the determination of iAs in food samples, are briefly summarized in the next few paragraphs, grouped by techniques.

Hydride generation–atomic absorption spectrometry (HG-AAS)

Several studies propose previous sample extraction and concentration before measurement of iAs. Among them Uluzolu et al. [119] develops a method based on solid-phase extraction (SPE) using *Streptococcus pyogenes* loaded on Sepabeads SP70 resin, for the speciation of As(III) and As(V). The method is applied to food samples of animal and plant origin. A method involving selective separation of As(III) and As(V) is proposed by Tuzen et al. [120]. That method is based on the selective adsorption of As(III) onto Diaion HP-2MG resin coated with *Alternaria solani*. The method is applied to CRMs of plant origin. Rasmussen et al [121] develops a method to determine iAs in food and feed samples of marine origin. The method involves off-line aqueous extraction and separation by SPE followed by HG-AAS (silica cell) detection. Optimized conditions during the extraction permit the selective separation of iAs

from organic arsenic species such as AB, MA and DMA; the method is validated in-house. The same author [122] also develops and validates another method based on the same extraction–pre-concentration system, optimized to obtain lower LOD and a higher throughput of sample extraction, to determine iAs in rice and rice products. Cerveira et al. [123] applies HG-AAS to measure iAs in several types of rice samples, after selective extraction with HNO_3. Sun and Liu [124] develops a method for analysis of As(III) and total iAs in dietary supplements by using a slurry in the presence of 8-hydroxiquinoline. After generation of hydride, As(III) is determine with HG-AAS using a gas–liquid separator and an electrothermal quartz atomizer. Total iAs is measured after reduction of As(V) to As(III). The authors check the recovery in the determination of total iAs by comparing it with the Chinese Standard Method [82] using HG-AFS for As measurement. The same method was applied for speciation of iAs in wheat and rice flours [125].

Among the applications of methods that already exist, several studies report iAs determination in food across different fields of interest. A method based on the determination of total As via dry ashing mineralization and quantification by FI-HG-AAS together with acidic digestion and chloroform extraction determines iAs from the back extraction [126]. This method is applied in Diaz et al. [127] to determine total As and iAs in several algae species, for both human consumption and production of phytocolloids, harvested from different regions of the Chilean coast. Several research groups in Thailand apply a similar analytical method in several studies with different objectives, but all based on the assessment of total As and iAs in samples collected from different regions of Thailand. Those studies include: marine fish, mollusks and crustaceans [128]; freshwater fish and prawns [129]; and a comparative study of total As in freshwater fish sampled from natural water sources and aquaculture systems [130]. Three types of rice and rice bran produced from them are also analyzed and the results compared [131]. Ubonnuch et al. [132] analyzes rhizomes of Zingiberaceae, a family of plants collected in Thailand, as a preliminary assessment of the risk of consuming natural products. Ruangwises et al. (2010) [133] and Ruangwises et al. (2011) [134] evaluate the intake of total As and iAs within populations from two contaminated areas of Thailand. Also, a study is developed to assess the risk of cancer due to exposure to iAs in Ronphibun, Thailand [135], by applying the guidelines in USEPA 2001.

Hydride generation–atomic fluorescence spectrometry (HG-AFS)

Several studies report using HG-AFS to measure total As and iAs in different food samples. In a study of the arsenic content of several commercial Spanish garlic samples, Sousa Ferreira et al. [136] proposes a method for screening of As(III) and As(V) based on extraction with H_2SO_4. In that study As is further measured in two aliquots in which the differences in the efficiency of HG with and without previous reduction is evaluated by means of two equations relating to the two oxidation states of As. G. Chen and T. Chen [137] proposes the quantification of iAs in rice via initial extraction with HNO_3 and H_2O_2 after which the resulting As(V) is selectively retained in a SPE cartridge (silica-based SAX) and iAs determined after elution and generation of arsine. The experimental conditions for acid-oxidizing extraction, absorption in an SPE cartridge and the generation of arsine are carefully optimized and discussed in depth. B. Chen et al. [138] describes a fast screening method for total As and iAs in a wide variety of rice

grains of different geographic origins, with the different matrices having no significant influence on the final measurements. For total As, UV-HG-AFS is used since the oxidative photolysis ensures quantitative oxidation of all the As species to As(V).

Hydride generation–inductively coupled plasma mass spectrometry (HG-ICPMS)

Several methods are proposed to suitable screening of iAs in food samples using an oxidative acidic extraction. Musil et al. [139] reports a method based on the extraction of iAs with HNO_3 and H_2O_2, and then on the use a selective HG coupled to ICPMS. To achieve this, HCl and $NaBH_4$ concentrations are optimized to volatilize almost exclusively arsines from the iAs, while minimizing possible volatile compounds generated from other organoarsenic compounds present in the samples. The method is applied to rice and seafood samples. The same method is further applied by Pétursdóttir et al. [140] for the analysis of a wide number of rice samples. Moreover, both methods are compared with the more widely used one involving HPLC-ICPMS for measurement and the results are shown to be comparable.

2.2 Methods using coupled techniques

Many proposals have been made for arsenic speciation by combining techniques that provide efficient separation of the species with suitable detection and quantification. These coupled techniques provide a high degree of automation, good reproducibility and offer application in different fields. Among them, here we mention some reviews that are specifically dedicated to arsenic speciation with coupled techniques [63,68,69,73,92,141]. In addition, some more general reviews of analytical techniques include arsenic speciation. Some of them describe food samples or summarize such aspects as pre-treatment, extraction and preservation of the arsenic species, pre-concentration, how to overcome matrix interference and specific instrumental conditions (such as types of nebulizers, the use of a dynamic reaction cell and internal standards) [66,67,72,76,78,79,142–144]. Some studies treat and discuss a specific subject in depth, as in the work of Pétursdóttir et al. [145] concerning the influence of the extraction step on the analysis of iAs in seafood, with measurement by coupled techniques. Next we summarize studies of applications of coupled techniques for iAs determination in several types of food, according to the separation technique.

2.2.A Coupled techniques that use HPLC as the separation technique

Most information corresponds to coupling techniques that use HPLC to separate As species. We consider applications based on HPLC-AAS, HPLC-HG-AFS and HPLC-ICPMS. No applications have been found of HPLC-ICPAES. Based on these coupling options, most studies use HPLC-ICPMS. Nevertheless, we also include studies using HPLC and detection systems other than ICPMS and that report iAs contents, along with some other species, to highlight interest in its toxicity. The vast majority of studies based on HPLC use strong anion exchange columns (SAX) and $NH_4H_2PO_4$, NH_4NO_3 or $NaHCO_3$ as the mobile phase. Thus, in

the following information, the type of chromatographic system is only reported in studies that use a system other than these.

The coupled technique HPLC-MS or HPLC-MS/MS, proposed for arsenic speciation in samples containing more complex compounds than those considered as iAs, has been applied to obtain molecular structure information on arsenic compounds of interest, although in general with no proved toxic effects, and has been shown not to be suitable for small molecules such as arsenate, arsenite and their methylated compounds.

HPLC–atomic absorption spectrometry (HPLC-AAS)

Since very few applications of this technique were found, each is mentioned here. Tian et al. [146] develops a gas–liquid separator for gradient arsenic HG, interfaced between HPLC coupled to the AAS detector, using a reversed-phase column and using sodium 1-butanesulfonate, malonic acid, tetramethylammonium hydroxide, MeOH and ammonium tartrate as the mobile phase. After optimizing the transport of the hydrides to the detector, the method is applied to the determination of arsenic species in hijiki algae. Niedzielski et al. [147] aims to determine iAs and DMA in species of mushrooms collected from forests in Poland with different degrees of contamination, as well as some that are commercially available. The extraction of arsenic species is performed with phosphoric acid with Triton X100 and the species are measured by HPLC-HG-AAS with a quartz atomizer and Ar as the carrier gas. Bergés-Tiznado et al. [148] analyzes cultured oyster samples from the SE Gulf of California in Mexico; although a non-coupled technique is used, since the corresponding fractions are collected from two HPLC columns (anionic and cationic) are finally measured by ETAAS. Only two samples are reported to have very low contents of iAs.

HPLC–Hydride generation–atomic fluorescence spectrometry (HPLC-HG-AFS)

A review by Y-W Chen et al. [149] describes relevant chemical and instrumental aspects, as well as applications, of this coupled technique for the speciation of some elements; among them arsenic. For this element, the literature on speciation in some food materials is included, among a wide number of matrices. Extraction systems as well as the stability of the chemical species throughout the overall chemical process are also included. Jesus et al. [150] proposes a method for arsenic speciation by adding sequential injection analysis: SIA-HPLC-AFS. In such a system, while the chromatographic detection operates in the usual way, the SIA module is programmed to inject sequentially the standard additions of the arsenic species. The method is applied to the analysis of seafood extracts to quantify the most toxic species: As(III), As(V), MA and DMA. Garcia-Salgado et al. [151] applies HPLC-HG-AFS using both anionic and cationic columns, which includes a photooxidation step, resulting in HPLC-(UV)-HG-AFS, to carry out arsenic speciation in edible algae extracts. The same authors in Garcia-Salgado et al. [152] use the same technique in a study of the stability of toxic arsenic species and arsenosugars in hijiki alga samples under several storage conditions. They highlight the predominance of As(V) in such food. Cano-Lamadrid et al. [153] applies HPLC-HG-AFS to determine iAs, together with MA and DMA, in rice samples collected from different provinces of Iran. Extraction of the arsenic

species is carried out using TFA and the iAs levels are found to be below the maximum FAO residue limit of 200 μg kg^{-1} for rice [51].

HPLC–inductively coupled plasma optical emission spectrometry (HPLC-ICPAES)

In a study of interference to the determination of iAs in seaweed by ion chromatography (IC)-ICPAES, Cui et al. [154] assays two extractants: HNO_3 and MeOH. That study concludes that suitable performance was not obtained with either system and the authors propose an alternative method for the determination of total iAs from seaweed. They add concentrated HCl and after separation, HBr and hydrazine sulfate are added to reduce As(V) to As(III); extraction of iAs with chloroform is finally carried out and measured by ICPAES.

HPLC–inductively coupled plasma mass spectrometry (HPLC-ICPMS)

As mentioned above, this technique has been the most commonly used over the last decade to determine arsenic species in several matrices. Here we summarize studies whose aim is the specific determination of iAs in food products. Furthermore, some studies to determine other arsenic species but that highlight the importance of obtaining information on iAs contents are also considered, reporting the suitability of this technique for arsenic speciation.

Thus, Prinkler et al. [155] compares different methods of signal treatment to improve the LOD of the different species, as an attempt to decrease the noise signal. The study obtained different signal-to-noise rations according to the convolution of the signal treatment systems with Gaussian distribution curves, de-noising via Fourier transform or wavelet transform. The study concludes that the last method was the most appropriate. Ammann [156] used a narrow-bore chromatographic system with low flow rates to optimize the efficiency of the nebulizers when using high resolving sector-field ICPMS as the detection system. Chromatographic performance for arsenic species separation and interference with the detection are discussed. Amaral et al [157] uses ICP-QMS in the coupled system and proposes the use of $^{83}Kr^{+}$ instead of Ar for the interference standard method (ISM) to overcome the most common sources of interference that occur in Ar plasma. The system improved both the accuracy and sensitivity of arsenic species determination. Some reviews and studies report sample preparation and extraction methods for arsenic speciation in food as a preliminary step before measurement [90]. Grotti et al. [158] discusses the influences of the arsenic species on the ICPMS signal when working at a low liquid flow rate (μHPLC-ICPMS). In general, different ICPMS responses are originated by differences in the volatility of the elemental species, as discussed by several authors. After assaying and comparing different nebulizers/spray chamber systems, this study supports this assumption and recommends species-specific calibration for the quantification of arsenic species. Jackson et al. [159] proposes a general approach for arsenic speciation by modifying the existing method and using carbonate eluents for a small particle size, short Hamilton PRP-X100 column which is interfaced with an ICPMS triple quadrupole, Agilent 8800 ICP-QQQ, using oxygen as the reaction gas and detection of AsO at m/z 91.

Among the types of food to which HPLC-ICPMS is applied for the determination of toxic iAs compounds, rice and rice-based products, and to a lesser extent other cereals, are the focus of increasing interest; as reported in studies this decade. Among the applications, the

optimization of extraction systems to obtain selective extraction of iAs is one of the main objectives, but when applying a separation–detection coupled system, information on methylated arsenic species in those types of samples is also obtained and reported. Thus, the studies using this technique report results for iAs as well as DMA and MA, and they differ mainly in the extraction systems for arsenic species. The variety of extraction systems and measurement conditions are summarized next, according to the target food type.

Rice and rice products

Huang et al. [160] studies several extraction systems that ensure suitable extraction of iAs compounds while preserving any possible transformation between As(III) and As(V) during the process, and finally proposes extraction with 0.28 mol L^{-1} HNO_3 at 95ºC for 90 min. The method was applied to several types of rice samples. Narukawa and Chiba [161] develops heat-assisted extraction with water for arsenic speciation in rice flour at 90ºC for 3 h. The authors discuss optimization of the extraction parameters in depth, as well as the influence of sample particle size on the extraction conditions, by considering information obtained from SEM (scanning electron microscopy) analysis of the surface of samples. For separation of arsenic species, a C18ODS L-column was used with sodium 1-butanesulfonate/malonic acid/tetramethylammonium hydroxide/MeOH as the mobile phase. Nishimura et al. [162] develops a partial digestion method using 0.15 mol L^{-1} HNO_3. After assaying 80ºC and 100ºC, the latter temperature was adopted for extraction, for 2 h, of iAs, MA and DMA from several varieties of rice from Japan. Paik et al. [163] proposes and validates a method based on ultrasonic extraction with MeOH:water (1:1) containing 1% HNO_3 in a study of arsenic speciation in eleven polished rice samples cultivated near areas of South Korea polluted by mining and for iAs finds a mean value of 25.5 μg kg^{-1} . Huang et al. [164] validates the method established before for iAs determination [160] by applying it to rice CRMs and through participation in the PT IMEP-107 [39,165], dedicated to the determination of iAs in rice. The validated method is applied to twelve types of rice samples of different origins. The concentrations of As(III) and As(V) increased with increasing total grain As concentration, and As(III) was predominant in almost all the samples analyzed, independent of the rice origin. Narukawa et al. [166] proposes specific monitoring test for iAs in rice, based on a previously developed and validated method, using water as the iAs extractant [161]. The method is applied to 20 white rice flour samples. For separation, a C18 column with sodium 1-butanesulfonate/malonic acid/tetramethylammonium hydroxide/MeOH as the mobile phase was used and arsenobetaine was used as the internal standard. Different percentages of iAs, with respect to total As, were found, depending on the geographical origin of the samples. Llorente-Mirandes et al. [33] optimizes and validates a method for the determination of arsenic species in rice. The arsenic species were extracted with a mixture of 0.2% HNO_3 and 1% H_2O_2 in a microwave (MW) system, to completely oxidize As(III) to As(V). Full validation is performed and the relative expanded uncertainty is estimated, based on the top-down method. The validated method is applied to the determination of arsenic species in 20 samples of rice and rice products. Sommella et al. [167] determines total As and iAs in several Italian rice samples. Extraction is performed with 1% HNO_3 and further addition of H_2O_2, while separation is by anion exchange column and quadrople ICPMS is used for detection. The iAs contents varied with the region of Italy the samples came from. Maher et

al. [168] extracts arsenic species using 2% HNO_3 before measurement by the coupled technique. Both cation and anion exchange columns are used for separation. The analysis is also carried out by XANES (X-ray absorption near edge structure) and the results of both measurement techniques compared, showing general agreement. The method is applied to rice samples from different countries. Kim et al. [169] uses 1% HNO_3 at 80ºC for 30 min for the extraction of arsenic species from 30 samples of rice grain collected from regions in South Korea known to contain arsenic, as well as 34 polished rice samples from the USA. The As(III) concentration in the American rice samples was slightly lower than that in the samples collected in Korea. Baba et al. [170] performs iAs, MA and DMA analysis by extracting them with 0.15 mol L^{-1} HNO_3 for 120 min at 100ºC. The authors summarize the chromatographic separation modes used for arsenic speciation; among them anion exchange columns are the most widely used although several other chromatographic systems are mentioned and discussed. They adopt the use of PFP (pentafluorophenyl) columns, after assaying and comparing some systems. The best results were obtained with a Discovery HS F5 column in isocratic mode and, after optimization of the elution conditions, 0.1% HCOOH and 1% MeOH, the latter as an organic modifier to enhance the signal. AB is used as the internal standard. The method is applied to several samples of rice purchased from markets in Japan. Narukawa et al. [171] assays various extraction systems for arsenic speciation in rice flour and the efficiencies are discussed in depth. Moreover, prevention of possible changes in the arsenic species during the processes, as well as the effects of the most common sources of interference on the separation and on the detection are also reported and discussed. A proposal for both As(III) and As(V) extraction from rice flour is based on 0.15 mol L^{-1} HNO_3 containing Ag in a heat block, and if only iAs is required, the proposal is based on extraction with 0.5 mol L^{-1} HNO_3 and H_2O_2 in a heat block. For separation, a C_{18} column with sodium 1-butanesulfonate/malonic acid/tetramethylammonium hydroxide/MeOH as the mobile phase is used.

Cereal-based food

As a part of a study of the distribution and speciation of arsenic in wheat grain from field-grown crops from European countries, Zhao et al. [172] determine iAs species in wholemeal and white wheat flour samples. The extraction of the species is performed with HNO_3 and H_2O_2 under MW. Tsai and Jiang [173] proposes an extraction system based on that established by Mar et al. [174] (which uses MW-assisted enzymatic digestion with Protease XIV and amylase) optimizing the conditions by extending the digestion time with respect to the proposed by Mar et al. [174], and applies it to the analysis of cereals. The final measurement is performed by IC-DRC-ICPMS (IC–dynamic reaction cell–ICPMS). D'Amato et al. [175] focuses on the sample treatment to obtain a good yield of arsenic species without degradation. After assaying different methods, MW extraction with HNO_3 was the most effective. The conditions are detailed in depth, including lyophilization and elimination of the residual humidity, and the method is applied to wheat and wheat products. Llorente-Mirandes et al. [32] performs a fully validated method, based on [33], for the determination of arsenic species in a large number and variety of samples of cereal-based food products and infant cereals. The method is used by the Laboratory of the Public Health Agency of Barcelona under accreditation by ENAC/Spanish National

Accreditation Entity, according the ISO/IEC 17025 standard, for its application in cereal-based food products.

Infant food

The method of Llorente-Mirandes et al. [32] mentioned above was applied to the determination of arsenic species in 9 samples of infant cereal products. Brockman and Brown IV [176] proposes an initial extraction with water at 98ºC for 3 h and later addition of hydrogen peroxide to the aqueous filtrate obtained. The resulting arsenate, MA and DMA from infant rice cereals are analyzed by this coupled technique. The authors conclude that iAs was found in all of the infant rice products in a large range between 33% and 77% of total As. Jackson et al. [30], in a broad study of iAs content in infant formulas and first foods, used an extraction with 1% HNO_3 following a progressive heating program with MW from 55ºC to 95ºC. For measurement, two chromatographic systems were used: both based on two anionic exchange columns, and with either phosphate at pH 6 as the mobile phase or with tetramethyl ammonium hydroxide. The samples, purchased from supermarkets, included 15 infant formulas, 41 fruit purees, and 18 second- and third-stage foods. As concentrations < 23 ng/g were found. Juskelis et al. [177], in a study for a survey of arsenic in rice cereals for infants, applied an extraction method for iAs, MA and DMA based on the use of 0.28 M HNO_3 at 95ºC for 90 min in a block digestion system. A total of 31 different samples of organic wholegrain rice, mixed-grain flour, organic rice and rice flour were analyzed and the results showed that the iAs levels varied among all the samples studied: values in the range of μg iAs per serving, for all the samples are reported (considering 15 g per serving, according to the reference amount customarily consumed (RACC) per 21 CFR 101.12).

Other types of food

The coupled technique HPLC-ICPMS has also been applied for arsenic speciation in types of food other than rice and cereals. In many cases, as for example in several types of food of marine origin, the number of arsenic species could be high. However, as mentioned above, in such samples there are drawbacks caused by the presence of polyatomic sources of interference arising from chloride. Several correction systems have been proposed such as high-resolution MS and quadrupole-based instruments with a reaction cell or collision cell [142]; or the use of the interference standard method (IFS) [157]. In complex food matrices, the selective extraction of iAs is more difficult than it is from rice and cereal samples. When analyzing complex matrices, a shift in the retention time of the iAs species (As(III) and As(V)) may be observed, and consequently co-elution with organic arsenic species (arsenobetaine, arsenosugars and others) may occur. Moreover, not all extractant reagents (MeOH/water, dilute HCl, HNO_3, TFA, NaOH, etc.) quantitatively extract iAs from the matrix. As a consequence, the analytical proposals reported in the literature are scarcer and here we summarize those applications in which the main goal is the selective determination of iAs.

Dufailly et al. [178] validates a method using IC-ICPMS for measurement, after ultrasound-assisted enzymatic extraction (UAEE) with protease XIV and α-amilase. The method is validated for a variety of food samples including rice, infant food and fish. Mao et al. [179]

develops highly polar stir bar sorptive extraction (SBSE) for arsenic species, coated with TiO_2-PPHF (polypropylene hollow fiber), coupled to HPLC-ICPMS. A C_{18} chromatographic column with MeOH/water, and sodium butanesulfonate/malonic acid is used as the mobile phase. The method is applied to determine arsenic species, including iAs, in chicken samples. Raber et al. [180] proposes an extraction method based on 0.02 mol L^{-1} trifluoroacetic acid with 30% H_2O_2 under sonication. In a second step, 95ºC of heat is applied for 60 min in an Ultraclave MW system. The method is applied to rice, wheat and tuna fish samples. Julshamn et al. [181] applies an extraction method for iAs based on 0.07 mol L^{-1} HCl and 3% H_2O_2 at 90ºC for 20 min. The method is applied to determine iAs in 25 fish samples from Norwegian seas. Pétursdóttir et al. [182] , in a study to establish a method to determine iAs in seafood, assayed three extraction methods based on 0.07 mol L^{-1} HCl in 3% H_2O_2; 2% HNO_3 or NaOH in 50% EtOH. The results are discussed, pointing out that some of them could influence the performance of the separation. HG was introduced for measurement in the coupled technique, resulting in HPLC-HG-ICPMS. This additional step, which uses $NaBH_4$ in an HCl medium as a reducing agent, enhances the sensitivity, since the volatile hydrides generated enter quantitatively into the plasma in a measurable fashion, and in this study LOD improved 10- to 100-fold, with respect to conventional nebulization systems. Narukawa et al. [183] studies extraction methods for As(III) and As(V) from several edible algae, including 15 samples of *Hizikia fusiforme*. They assay MeOH, HNO_3, THAH, pepsin and α-amylase, under three extraction conditions: ultrasonic, heat-assisted and MW-assisted, and conclude that extraction with water under ultrasonic conditions is the most useful for monitoring iAs in hijiki and the other algae studied. For separation, a C_{18} chromatographic column is used, with sodium 1-butanesulfonate/malonic acid/tetramethylammonium hydroxide/MeOH as the mobile phase. In a study of the iAs content of dietary supplements, considering that no maximum levels for As are included in the recent EU regulations, Hedegaard [184] studies 16 different dietary supplements based on herbs, other botanicals and algae collected from stores in Denmark, with origins in China (9), Taiwan (1), Denmark (5) and the USA (1). Extraction with 0.006 mol L^{-1} and 3% H_2O_2 at 90ºC for 20 min is applied. For measurement, a polymer strong anion exchange column with 3% ammonium carbonate adjusted to pH 10.3 is used. To estimate the exposure, the corresponding daily dose is considered for each supplement. In work on the shiitake species *Lentinula edodes* [29], several types of edible shiitake mushrooms are extracted with 0.02% HNO_3 and 1% H_2O_2 in a MW system; the results show that iAs is the predominant As species. Piras et al. [185] determines tAs and iAs in samples of several marine organisms collected from the Boi Cerbus Lagoon in Sardinia (Italy): an important fishing area. The iAs is determined using HPLC-ICPMS after extraction with HCl 0.07 mol L^{-1} and 3% H_2O_2.

Some studies determine iAs in fruit juices, following the recommendations of the FDA [28]. Wang et al. [186] proposes iron-pairing chromatography with a ODS column and malonic acid/TBA/MeOH as the mobile phase, to determine iAs, MA and DMA in fruit juice samples, and fruit-based beverages: iAs is the major arsenic compound found.

As a summary of results for iAs by HPLC-ICPMS in various types of food**,** several chromatograms are shown in Figure 4 (a-f): a) rice, b) infant multicereals, c) hijiki seaweed (*Sargassum fusiforme*), d) mushroom supplement (*Grifola frondosa*, commercially known as Maitake) e) tuna fish, and f) mussel. The chromatograms are unpublished results of research by our working group.

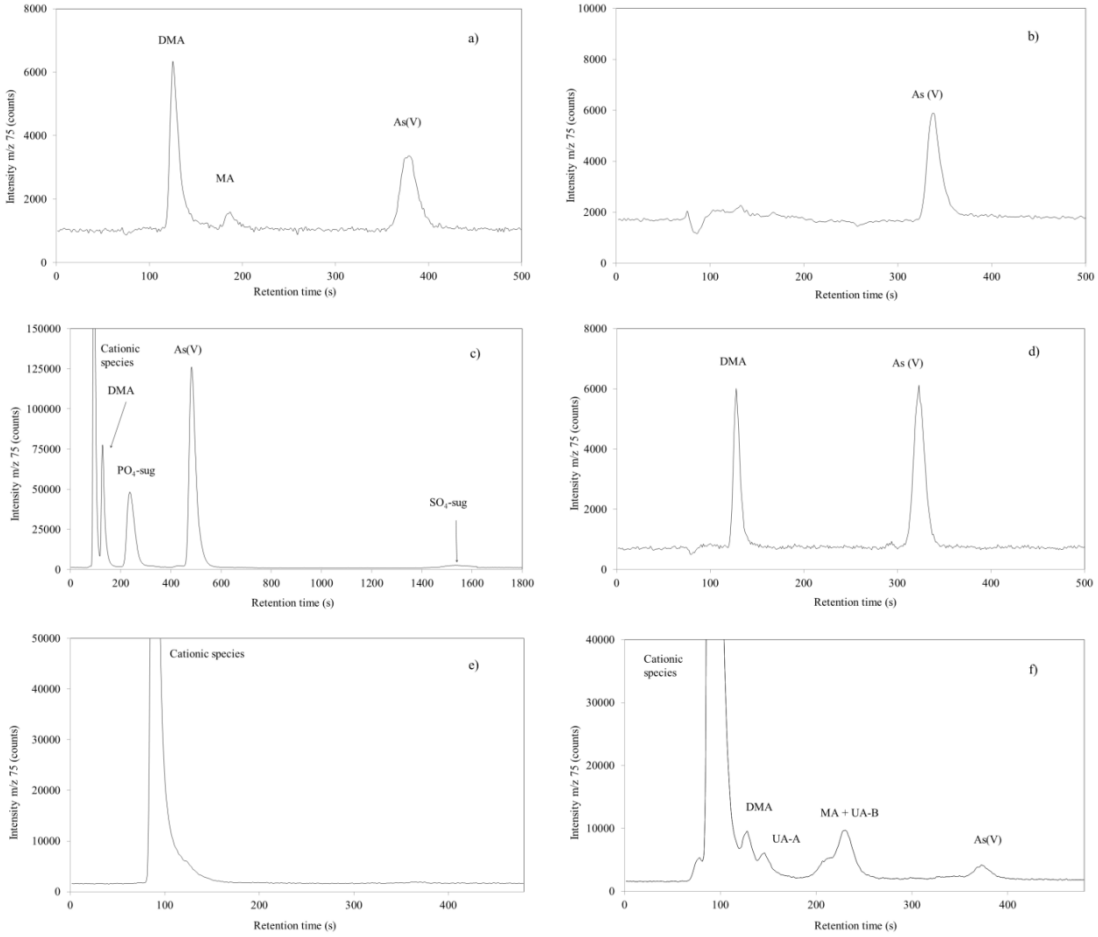

Figure 4. Anion exchange HPLC-ICPMS chromatograms of rice (a), infant multicereals (b), Hijiki seaweed (*Sargassum fusiforme*) (c), mushroom supplement (*Grifola frondosa*, commercially known as Maitake) (d), tuna fish (e), and mussel (f).

2.2.B Coupled techniques that use capillary electrophoresis (EC) as the separation technique

Capillary electrophoresis (CE) has been proposed as a coupled technique for element speciation, but fewer contributions are reported than for than HPLC. Previous problems associated with the interface with the different detection systems have recently been overcome [187]. Very few contributions have been found that deal with arsenic speciation in general over the last five years [188,189]. We now summarize those reports with applications to arsenic speciation in

food samples; some of them include iAs results, although with no specific determination of iAs species.

Hsieh et al. [190] couples CE with dynamic reaction cell ICPMS as the detector for arsenic speciation, with application to the CRM NRCC DOLT-3, in which the iAs value found was lower than the LOD, and to dietary supplements. Niegel et al. [191] develops a method based on CE-ESI-TOF-MS (CE coupled to electrospray ionization time-of-flight mass spectrometry) for arsenic speciation, with application to the analysis of some algae extracts; although no results for iAs compounds are obtained. Liu et al. [192] proposes a novel interface (the commercial CE-ESI-MS sprayer kit) for CE-ICPMS and applies it to arsenic speciation in the CRMs TORT-2 and DORM-3, as well as to herbal plants and chicken meat, the results from which include iAs compounds. More recently, Qu et al. [193] develops a method for arsenic speciation in rice and cereals. It is based on the extraction of arsenic compounds by means of direct enzyme-assisted MW digestion, to reduce matrix effects in the final measurement by CE-ICPMS. The method is validated by applying it to the rice CRMs: NIST SRM 1568b and NMIJ CRM 7503-a.

2.3 Other analytical techniques

Non-spectrometric analytical techniques have been reported for iAs determination; although few of them report applications to food samples. Gürkan et al. [194] describes a method to determine iAs by means of a CPE (cloud point extraction) procedure based on the formation of a complex with neutral red as the ion-pair reagent and using UV-vis detection (CPE-UV-Vis). The method allows the determination of As(III), total As and As(V), and is applied to alcoholic and non-alcoholic beverage samples. The same authors [195] propose Acridine Orange, AOH^+ using Triton X-114 with tartaric acid pH 5.0 as a new ion pairing complex formation of As(V), for applying it to the method above described, which is applied to determine iAs in beverage and rice samples.

Some electrochemical techniques have been developed for the measurement of iAs. Liu and Huang [196] reviews recent contributions of voltammetric methods for the determination of iAs. That review considers types of electrode systems, including electrodes based on nanomaterials, and highlights the increased demand by researchers for sensors to measure *in situ*. The vast majority of applications of such systems have been applied to the analysis of iAs in water and waste water, and no applications to the measurement of iAs in food samples have been found. Several biosensors for the detection iAs have been developed. They involve the coupling of a biologically engineered system with a sensitive analytical technique; they can be based on fluorescence response [197] , luminescence or electrochemical response [198]. Different developments in this field are reviewed by [199]. The literature warns that the application of these techniques to complex matrices, such as environmental or food samples, is still a challenge.

3. ASSESSMENT OF QUALITY CONTROL

Quality control (QC) and quality assurance (QA) are still not widely implemented in element speciation in food products. Nevertheless, noticeable efforts have been made in recent

years to develop strategies to support the quality of results in this field of analysis. The preparation of suitable CRMs of different types of food and the organization of PT form the basis of these efforts; the use and application of both are mandatory in food control laboratories, as regulated by ISO/IEC Standard 17025 [85]. A comprehensive scheme of QA in analytical chemistry laboratories would include the following elements: validation of analytical methods; use of CRMs; routine application of internal QC; and participation in PT [200]. Method validation is an essential component of the measures that a laboratory should implement to allow it to produce reliable analytical data and demonstrate whether the method is fit for a particular analytical purpose. Typical performance characteristics of analytical methods are: applicability, selectivity, calibration, trueness, accuracy, precision, recovery, operating range, LOD and limits of quantification (LOQ), sensitivity, uncertainty, ruggedness and fitness-for-purpose [201].

The following subsections specifically focus on the evaluation of the accuracy of the method by means of use of certified reference materials (CRMs) (3.1), and on participation in PT (3.2) as external QC of method validation. Besides, section 3.1 is subdivided and the text focuses on: CRMs available for iAs (3.1.1); other CRMs available with a certified total arsenic value (3.1.2); other strategies to evaluate accuracy (3.1.3).

3.1. Use of certified reference materials (CRMs)

CRMs are useful to evaluate the accuracy of the analytical method; both for validation and QC purposes. Sample treatment (digestion, extraction, etc.), separation and measurement processes are all subject to errors such as contamination, degradation, matrix effects, instability and interconversion of arsenic species, and calibration errors. Recovery, mass balance and QA/QC of the analytical method should be determined in all the steps of the procedure (Figure 3). CRMs are traceable to international standards with a known uncertainty and therefore can be used to address all aspects of bias, assuming that there is no matrix mismatch. CRMs should be of similar composition of real samples and have concentration levels similar to those of the samples analyzed [201]. CRMs are provided by various organizations, such as: the Institute for Reference Materials and Measurements (IRMM), the National Institute for Environmental Studies (NIES), the National Institute of Standards and Technology (NIST), the National Metrology Institute of Japan (NMIJ), the National Research Council of Canada (NRC-CNRC), the Chinese Academy of Geological Sciences (CAGS), the China National Analysis Center for Iron and Steel (CNCIS), the Korea Research Institute of Standards and Science (KRISS) and the Antarctic Environmental Specimen Bank (BCAA) all produce CRMs for different matrices.

The first food CRMs were certified for tAs content and were produced several decades ago. Later, since the toxicological effects of arsenic species differ markedly between them, some analytical methods were developed to quantify the mass fraction of the species in various matrices. The start was made with environmentally and food matrices of relevant species. Feasibility studies of some arsenic species (e.g. AB and DMA) were performed in the 1990s and 2000s. In the last years, efforts on the production of CRMs with inorganic arsenic value in food, especially rice, are performed. Although considerable progress has been made regarding the

establishment of specific and sensitive analytical methodology for arsenic species, few CRMs are available with certified values for arsenic species in food samples.

At present, only four CRMs are available with certified values for arsenic species other than iAs (AB and/or DMA). The CRM BCR-627 Tuna Fish was one of the first materials certified for As species and it was produced by IRMM in 1999 [202]. The material was certified for tAs, DMA and AB values. Years after certification, the material is still available from the IRMM website [203], which means that AB and DMA species are stable over time and no transformation or degradation is produced [204]. More recently, three other marine food materials have been produced, extending the availability of suitable fish and shellfish CRMs with certified AB value: TORT-3 Lobster Hepatopancreas (NRC-CNRC), CRM 7402-a Cod Fish Tissue and CRM 7403-a Swordfish Tissue (both from NMIJ).

3.1.1 CRMs available for inorganic arsenic

The commercially available food matrix CRMs with a certified iAs value are summarized in this section. Although some advances have been made in specific analytical methods for iAs determination in recent years, very few CRMs have been developed. Only rice and seaweed CRMs are available with a certified value for the iAs content. Five CRMs for iAs have been produced since 2009 by different institutions including NMIJ, NIST and IRMM. Four of them are rice matrices: NIST SRM 1568b, ERM-BC211, NMIJ CRM 7503a and NMIJ CRM 7532a, which are also certified for tAs and DMA. The other is hijiki seaweed (NMIJ CRM 7405a) which is also certified for tAs, and other arsenic species have been reported [205]. Inorganic arsenic results reported in the literature for these CRMs in the period 2010-2015 are shown in Table I. The type of food, supplier, certified values, tAs reported, method and measurement technique for iAs determination are also shown. No data for iAs content in NMIJ CRM 7532a brown rice has been found in the literature. Therefore, the certified iAs value cannot be compared with other researcher and this CRM is not included in Table I. The iAs certified value for this material is 0.298 ± 0.008 mg As kg^{-1} ± uncertainty. Based on the information provided in Table I, the need to produce more CRMs with a certified iAs value in different food matrices can be appreciated. Some aspects should be considered to select and analyze a representative CRM: the origin and type of the matrix, the type of iAs species and the level of concentration.

Some thermal process is generally applied before the pre-treatment of the CRMs. For example, SRM 1568b was dried for 24 h at 101°C while NMIJ 7532a was dried at 60°C for 8 h; in contrast, BC-211 was stored at -20°C before being processed. All the rice CRMs were milled and sieved or pulverized and mixed to ensure homogeneity. The hijiki CRM was washed, freeze-dried, freeze-pulverized, sieved and mixed for homogenization. For all of the CRMs, a sterilization step was applied by γ-irradiating the material at a range of doses in order to eliminate active bacteria as a potential source of instability for arsenic species. The producers of CRMs usually recommend storing the materials shielded from sunlight or UV-radiation, in a clean place at room temperature or below. Only in the case of BC211 is it specified that the material should be stored at -20ºC ± 5ºC, in the dark.

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Table I. Available food CRMs with an inorganic arsenic certified value. Results obtained from literature (2010-2015) and expressed as mg As kg^{-1}.

CRMs Code	Type of food	Supplier	Certified value	tAs reported	iAs method	iAs technique	iAs reported value	References
CRM 7503-a	Rice	NMIJ	tAs= 0.098 ± 0.007 As(III)= 0.0711 ± 0.0029 DMA= 0.0133 ± 0.0009 As(V)= 0.013 ± 0.0009	0.098 ± 0.005	MAE/(HNO_3/H_2O_2)	HPLC-ICPMS	iAs= 0.0849 ± 0.0007	Llorente-Mirandes et al. [33]
				0.095 ± 0.005	MAE/(HNO_3/H_2O_2)	HPLC-ICPMS	iAs= 0.0837 ± 0.0016	Llorente-Mirandes et al. [32]
				0.095 ± 0.001	HEAT (block)/(HNO_3)	HPLC-ICPMS	As(III)= 0.067 ± 0.001 As(V)= 0.015 ± 0.002	Huang et al. [164]
				0.101 ± 0.005	HEAT (block)/(H_2O)	HPLC-ICPMS	As(III)= 0.0740 ± 0.0023 As(V)= 0.0140 ± 0.0005	Narukawa et al. [161]
				0.096 ± 0.002	MAE/(H_2O)	HPLC-ICPMS	As(III)= 0.0130 ± 0.0005 As(V)= 0.0711 ± 0.0008	Narukawa et al. [171]
				0.096 ± 0.002	HEAT (block)/(HNO_3)	HPLC-ICPMS	As(III)= 0.0133 ± 0.0005 As(V)= 0.0717 ± 0.0007	
				0.096 ± 0.002	HEAT (block)/(HNO_3/Ag)	HPLC-ICPMS	As(III)= 0.0712 As(V)= 0.0135	
				0.096 ± 0.002	HEAT (block)/($HClO_4$)	HPLC-ICPMS	As(III)= 0.0714 As(V)= 0.0138	
				0.099 ± 0.001	HEAT (block)/(HNO_3)	HPLC-ICPMS	As(III)= 0.0714 ± 0.0004 As(V)= 0.0134 ± 0.0002	Narukawa et al. [206]
				No reported	Shaking/(HCl) /extraction ($CHCl_3$/back extr. 1 M HCl)/	ICPMS	iAs= 0.080 ± 0.008	Fontcuberta et al. [207]
				0.096 ± 0.002	HEAT (block)/(HNO_3)	HPLC-ICPMS	As(III)= 0.057 ± 0.002 As(V)= 0.017 ± 0.003	Kuramata et al. [208]
				No reported	Shaking/(HCl) /extraction ($CHCl_3$/back extr. 1 M HCl)/)	ICPMS	iAs= 0.0815 ± 0.0085	Wu et al. [209]
				No reported	Heat block/HNO_3	HPLC–ICPMS	As (V)= 0.013 ± 0.001 As (III)= 0.068 ± 0.003	Baba et al. [170]
				No reported	Heat with water/Enzymatic ext.(amylase)	HPLC-ICPMS	As(III)= 0.0602 ± 0.0025 As(V)= 0.0145 ± 0.0017	Nookabkaew et al. [210]
				No reported	Shaking/HCl/pepsin (bioaccessible extracts)	HPLC-HEPO-HG-ICPMS	As(III)= 0.0594 ± 0.0028 As(V)= 0.0226 ± 0.0004	Oguri et al. [211]
				No reported	MAE/ Enzymatic ext.(amylase)	CE-ICPMS	As(III)= 0.0621 ± 0.00173 As(V)= 0.01927 ± 0.0011	Qu et al. [193]

ERM-BC211	Rice	IRMM	tAs= 0.260± 0.013 DMA= 0.119 ± 0.013 iAs= 0.124 ± 0.011	0.256 ± 0.009	MAE/(HNO_3/H_2O_2)	HPLC-ICPMS	iAs= 0.122 ± 0.006	Llorente-Mirandes et al. [29]
				0.263 ± 0.011	MAE/(HNO_3/H_2O_2)	HPLC-ICPMS	iAs= 0.119 ± 0.005	Zmozinski et al. [212]
				No reported	MAE/(HNO_3/H_2O_2)	SPE-HG-AFS	iAs= 0.124 ± 0.002	Chen. G et al. [137]
				No reported	MAE/(HNO_3/H_2O_2)	HG-AFS	iAs= 0.1214 ± 0.0048	Chen. B et al. [138]
				0.256 ± 0.008	HEAT/(TFA)	HPLC-HG-AFS	iAs= 0.129 ± 0.012	Cano-Lamadrid et al. [153]
				0.257 ± 0.015	MAE/(HNO_3)	HG-AAS	iAs= 0.116 ± 0.003	Cerveira et al. [123]
SRM 1568b	Rice	NIST	tAs= 0.285 ± 0.014 DMA= 0.180 ± 0.012 MA= 0.0116 ± 0.0035 iAs= 0.092 ± 0.010	No reported	MAE/ Enzymatic ext.(amylase)	CE-ICPMS	As (III)= 0.05542 ± 0.0019 As(V)= 0.04092 ± 0.00678	Qu et al. [193]
				No reported	MAE/(HNO_3/H_2O_2)	HPLC-ICPMS	iAs= 0.087 ± 0.002	Signes-Pastor et al. [31]
CRM 7405-a	*Hizikia fusiforme*	NMIJ	tAs= 35.8 ± 0.9 As(V)= 10.1 ± 0.5	No reported	Shaking/HCl/pepsin (bioaccessible extracts)	HPLC-HEPO-HG-ICPMS	As(V)= 10.2 ± 0.1	Oguri et al. [211]

Notes. The ± terms are as provided by the original publications. They are predominantly standard deviations for some number of replicates or in some cases uncertainties. MAE; microwave assisted extraction.

Different approaches have been adopted by the producers to express the iAs mass fraction or concentration in the CRMs: three of the rice CRM (NIST 1568b, ERM-BC211 and NMIJ 7532a) are certified with iAs values (the sum of As(III) + As(V)); the other one is certified for As(III) and As(V) separately (NMIJ 7503a); and the seaweed (NMIJ 7405a) as arsenate. The inorganic species present in these CRMs are of natural origin, according to the certification reports, no spiking experiments were performed. The iAs level in the four rice CRMs ranged from 0.084 to 0.298 mg As kg^{-1} ; the typical range for rice samples [213]. Typically, the iAs content in the brown rice CRM is higher than in the white rice CRMs, as commonly reported [214–216].

The first CRM released with a certified iAs value was CRM 7503-a rice and it was produced by NMIJ. The certificate is dated August 2009 and it is the most analyzed CRM. Several authors use it to assess the accuracy of iAs methods [32,33,161,164,170,171,193,206–211]. The mean value for iAs content of the values reported in Table I is 0.0823 ± 0.0037 mg As kg^{-1} (mean value ± standard deviation, n=16 reported results) which is in perfect agreement with the certified value of iAs: 0.0841 ± 0.0030 mg As kg^{-1} (the sum of the certified As(III) and As(V) values ± the square root of the sum of their squared uncertainties). Nine of the published values use different extraction methods, such as MW-assisted extraction (MAE) or heating in a block with several extractants such as HNO_3, HNO_3/H_2O_2, $HClO_4$, H_2O or enzymes; and with final measurement via the coupled HPLC-ICPMS technique, which allows iAs to be separated from methylated species and the iAs species to be determined satisfactorily [32,33,161,164,170,171,206,208,210]. A study of bioaccessible extracts (0.07 mol L^{-1} HCl and 0.01 % pepsin) was performed using (HPLC-ICPMS) with a high-efficiency photooxidation (HEPO) and HG system [211]. A bioaccessible iAs value close to the certified one was obtained: 0.0821 ± 0.0024 mg As kg^{-1} . Two authors selectively extract the iAs with HCl and subsequent extraction with chloroform of the iAs present in the acid medium [207,209], based on the method of Muñoz et al. [126]. The final determination is performed by ICPMS and results comparable to the certified value were obtained. Although CE-ICPMS is not usual in iAs determination, Qu et al. [193] extract iAs with an enzyme-assisted water-phase MAE and quantify by CE−ICPMS, reporting a satisfactory iAs value for the NMIJ 7503-a rice material.

Very recently, EC-JRC-IRMM has produced a rice CRM (ERM-BC211) which is certified for DMA and iAs as well as for tAs. Six studies analyze this material [29,123,137,138,153,212] and the mean value for the reported iAs results is 0.122 ± 0.004 (mean ± standard deviation, n=6 results) which is in agreement with the certified value: 0.124 ± 0.011 mg As kg^{-1} . Five studies use MAE with HNO_3 or HNO_3/H_2O_2 as the extractant solvent; two of them with determination of iAs by HPLC-ICPMS [29,212] and two by HG-AFS [137,138] and the other by HG-AAS [123]. Another study extracts iAs with TFA and determination is by HPLC-HG-AFS [153].

SRM 1568b white rice was recently released by NIST and it is certified for arsenic speciation (DMA, MA and iAs).To date, two studies analyzes it to evaluate the accuracy of their methods; one is based on As species in rice by CE-ICPMS [193] and the other is focused on rice-based products for infants and young children by HPLC-ICPMS [31]. Finally, only one study was found that analyzes the NMIJ 7405a hijiki and the reported iAs value is in agreement with the certified one [211]. The high content of iAs (10.1 ± 0.5 mg As kg^{-1}) in this seaweed is usually

found in studies of hijiki (*Hizikia fusiforme*), which is known to bioaccumulate arsenic as iAs [26,217]

3.1.2 Other CRMs available with certified total arsenic value

Due to the lack of CRMs with a certified iAs value, many authors perform arsenic speciation analysis on CRMs in which the tAs content or other arsenic species are certified. For validation purposes, the data obtained is compared with data reported in the literature by different researchers. This is one of the most commonly used practices within the scientific community to evaluate accuracy without a certified iAs value. Furthermore, the sum of As species is usually compared with the certified total As content (a so-called mass-balance study) or with tAs determined in the sample extract (column recovery). Mass balances or column recoveries of 80%–110% of total As are considered acceptable. Values close to 100% indicate full quantification of the As species present in the sample and guarantee the correctness of the chromatographic separation.

Therefore, the following paragraphs focuses only on reported iAs values in food matrix CRMs; so studies reporting tAs or arsenic species in a CRM but not iAs results are not included in this section. The reported values are summarized in Table II, which includes type of food, supplier, certified values, total arsenic reported, iAs method, measurement technique and iAs value.

The authors wish to summarize the ability of the analytical community to perform iAs analysis in different food matrices CRMs. For this, we focus on reported iAs results in the most commonly CRMs analyzed: SRM 1568a rice, TORT-2 lobster and DOLT-4 fish. The reported results in these CRMs are shown in Figure 5 and Figure 6 for SRM 1568a and TORT-2, respectively; and in Table II for DOLT-4. Furthermore, specific highlights of iAs analysis in these CRMs are summarized in the following paragraphs.

In the case of SRM 1568a (Figure 5) and TORT-2 (Figure 6), reported results are tabulated according to the iAs value, from low to high, illustrating the capacity of the analytical community to measure the iAs content in these CRMs. There are different ways to express and publish iAs results for these CRMs in the original publications: total iAs; only arsenite or only arsenate; or both species separately. We express and summarize all the results as iAs, i.e., the sum of arsenite plus arsenate, in order to facilitate comparison of the data. Therefore, in the Figures, the continuous lines represent the average concentration of iAs and the dashed lines delimit the target interval X ± SD in mg As kg^{-1}. The individual error bars represent the errors reported in the original publications. Where arsenite and arsenate were reported separately, the iAs value (the sum of arsenite and arsenate) and the error bar are calculated (the square root of the sum of their squared uncertainties or standard deviations). We note that researchers usually report results as mean value ± error, which is predominantly SD for a number of replicates and in a few cases it is referred to the associated U value.

Table II. Food CRMs with published results of an inorganic arsenic content. Results obtained from literature (2010-2015) and expressed as mg As/kg.

CRMs Code	Type of food	Supplier	Certified tAs value	tAs reported	iAs method	iAs technique	iAs reported value	References
NCS ZC73008	Rice	CNCIS	0.102 ± 0.008	0.105 ± 0.006	MAE/(HNO_3/H_2O_2)	HPLC-ICPMS	iAs= 0.080 ± 0.003	Llorente-Mirandes et al. [33]
				No reported	MAE/(HNO_3/H_2O_2)	HPLC-ICPMS	iAs= 0.084 ± 0.001	
GBW 10010	Rice	CAGS	0.102 ± 0.008	0.1099 ± 0.0072	Incubation 80ºC/Ultra-pure water	HPLC-ICPMS	As(III)= 0.0461 ± 0.0024 As(V)= 0.156 ± 0.0016	Liang et al. [218]
					Incubation 80ºC/(Acetic acid (1%)	HPLC-ICPMS	As(III)= 0.0477 ± 0.0009 As(V)= 0.0152 ± 0.0004	
					Incubation 80ºC/(Nitric acid (1%)	HPLC-ICPMS	As(III)= 0.0616 ± 0.0045 As(V)= 0.0079 ± 0.0051	
					Incubation 80ºC/(TFA (0.2 M)	HPLC-ICPMS	As(III)= 0.0645 ± 0.0009 As(V)= 0.0110 ± 0.0003	
					Incubation 80ºC/(TFA (2 M)	HPLC-ICPMS	As(III)= 0.0619 ± 0.004 As(V)= 0.017.4 ± 0.003	
					Incubation 80ºC/(Methanol (50%)	HPLC-ICPMS	As(III)= 0.0536 ± 0.0077 As(V)= 0.0128 ± 0.002	
					Incubation 80ºC/Methanol (50%)/TFA (0.2M)	HPLC-ICPMS	As(III)= 0.0626 ± 0.0056 As(V)= 0.0118 ± 0.0029	
SRM 1567a	Wheat flour	NIST	0.006	0.0065 ± 0.0006	MAE/(enzymatic extraction)	HPLC-ICPMS	As(III)= 0.0032 ± 0.00004 As(V)= 0.0027 ± 0.00005	Tsai et al. [173]
SRM 8436	Durum Wheat Flour	NIST		0.013 ± 0.001	SON/(MeOH/H_2O)	HPLC-ICPMS	As(III)= 0.0012 ± 0.0002 As(V)= 0.00723 ± 0.00008	D'Amato el al. [175]
				0.013 ± 0.001	Ultrasonic probe/(H_2O)	HPLC-ICPMS	As(III)= 0.00318 ± 0.00009 As(V)= 0.0027 ± 0.00025	
				0.013 ± 0.001	MAE/(HNO_3)	HPLC-ICPMS	As(V)= 0.0109 ± 0.0006	
				0.013 ± 0.001	MAE/(enzymatic extraction)	HPLC-ICPMS	As(III)= 0.00216 ± 0.00044 As(V)= 0.00169 ± 0.0003	
SRM 1570a	Spinach leaves	NIST	0.068 ± 0.012	No reported	Shaking/(HCl) /extraction ($CHCl_3$/back extr. 1 M HCl)/	FI-HG-AAS	iAs= 0.038 ± 0.005	de la Calle et al. [41]
					MAE/(HNO_3/H_2O_2)	HPLC-ICPMS	iAs= 0.075 ± 0.004	
					Shaking/(HCl) /extraction ($CHCl_3$/back extr. 1 M HCl)/	ICPMS	iAs= 0.074 ± 0.010	
					MAE/(HNO_3/H_2O_2)	HPLC-ICPMS	iAs= 0.060 ± 0.002	
					MAE/(HNO_3 + H_2O_2)	HPLC-ICPMS	iAs= 0.055 ± 0.003	

					MAE/(HCl /H_2O_2)	HPLC-ICPMS	iAs= 0.034 ± 0.005	
					MAE/(TFA /H_2O_2)	HPLC-ICPMS	iAs= 0.045 ± 0.003	
				0.069 ± 0.005	MAE/(HNO_3/H_2O_2)	HPLC-ICPMS	iAs= 0.059 ± 0.005	Llorente-Mirandes et al. [29]
SRM 1573a	Tomato leaves	NIST	0.112 ± 0.004	No reported	UAE/(H_2SO_4/EDTA)	HG-AFS	As(V)= 0.0879 ± 0.0021 As(III)= 0.0226 ± 0.0003	Sousa-Ferreira et al. [136]
NCS ZC73012	Cabbage	CNCIS	0.062 ± 0.014	0.0603 ± 0.0007	MAE/(HNO_3/H_2O_2)	HPLC-ICPMS	iAs= 0.0519 ± 0.0035	Norton et al. [219]
SRM 1577	Bovine Liver	NIST	0.055 ± 0.005	0.053 ± 0.002	SON/ HNO_3/MeOH	HPLC-ICPMS	As(V)= 0.012 ± 0.001	Batista et al. [90]
SRM 1566a	Oyster tissue	NIST	14.0 ± 1.2	No reported	Shaking/(HCl) /extraction ($CHCl_3$/back extr. 1 M HCl)/	FI-HG-AAS	iAs= 0.586 ± 0.049	Ruangwises et al. [130]
				No reported	Shaking/(HCl) /extraction ($CHCl_3$/back extr. 1 M HCl)/	FI-HG-AAS	iAs= 0.601 ± 0.037	Ruangwises et al. [133]
				No reported	Shaking/(HCl) /extraction ($CHCl_3$/back extr. 1 M HCl)/	FI-HG-AAS	iAs= 0.598 ± 0.035	Ruangwises et al. [128]
				No reported	Shaking/(HCl) /extraction ($CHCl_3$/back extr. 1 M HCl)/	FI-HG-AAS	iAs= 0.581 ± 0.050	Saipan et al. [129]
				No reported	Shaking/(HCl) /extraction ($CHCl_3$/back extr. 1 M HCl)/	FI-HG-AAS	iAs= 0.601 ± 0.037	Ruangwises et al. [134]
				No reported	Shaking/(HCl) /extraction ($CHCl_3$/back extr. 1 M HCl)/	FI-HG-AAS	iAs= 0.601 ± 0.037	Saipan et al. [135]
SRM 1566b	Oyster tissue	NIST	7.65 ± 0.65	6.94 ± 0.2 and 7.2 ± 0.3	MAE/($MeOH$/H_2O)	HPLC-ICPMS	As(V)= 1.16 ± 0.01	Santos et al. [220]
				7.67 ± 0.13	MAE/(HNO_3/H_2O_2)	HPLC-ICPMS	iAs= 0.05 ± 0.001	Zmozinski et al. [212]
				No reported	MAE/(H_2O)	IEC-ICPMS	As(III)= 0.357 ± 0.057 As(V)= 0.427 ± 0.038	Leufroy et al. [221]
				8.06 ± 0.08	MAE/($MeOH$/H_2O)	IC-ICPMS	As(V)= 0.05 ± 0.01	Nam et al. [222]
CRM 108-04-001	Oyster tissue	KRISS	13.51 ± 0.30	14.19 ± 0.09	MAE/($MeOH$/H_2O)	IC-ICPMS	As(V)= 0.03 ± 0.01	Nam et al. [222]
MURST-ISS-A2	Antarctic Krill	BCAA	5.02 ± 0.44	5.29 ± 0.4	Shaking/($MeOH$/H_2O)	HPLC-ICPMS	As(V)= 0.03 ± 0.01	Grotti et al. [223]
SRM 2976	Mussel tissue	NIST	13.30 ± 1.8	13.7 ± 0.25	MAE/(HNO_3/H_2O_2)	HPLC-ICPMS	iAs= 0.11 ± 0.013	Zmozinski et al. [212]

ERM-CE278	Mussel tissue	IRMM	6.07 ± 0.13	6.09 ± 0.21	MAE/(HNO_3/H_2O_2)	HPLC-ICPMS	iAs= 0.07 ± 0.003	Zmozinski et al. [212]
				5 ± 0.6	SON/ (HNO_3/MeOH)	HPLC-ICPMS	As(III)= 0.2 ± 0.02 As(V)= 0.4 ± 0.04	Batista et al. [90]
BCR 627	Tuna fish tissue	IRMM	4.8 ± 0.3	5.2 ± 0.5	MAE/(H_2O)	IEC/ICP-MS	As(III)= 0.054 ± 0.014	Leufroy et al. [224]
				4.8 ± 0.3	MAE/(MeOH/H_2O)	IEC/ICP-MS	As(III)= 0.172 ± 0.071	
				4.68 ± 0.03	MAE/(MeOH/H_2O)	HPLC-ICPMS	As(III)= 0.29 ± 0.04 As(V)= 0.035 ± 0.001	Santos et al. [220]
				4.1	SON/(Enzymatic solution)	IC-ICPMS	iAs= 0.036	Dufailly et al. [178]
				4.84 ± 0.13	MAE/(HNO_3/H_2O_2)	HPLC-ICPMS	iAs= 0.02 ± 0.002	Zmozinski et al. [212]
				No reported	MAE/(H_2O)	IEC-ICPMS	As(III)= 0.068 ± 0.003 As(V)= 0.041 ± 0.001	Leufroy et al. [221]
				4.20 ± 0.03	Shaking (two-step sequential extraction)/acetone and MeOH/water	HPLC-HR-ICPMS	As(III)= below LOD As(V)= below LOD	Ruiz-Chancho et al. [225]
				No reported	Cell clean-up – PAEH	HPLC-ICPMS	As(III)= 0.075 ± 0.0006	Moreda-Piñeiro et al. [226]
				4.82 ± 0.41	Shaking/(HCl) /extraction ($CHCl_3$/back extr. 1 M HCl)/	HR-ICPMS	iAs= 0.82 ± 0.049	Lewis et al. [105]
DOLT-3	Dogfish Muscle	NRC-CNRC	10.2 ± 0.5	10.0 ± 0.4	MAE/(H_2O)	IEC-ICPMS	As(III)= 0.074 ± 0.011 As(V)= 0.073 ± 0.007	Leufroy et al. [224]
				9.6 ± 1.1	MAE/(MeOH/H_2O)	IEC-ICPMS	As(III)= 0.136 ± 0.004	
				No reported	MAE/(Enzymatic extraction)	CE-ICPMS	iAs below LOD	Hsieh et al. [190]
				10 ± 0.4	SON/(HNO_3/MeOH)	HPLC-ICPMS	As(III)= 0.3 ± 0.1 As(V)= 0.4 ± 0.2	Batista et al. [90]
DOLT-4	Dogfish Muscle	NRC-CNRC	9.66 ± 0.62	No reported	MAE/(HCl/H_2O_2)	HPLC-ICPMS	iAs= 0.039 ± 0.001	Pétursdóttir et al. [182]
					MAE/(HCl/H_2O_2)	HPLC-HG-ICPMS	iAs= 0.011± 0.002	
					MAE/(HNO_3)	HPLC-ICPMS	iAs= 0.028 ± 0.003	
					MAE/(HNO_3)	HPLC-HG-ICPMS	iAs= 0.011 ± 0.002	
					MAE/(NaOH/EtOH)	HPLC-ICPMS	iAs= 0.027 ± 0.003	
					MAE/(NaOH/EtOH)	HPLC-HG-ICPMS	iAs= 0.010 ± 0.003	
				No reported	MAE/(HCl/H_2O_2)	HPLC-ICPMS	iAs=<0.040	Baer et al. [40]
					MAE/(MeOH/H_2O)	HPLC-ICPMS	iAs=ND	
					SON/(TFA/H_2O_2)	HPLC-ICPMS	iAs= 0.047 ± 0.006	
					Shaking/(HCl) /extraction	FI-HG-AAS	iAs= 0.075 ± 0.005	

					(CHCl$_3$/back extr. 1 M HCl)/ Shaking/(HCl) /extraction (CHCl$_3$/back extr. 1 M HCl)/	HR-ICPMS	iAs= 0.152 ± 0.010	
				No reported	MAE/(HCl/H$_2$O$_2$)	HPLC-HG-ICPMS	iAs= 0.011 ± 0.002	Pétursdóttir et al. [145]
					MAE/(H$_2$O/MeOH)	HPLC-HG-ICPMS	iAs= 0.012 ± 0.003	
					SON and MAE/(TFA/H$_2$O$_2$)	HPLC-HG-ICPMS	iAs= 0.011 ± 0.004	
					Shaking/(HCl) /extraction (CHCl$_3$/back extr. 1 M HCl)/	HPLC-HG-ICPMS	iAs= 0.036 ± 0.007	
					MAE/(HNO$_3$)	HPLC-HG-ICPMS	iAs= 0.011 ± 0.002	
					MAE/(HNO$_3$/H$_2$O$_2$)	HPLC-HG-ICPMS	iAs= 0.017 ± 0.003	
					MAE/(H$_2$O)	HPLC-HG-ICPMS	iAs= 0.011 ± 0.003	
					SON/(H$_2$O)	HPLC-HG-ICPMS	iAs= 0.010 ± 0.001	
					MAE/(NaOH/EtOH)	HPLC-HG-ICPMS	iAs= 0.010 ± 0.003	
				9.64 ± 0.11	MAE/(HNO$_3$/H$_2$O$_2$)	HPLC-ICPMS	iAs= 0.02 ± 0.003	Zmozinski et al. [212]
				No reported	MAE/(H$_2$O)	IEC-ICPMS	As(III)= 0.253 ± 0.019 As(V)= 0.134 ± 0.006	Leufroy et al. [221]
DORM-2	Dogfish Muscle	NRC-CNRC	18.0 ± 1.1	18.75 ± 0.66	MAE/(MeOH/H$_2$O)	HPLC-ICPMS	As(III)= 0.61 ± 0.04	Santos et al. [220]
				17.9 ± 0.9	MAE/(H$_2$O)	IEC-ICPMS	As(III)= 0.031 ± 0.014 As(V)= 0.029 ± 0.018	Leufroy et al. [224]
				19.7 ± 0.4	MAE/(MeOH/H$_2$O)	IEC-ICPMS	As(III)= 0.064 ± 0.011 As(V)= 0.026 ± 0.002	
				17.0 ± 0.7	Shaking (two-step sequential extraction)/acetone and MeOH/water	HPLC-HR-ICP-MS	As(III)= below LOD As(V)= below LOD	Ruiz-Chancho et al. [225]
				17.9 ± 0.98	Step 1: MAE/(HClO$_4$/Fe$_2$(SO$_4$)$_3$/HCl Step 2: (As(III)):SON/HCl/CHCl$_3$/HCl	ETTAS	As(III)= 0.053 ± 0.001 As(V)= 0.051 ± 0.002	Shah et al. [96]
				16.9 ± 0.3 (as sum of As species)	Cell clean-up – PAEH	HPLC-ICPMS	As(III)= 0.081 ± 0.0005	Moreda-Piñeiro et al. [226]
				19.5 ± 1.3	Shaking/(HCl) /extraction (CHCl$_3$/back extr. 1 M HCl)/	HR-ICPMS	iAs= 0.131 ± 0.010	Lewis et al., 2012 [105]
				No reported	Ultrasonic bath/H$_2$O	SIA-HPLC-AFS	As(III)= 0.037 ± 0.02	Jesus et al. [150]
DORM-3	Dogfish Muscle	NRC-CNRC	6.88 ± 0.30	5.8 ± 0.4	MAE/(H$_2$O)	IEC-ICPMS	As(III)= 0.085 ± 0.014 As(V)= 0.243 ± 0.023	Leufroy et al. [224]
				7.1 ± 0.4	MAE/(MeOH/H$_2$O)	IEC-ICPMS	As(III)= 0.129 ± 0.018 As(V)= 0.276 ± 0.036	

				No reported	MAE/(EtOH/NaOH)	HPLC-ICPMS	iAs= 0.073 ± 0.008	Pétursdóttir et al. [227]
				No reported	MAE/(H_2O) MAE/(H_2O/H_2O_2) MAE/(HNO_3/H_2O_2)	HPLC-HG-ICPMS HPLC-HG-ICPMS HPLC-HG-ICPMS	iAs= 0.11 ± 0.01 iAs= 0.12 ± 0.01 iAs= 0.16 ± 0.01	Pétursdóttir et al. [145]
				No reported	MAE/(HCl/H_2O_2) MAE/(HCl/H_2O_2)	HPLC-ICPMS SPE-HG-AAS	iAs= 0.19 ± 0.01 iAs= 0.18 ± 0.02	Rasmussen et al. [121]
				No reported	MAE/(H_2O)	IEC-ICPMS	As(III)= 0.134 ± 0.008 As(V)= 0.263 ± 0.009	Leufroy et al. [221]
				7 ± 0.8	SON/(HNO_3/MeOH)	HPLC-ICPMS	As(V)= 0.4 ± 0.06	Batista et al. [90]
				No reported	Shaking/SON/(H_2O)	CE-ICPMS	As(V)= 1.40 ± 0.04	Liu et al. [192]
CRM n 9	*Sargassum fulvellum*	NIES	115 ± 9	110.3 ± 0.7	Shaking/(Water)	HPLC-ICPMS	As (V) = 69.9 ± 1	Llorente-Mirandes et al. [228,229]
				117 ± 2	Shaking/(Water)	HPLC-ICPMS	As(V)= 68.5 ± 6.6	Ruiz-Chancho et al. [230]
				109 ± 2	MAE/(Water)	HPLC-(UV)-HG-AFS	As(V)= 70 ± 1	Garcia-Salgado et al. [151]
BCR-279	*Ulva lactuca*	IRMM	3.09 ± 0.21	2.9 ± 0.3	Shaking/(Water)	HPLC-ICPMS	As(III)= 0.06 ± 0.03 As(V)= 0.53 ± 0.04	Pell et al. [231,232]
				3.4 ± 0.1	SON/(Water)	HPLC-ICPMS	As(V)= 0.7	Caumette et al. [233]

Notes. The ± terms are as provided by the original publications. They are predominantly standard deviations for some number of replicates or in some cases uncertainties. MAE; Microwave Assisted Extraction; SON: Sonication; PAEH: Pressurized Assisted Enzymatic Hydrolysis Extraction; UAE: Ultrasound-Assisted Extraction.

Highlights of inorganic arsenic analysis in SRM 1568a rice

For several years, NIST SRM 1568a rice has been analyzed as part of the method validation for the determination of As(III), As(V), MA, and DMA in rice. Although it is only certified for tAs content (0.290 ± 0.030 mg As kg^{-1}) and not for arsenic species, it is routinely used to assess the accuracy of As species by comparing measured results with the literature. Almost no studies report results for more than 4 species and there seems to be agreement that the material only contains iAs and the two methylated species, as these are what are detectable by the majority of the methods employed in the literature reviewed.

Several authors analyze the rice material and dataset includes 46 iAs results, as shown in Figure 5. Plotting the results chronologically does not lead to any further conclusion: there is no obvious change in the reported values as a function of time, although the time covered is short (2010-2015). The dataset includes one result outside the ± 3 standard deviations range, 0.204 mg As kg^{-1}, so this is considered an outlier. If this value is excluded, the mean value for iAs is 0.098 ± 0.009 mg As kg^{-1} (X ± SD, n=46 results, corresponding to 34% of the certified tAs), where the ± term is the standard deviation (SD) of all the reported values. Although several methods and techniques are used by different researchers, it is worth noting that little dispersion of the iAs results was found. The iAs results range from 0.074 to 0.113 mg As kg^{-1}. Satisfactory agreement between the reported values and the calculated mean value is observed. If the reported values are expressed in terms of error, considering the mean value as a reference value, they would range from 76% to 116%.

Different measurement techniques are used to determine iAs content, with HPLC-ICPMS being the most common (with different HPLC columns, different eluents, etc.): 36 results were found from several authors [30,32,33,144,161–164,167,170,173,175,177,178,180,208,210,219,234–242] whereas only one researcher used the HPLC-HG-AFS coupled system [243]. Several authors use non-coupled HG as a previous step to measuring iAs with different techniques. Five publications from the same group use FI-HG-AAS to determine iAs content [131–135]; while two authors apply an HG-AFS system, one of them with a prior SPE step [137] and the other without SPE [138]. Furthermore, a validated method using an SPE-HG-AAS system is applied [122]; and also a speciation method using selective HG conditions and measuring by ICPMS is reported [139]. In addition, a method for determination of inorganic arsenic by CPE-UV-Vis is used [195]. Meanwhile, Lopez-Garcia et al. [94] reports a value for As(III) + As(V) + MA = 0.099 mg As kg^{-1} by ETAAS using suspensions prepared in 0.01 mol L^{-1} TMAH, which is in close to the mean calculated value.

Different extraction solvents are used, supported by sonication, shaking, MAE or heating in a water bath, etc. Some of these cause redox changes in the inorganic species producing a high dispersion in the values reported for arsenite or arsenate, and high uncertainty over the reported concentrations. In spite of high interconversion between arsenite and arsenate, the total iAs content remains constant and unaltered with no loss of analytes observed. This can be seen in Figure 5, in which the results are tabulated as iAs, and the majority of the data are inside the target interval X ± SD. The most commonly used extraction solvent is dilute HNO_3 [30,162,164,170,175,177,180,208,234–236,239–241]. Other studies combine the use of HNO_3 with the addition of H_2O_2 to oxidize As(III) to As(V) and quantify the total iAs as As(V) [32,33,122,137–139,144,167,219]. Also,

a specific extraction method such as selective extraction of iAs with HCl and subsequent extraction with $CHCl_3$ of the iAs present in the acid medium is applied by several authors [131–135]. Meanwhile, other extraction methods are also used to extract iAs from the rice material, including: enzymatic extraction [173,178,210]; H_2O [144,161]; MeOH/H_2O [163,237]; TFA [180,242,243]; and suspensions of TFA in H_2O_2 [180], NH_3 [180] or TMAH [94].

Despite the use of different extraction methods and measurement techniques, the values reported show no clusters related to the analytical approach. The concentration of iAs determined in this CRM does not seem to depend on the analytical methodology. The NIST website indicates SRM 1568a is not available at present (last access: May 2015): this material is currently "out of stock" and was superseded by SRM 1568b, which was certificated in October 2013. As specified in the certificate of analysis, the existing material from production of SRM 1568a was used to produce the new SRM 1568b. The certified mass fraction value for iAs in the new SRM is 0.092 ± 0.010 mg As kg^{-1} , which is in perfect agreement with the data previously reported for the analysis of the former NIST 1568a (iAs= 0.097 ± 0.009 mg As kg^{-1}). The expanded uncertainty for SRM 1568b (0.010 mg As kg^{-1}) does include the mean of the values reported for SRM 1568a, and thus it is likely that the means are not significantly different. Therefore, we seem to be able to claim that the international analytical chemistry community is capable of measuring iAs content in rice.

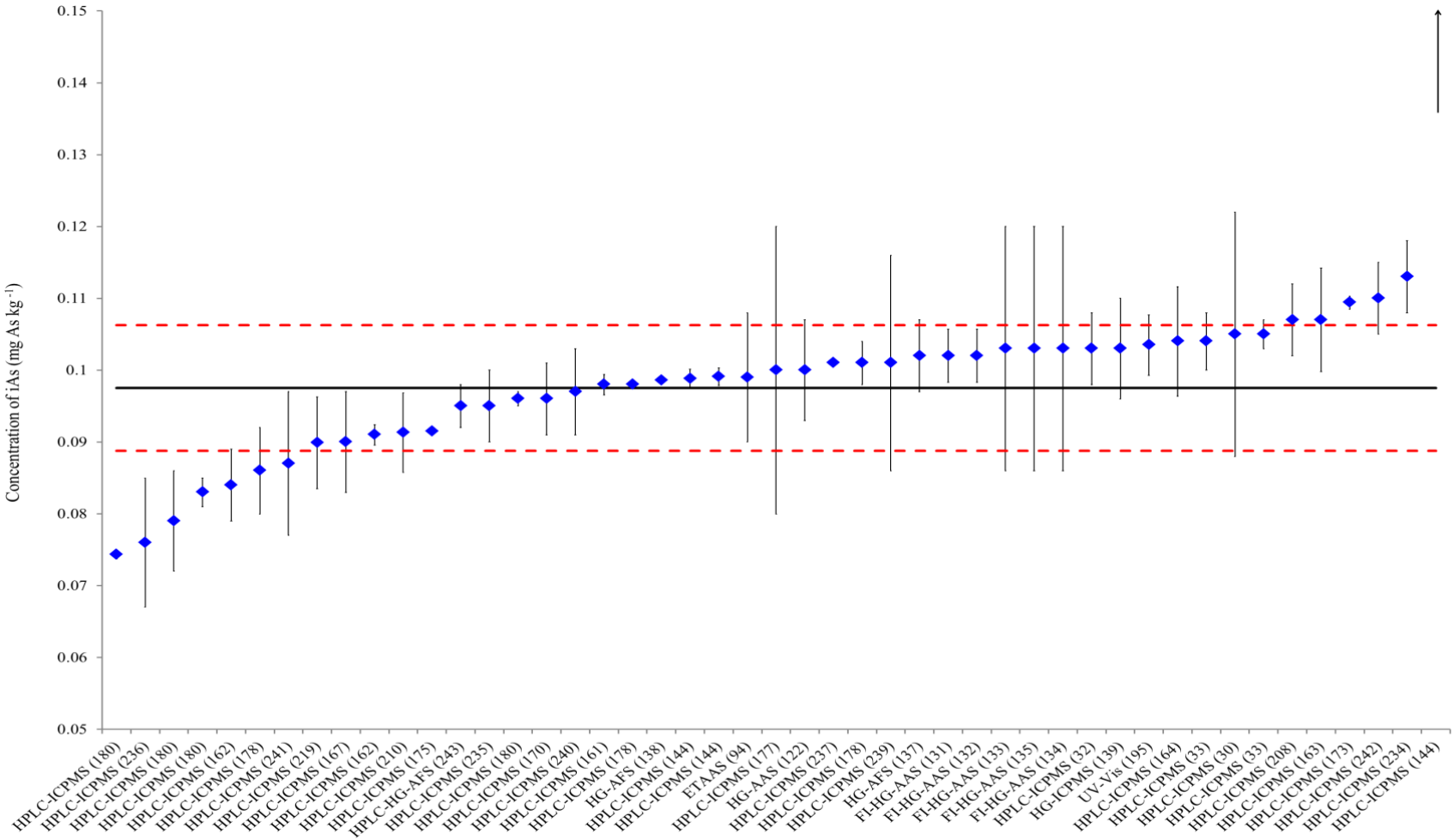

Figure 5. Inorganic arsenic concentration in NIST SRM 1568a reported in the literature (blue rhombus, 2010-2015). The continuous black line represents the average concentration and the red dashed lines delimit the target interval (X ± SD = 0.098 ± 0.009 mg As kg^{-1} of inorganic arsenic). X axis shows the measurement technique and reference.

Highlights of inorganic arsenic analysis in TORT-2 Lobster Hepatopancreas

Among the marine food CRMs, TORT-2 Lobster Hepatopancreas is one that is commonly analyzed in the literature. The material was produced by NRC-CNRC and the certificate is dated December 1994. It is certified for tAs content (21.6 ± 1.8 mg As kg^{-1}, mean value ± uncertainty) but not for arsenic species. Several As species have been reported in this material, with AB being the major species and DMA, MA and TMAO minor components [212,224].

Thirty-four published iAs contents [121,145,182,192,212,224,227,244,245] are tabulated and shown in Figure 6. The dataset includes an outlier: 4.46 mg As kg^{-1}, which is excluded from our further calculations. Reported values range from 0.230 to 1.233 mg As kg^{-1} for iAs; and the calculated mean value is 0.606 ± 0.215 mg As kg^{-1} (X ± SD, n=33 reported data), where the ± term is the standard deviation of all the reported values. High variability of results is found, the RSD of the reported values is 36%. As expected, iAs corresponds to a low proportion (2.8%) of the certified tAs content. Classifying the results chronologically does not lead to any further conclusion about the high dispersion of the published results. If we assume that the calculated mean value is the "true value", values ranges from 38% to 204% which not desirable from the analytical point of view.

Several techniques are employed to determine iAs content, with HPLC-HG-ICPMS being the most commonly used with different HPLC columns, mobile phases, extraction solvents, etc. Sixteen values for iAs have been found, resulting in an iAs value of 0.551 ± 0.142 mg As kg^{-1} (mean ± SD, n=16) [145,182,211,227]. Fourteen results are obtained using a coupled HPLC-ICPMS system, resulting in an iAs value of 0.652 ± 0.275 mg As kg^{-1} (mean ± SD, n=14) [121,145,182,212,224,227,244,245]. Differences were observed when comparing the mean HPLC-HG-ICPMS values with those obtained by HPLC-ICPMS; however, in both cases the standard deviation is quite high and the intervals (i.e., mean ± SD) overlap, which leads us to consider that no differences are observed between the means for the two techniques. Only one author used another coupled technique: HPLC-HG-AFS, with an iAs value of 0.369 ± 0.018 mg As kg^{-1} [227]. A study analyzing iAs content by CE-ICPMS obtained the highest value for iAs: 4.46 ± 0.03 mg As kg^{-1} [192]. Few data using non-coupled techniques are reported: two results obtained by SPE-HG-AAS, iAs = 0.90 ± 0.07 mg As kg^{-1} [121] and iAs = 0.544 ± 0.162 mg As kg^{-1}, as a value obtained from an inter-laboratory comparison (IMEP-32) [245]. Furthermore, one researcher found an iAs value of 0.669 ± 0.034 mg As kg^{-1} by high resolution (HR)-ICPMS [145].

A wide range of solvents supported by sonication, shaking, MAE or heating in a water bath are used to extract iAs from the CRM matrix. The most commonly used extraction solvents are: HCl with or without H_2O_2 [121,145,182,245]; HNO_3 with or without H_2O_2 [145,182,212]; NaOH in 50% EtOH [145,182,227,244]; and H_2O [145,224]. According to the reported values, mean values for iAs are: 0.674 ± 0.126 (n=8), 0.682 ± 0.097 (n=7) and 0.670 ± 0.264 (n=6) mg As kg^{-1} (mean ± SD) for HCl, HNO_3 and H_2O extractions, respectively. No differences in iAs content are observed between the three extraction solvents. However, mean data for NaOH in 50% EtOH extractions result in a lower value: 0.390 ± 0.085 mg As kg^{-1} (mean ± SD, n=7). To a lesser extent, other solvents are used, such as 50% MeOH or TFA extractions. In some cases, there are large differences between data obtained using the same extractant, with the measurement technique possibly being responsible for such dispersion. For example, using 50% MeOH, the differences

between reported values are notable: the iAs value is 0.676 by HPLC-HG-ICPMS [145] and 1.233 mg As kg^{-1} by IC-ICPMS [224]. Similarly with TFA extractions the iAs values are 0.315 (with the addition of H_2O_2) and 0.514 mg As kg^{-1} (without H_2O_2) [145]; with there being differences in the use of H_2O_2 and also in the measurement technique: the former using HPLC-HG-ICPMS and the latter HPLC-ICPMS. In another example, applying selective solubilization of iAs with HCl, subsequent extraction with $CHCl_3$ and further back-extraction with HCl, differences were also observed in the iAs content: 0.669 vs 0.331 mg As kg^{-1} [145]. The higher value is obtained by HR-ICPMS while the lower value corresponds to using HPLC-HG-ICPMS.

As an overview of iAs content in TORT-2, and in accordance with the values in Figure 6, we can say that highly variable iAs data have been published, which illustrates that it is difficult to obtain a consistent value for iAs in this seafood CRM. Comparing values in the literature according to the extraction method used leads us to state that NaOH extractions show lower concentrations than other solvents (i.e., HCl, H_2O or HNO_3). The large differences in the literature between concentrations of iAs in this seafood material reinforce the need to develop more and more reliable methods for its determination.

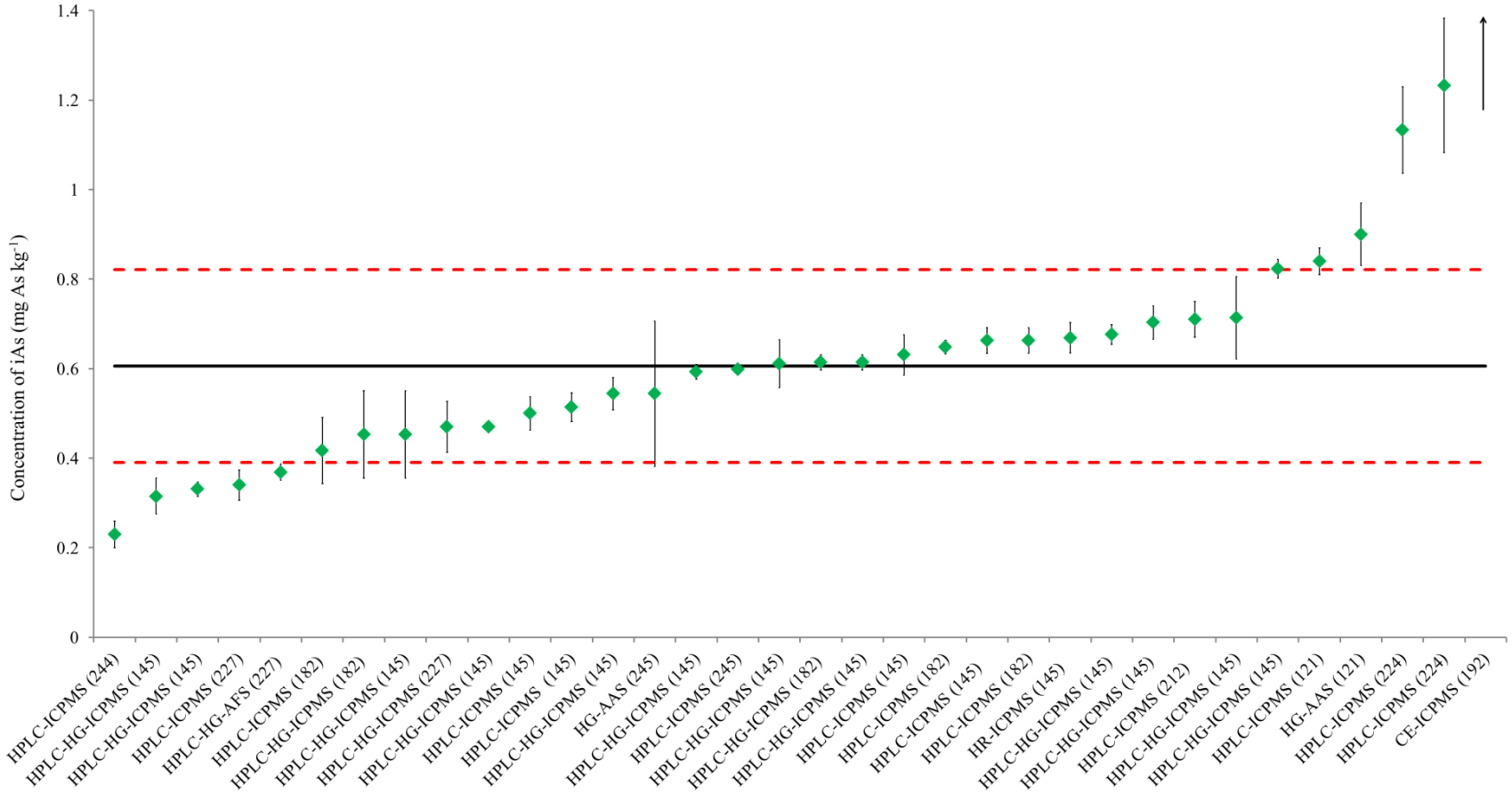

Figure 6. Inorganic arsenic concentration in NRC-CNRC TORT-2 reported in the literature (green rhombus, 2010-2015). The continuous black line represents the average concentration and the red dashed lines delimit the target interval (X ± SD= 0.606 ± 0.215 mg As kg^{-1} of inorganic arsenic). X axis shows the measurement technique and reference.

Highlights of inorganic arsenic analysis in DOLT-4 dogfish

The dogfish (*Squalus acanthias*) liver DOLT-4 is one of most analyzed of seafood CRMs. The material was produced by NRC-CNRC and the certificate is dated May 2008. It is certified for tAs content (9.66 ± 0.62 mg As kg^{-1}, mean value ± uncertainty) but not for iAs. AB is the major As compound followed by DMA, iAs, MA, TMAO, etc., as minor compounds [212].

Studies analyzing this dogfish liver material produce 17 published values for iAs in the literature (Table II). Some of the data correspond to values reported from PT, IMEP-109/30 [40]. From the results reported, the values range from 0.010 to 0.387 mg As kg^{-1} for iAs; and two of them could be considered as outliers (0.387 and 0.152 mg As kg^{-1}). Excluding those two values, the calculated mean is 0.024 ± 0.019 mg As kg^{-1} (X ± SD, n=15, ranging from 0.010 to 0.075), where the ± term is the standard deviation of all the reported values. Very high dispersion of results is reported and the RSD of the reported values is 76%. As usual in fish, the iAs content corresponds to a low proportion (0.3%) of the tAs content. There are few data in the literature, and a classification chronologically does not lead any conclusion about the high variability of the published iAs results. Range of values, considering the mean value as true value, ranged from 41% to 308%; again highlighting the considerable variability of the iAs results in the literature.

Tabulating the results by measurement techniques shows that the iAs mean values are: 0.014 ± 0.008 (n=9) and 0.031 ± 0.010 (n=6) mg As kg^{-1} (mean ± SD) for the coupled techniques HPLC-HG-ICPMS [145,182] and HPLC-ICPMS [40,182,212,221], respectively. Only two results obtained using non-coupled techniques have been published: iAs = 0.075 ± 0.005 mg As kg^{-1} by FI-HG-AAS [40]; and iAs = 0.152 ± 0.010 mg As kg^{-1} by HR-ICPMS [40].

Sorting the results by extraction method shows that several different solvents supported by sonication, shaking, MAE or heating in a water bath, are used to extract iAs from the fish matrix. For example, the following extractants were used: H_2O (n=3) [145,221]; NaOH in 50% EtOH (n=2) [182]; MeOH (n=1) [145]; HCl with H_2O_2 (n=2) [182]; and TFA (n=2) [40,145]. Extractions based on HNO_3 provide a mean value of 0.019 ± 0.007 mg As kg^{-1} (mean ± SD, n=4). There is high variability between selective extractions of iAs based on the method of Muñoz et al. [126], depending on the measurement technique employed; the iAs values are 0.036, 0.075 and 0.152 mg As kg^{-1} using HPLC-HG-ICPMS [145], FI-HG-AAS and HR-ICPMS [40], respectively.

It should be noted that a low iAs concentration is found in DOLT-4: 0.024 ± 0.018 mg As kg^{-1} (excluding the two outliers), with high dispersion between the reported values (Table II). It is not possible to show whether the extraction method or the measurement technique are significant influential factors; however, most reported methods show a low concentration of iAs in the material (<0.080 mg As kg^{-1}). Further developments and improvements of the analytical methods to determine iAs in seafood are needed in order to provide reliable iAs results.

3.1.3 Other strategies to evaluate accuracy

Although some CRMs with a certified iAs value have been produced in recent years, this does not seem to cover the wide range of the foodstuffs usually consumed in common diets. Some alternative approaches to evaluate accuracy without the appropriate and representative CRMs are: performing spiking experiments; compare the method with a reference method and

comparing different sample preparations with each other. In the following paragraphs we summarize some alternatives found in the literature to assess accuracy without a certified reference value.

Spiking experiments

An alternative, to assess accuracy in the absence of CRMs, is to perform spiking experiments and then calculate the recovery. Typically, a test material is analyzed by the method under validation both in its original state and after the addition (spiking) of a known mass of iAs to the test sample. Spiking (also known as fortification) procedures must be carefully planned in order to select the most suitable strategy to introduce a single iAs species or mixture of both (i.e., arsenite and arsenate) into the matrix. Some other variables that should be checked in order to prepare a spiked sample with a similar matrix to the original sample are: the maximum volume or weight to be added to the matrix; the contact time and conditions; and further pre-treatment steps (e.g. drying, sieving, milling, etc.). Furthermore, the homogeneity of the distribution of the species within the matrix should be addressed. In the case of the incorporation of a spiking solution into a liquid homogeneity is relatively easy to achieve; whereas, the process can be much more difficult when working with a solid matrix. Spiked samples, or sometimes a blank sample, are subjected to the respective sampling procedures and the contents measured [29,32,33,97,121,122,137,139,160,164,167,169,178,180,207,210,212,227]. The recoveries obtained are usually compared to CODEX criteria: 60%–115% for 10 μg kg^{-1} and 80%–110% for 0.1–10 mg kg^{-1} [246]. Recoveries in these ranges are considered acceptable and demonstrate the reliability of the sample preparation method. Sometimes spiking experiments are carried out by adding standards of As species to CRMs before analysis. Although the iAs content is not certified, the spiking of iAs has been performed on SRM 1568 rice [144,178] and also BCR-627 tuna fish [178].

Methods comparison

Another approach to evaluating accuracy is to compare the results achieved with a fully validated method to test for bias in the proposed method. This is a useful option when checking an alternative to an established standard method already validated and in use in the laboratory. Some studies of iAs determination compare methods in rice samples: SPE HG-AAS with HPLC-ICPMS [122]; HG-ICPMS with HPLC-HG-ICPMS [139]; HG-AFS with HPLC-ICPMS [138]; a slurry sampling-HG-AAS method [125] with the Chinese standard HG-AFS method [82]. Few studies comparing iAs results in on seafood samples were found, but one example of such a study compares SPE HG-AAS with HPLC-ICPMS [121]. Another study used MAE extraction with NaOH (1.5 mg/mL) in 50% ethanol to extract iAs from seafood samples and CRMs; the results were compared using different techniques: HPLC-ICPMS vs HPLC-HG-ICPMS vs HPLC-HG-AFS [227].

Another strategy to check the reliability of results is to compare different sample preparation procedures followed by measurements using the same detection technique. For example, three extraction methods are compared in seafood samples and CRMs, and the results are discussed according to the use of HPLC-ICPMS with and without HG [182]. The same authors extend the study to nine extraction methods for iAs determination in seafood (i.e., the most

commonly used in the literature) followed by measurements using HPLC-HG-ICPMS and the results are extensively discussed [145]. Different extraction methods are also applied, followed by measurements using HPLC-ICPMS, to compare the results in cereal-based food [175] and in rice [144,218].

3.2. Proficiency testing (PT)

As external QC, PT or inter-laboratory comparisons, is a valuable tool to test the reliability of a method by comparing results with an assigned reference value. Some institutions, organizations and laboratories regularly organize PT to evaluate the performance capabilities of analytical laboratories. In the following section we summarized PT focused on the determination of iAs in food matrices.

3.2.1 EC-JRC-IRMM proficiency testing (PT)

The Institute for Reference Materials and Measurements (IRMM) of the Joint Research Centre (JRC), a Directorate General of the European Commission, operates the International Measurement Evaluation Program (IMEP). It organizes inter-laboratory comparisons in support of European Union policies. The Directorate General for Health and Consumers (DG SANCO) of the European Commission (EC) has requested the European Union Reference Laboratory for Heavy Metals in Feed and Food (EU-RL-HM) to evaluate the performance of European laboratories with regards to total As and iAs analysis in food, with a view to future discussions on the need for regulatory measures. With that brief, several PT protocols have been organized in recent years by the IMEP on behalf of the EU-RL-HM. In the following paragraph we focus on PT organized within the IMEP, as summarized in Table III.

Table III. Proficiency tests and method validation focused on the determination of iAs in foodstuffs organized by EC-JRC-IRMM.

Proficiency test	Type of food	Objective	Analyte	Assigned values (mg As kg^{-1}) [a]	Results of participants [b]	Comments	References
IMEP-107 (2010)	Rice (produced by IRMM)	Judge the state of the art of analytical capability for the determination of total and iAs	tAs and iAs	tAs= 0.172 ± 0.018 and iAs= 0.107 ± 0.014	tAs, z= 77% (n=71) and iAs, z= 75% (n=21)	· Satisfactory performance of the participating labs for iAs. · The number of labs that determined iAs was lower than that determined tAs. · An extra effort is needed in the evaluation of uncertainties.	de la Calle et al. [39,165]
IMEP-30/109 (2010)	Dogfish liver (NRC CRM DOLT-4)	· To evaluate the analytical capabilities of nominated NRL and other laboratories	Cd, Pb, As, Hg, iAs and MeHg	tAs= 9.66 ± 0.62 and iAs= not assigned	· IMEP-30: tAs, z= 89% (n=42) and no scored for iAs · IMEP-109: tAs, z= 85% (n=28) and no scored for iAs	· Few participants reported values for iAs (23%) and iAs results were spread over a wide range. · Underestimation of tAs content	Baer et al. [40,247] de la Calle et al. [248]
IMEP-112 (2011)	Wheat (produced by IRMM)	To judge the state of the art of the determination of total and iAs in food	tAs and iAs	tAs= 0.177 ± 0.012 and iAs= 0.169 ± 0.025	tAs, z= 84% (n=51) and iAs, z= 58% (n=23)	· Satisfactory performance of iAs in wheat	de la Calle et al. 2012 [41,249]
	Vegetable food (NIST SRM 1570a spinach leaves)			tAs= 0.068 ± 0.012 and iAs= 0.054 ± 0.012	tAs, z= 74% (n=35) and iAs, z= 77% (n=23)	· Satisfactory performance of iAs in vegetable food	
	Algae (produced by IRMM)			tAs= 58.3 ± 7.0 and iAs= 0.188 ± 0.025	tAs, z= 82% (n=41) and iAs, z= 16% (n=6)	· Low number of laboratories obtaining a satisfactory ζ score for iAs in algae matrix. · Two standards for the determination of iAs did provide biased results (EN 15517:2008 and GB/T 5009.11-2003). · Underestimations of tAs content due to the incomplete digestion of sample.	

IMEP-39/116 (2013)	Mushroom (produced by IRMM)	To test the analytical capabilities of laboratories to determine heavy metals and tAs and iAs in mushrooms.	Cd, Pb, As, Hg and iAs	iAs= 0.321 ± 0.026	· IMEP-116: tAs, z= 91% (n=29) and iAs, z= 81% (n=13) · IMEP-39: tAs, z= 65% (n=35) and iAs, z= 64% (n=7)	· In IMEP-116, high percentage of NRLs obtained a satisfactory z -score for iAs. · In IMEP-39, several labs obtained satisfactory results for iAs using AAS-based techniques. · For tAs, participants using AAS-based techniques reported lower values than participants using ICPMS or ICPOES. · A high percentage of labs reported uncertainties which were likely underestimated	Cordeiro et al. [42,250,251]
IMEP-118 (2014)	Canned food (peas in brine) (produced by IRMM)	· To assess the analytical capabilities of participating laboratories · To evaluate the various sample preparation approaches when analyzing canned vegetables using the drained product or the the solid/liquid composite	As, Cd, Pb, Hg, Sn and iAs	Drained product: tAs= 0.117 ± 0.018 and iAs= 0.098 ± 0.020	tAs, z= 92% (n=47) and iAs, z=84% (n=16).	· iAs, drained product: the reported results are in good agreement with the assigned value. · iAs, solid/liquid composite: a tendency of overestimation. · tAs: the performance of the participants analyzing the drained product was better than those analyzing the solid/liquid composite. · A tendency to underestimate the tAs mass fraction. · An extra effort was identified in the evaluation of uncertainties associated to the results. · Significant discrepancies were observed for the LOD reported. Clear confusion between the LOD of the method and the instrumental LOD.	Fiamegkos et al. [44]
				Solid/liquid composite: tAs= 0.121 ± 0.014 and iAs= 0.082 ± 0.008	tAs, z= 82% (n=42) and iAs, z=74% (n=17).		
IMEP-41 (2014)	Rice (IMEP-107)	· To determine the performance characteristics of an analytical method for the quantification of inorganic arsenic in food by FI-HG-AAS	· Inorganic arsenic	iAs= 0.108 ± 0.011	· RSD_r= 7.8% · RSD_R= 15.6 · Overall mean= 0.096 ± 0.030 · Rec= 88.9 ± 29.4		Fiamegkos et al. [43]
	Wheat (IMEP-112)			iAs= 0.165 ± 0.021	· RSD_r= 10.1% · RSD_R= 10.9% · Overall mean= 0.146 ± 0.032 · Rec= 88.7 ± 22.5		
	Mussels (ERM-CE278k)			iAs= 0.0863 ± 0.008	· RSD_r= 8.6% · RSD_R= 18.2% · Overall mean= 0.133 ± 0.048 ·Rec= 153.7 ±		

Cabbage (IAEA-359)	iAs= 0.091 ± 0.016	· RSD_r= 9.6% · RSD_R= 22.1% · Overall mean= 0.074 ± 0.033 · Rec= 81.6 ± 38.7	· The results obtained by an expert lab using HG-ICPMS, in the case of mussels, cabbage and fish, were not in agreement with the rest of the results delivered by the experts. Determination of iAs by HG-ICP-MS is affected by interferences in samples with a high percentage of organic species of As, if no previous separation of those species is done.
Mushroom (IMEP-116)	iAs= 0.321 ± 0.026	· RSD_r= 4.1% · RSD_R= 6.1% · Overall mean= 0.275 ± 0.034 · Rec= 85.8 ± 12.6	
Seaweed (NMIJ-7405a)	iAs= 10.10 ± 0.50	· RSD_r= 4.7% · RSD_R= 15.2% · Overall mean= 7.548 ± 2.301 · Rec= 74.7 ± 23.1	
Fish (DORM-4)	iAs= 0.271 ± 0.061	· RSD_r= 10.3% · RSD_R= 22.8% · Overall mean= 0.295 ± 0.134 · Rec= 108.8 ± 55.4	Point 9.3.1 of the SOP is critical in samples with high fat content (mussels and fish). Some laboratories could not separate the two phases due to formation of an emulsion.
Rice (ERM-BC211)	iAs= 0.124 ± 0.011	Pre-test item of participants laboratories	Laboratories having reported results in agreement with the certified value were allowed to continue with the IMEP-41 analysis

[a] Assigned value for expert laboratories as $X_{ref} \pm U_{ref}$ (k = 2); [b] In IMEP-107, IMEP-30/109, IMEP-112, IMEP-39/116 and IMEP-118: results of participants are referred to % of z-score to z ≤2 (n=number of laboratories). RSD_r= repeatability relative standard deviation; RSD_R= reproducibility relative standard deviation; Rec=Recovery= X participants· 100/X assigned value.

IMEP-107: Determination of total and inorganic As in rice

The first PT to include iAs as an analyte was organized in 2009 and focused on the determination of total As and iAs in rice (IMEP-107) [39,165]. Reference values for total As and iAs were satisfactory assigned by several expert laboratories. A wide range of sample pre-treatment methods, and instrumental set-ups were applied by participants and the expert laboratories. Despite the use of these different methods, the results were not observed to cluster in relation to the analytical approach. The organizers comment that no particular problem related to the determination of iAs in rice was detected in the PT, and the performance of the participating laboratories was satisfactory. Finally, they conclude that the concentration of iAs determined in rice does not depend on the analytical method applied and that introduction of a maximum level for iAs in rice should not be postponed due to analytical concerns [39]. In addition, the IMEP-107 rice test material has been used as RMs in several studies and was analyzed to assess the accuracy of iAs results obtained using the specific method [33,97,122,164].

IMEP-109/30: Analysis of total Cd, Pb, As and Hg, as well as MeHg and iAs in seafood

Encouraged by the satisfactory results for iAs in rice, two inter-laboratory comparisons, IMEP-109 and IMEP-30, were performed in 2010 of the measurement of some trace elements, in addition to iAs, in seafood [40]. Only the EU NRL took part in IMEP-109 [248], while IMEP-30 was open to all laboratories [247]. The commercially available CRM DOLT-4 from NRC-CNRC was used as the test material for all this PT. Five expert laboratories, analyzed the test material to establish the reference value for iAs. The expert laboratories were not able to agree on a value for the iAs within a reasonable degree of uncertainty. For this reason, it was not possible to establish an assigned value for iAs and therefore the results from the laboratories for iAs could not be scored. The organizers concluded that the results were spread over a wide range, but 75% of the laboratories agreed that the iAs content of the test material did not exceed 0.25 mg kg^{-1}. Despite the spread, they stated that there seems to be no clear clustering of results according to the methods used. According to the results, the determination of iAs in seafood presented serious analytical problems and iAs is clearly more difficult to analyze in this seafood matrix than in rice (IMEP-107). Further information and possible causes for the dispersion of the results, attributed to the extraction and/or detection steps as the most likely cause, are widely discussed in the IRMM reports [247,248] and summarized in Baer et al. [40]. Additionally, it was concluded that more research is needed in the future to find appropriate and effective extraction procedures, as well as chromatographic conditions for reliable separation and quantification of iAs.

IMEP-112: Determination of total and inorganic in wheat, vegetable food and algae

IMEP-112 focused on the determination of total and inorganic arsenic in wheat, vegetable food and algae [41,249]. The assigned values (total As and iAs in wheat, and iAs in vegetable food and algae) were satisfactorily provided by a group of expert laboratories in the field. The organizers concluded that the concentration of iAs determined in any of the matrices does not depend on the analytical method applied, as proven by the results submitted by the seven expert laboratories and by the participants. A wide range of sample pre-treatment methods

and instrumental setups were applied and despite this, clustering of results related to the analytical approach was not observed. Furthermore, the participating laboratories performed, in general, satisfactorily for the determination of iAs in wheat and vegetable food; however, only a few laboratories obtained a satisfactory score for iAs in algae. Finally, it was also highlighted that, purely from the analytical point of view, there is no reason not to consider the option of introducing maximum levels for iAs in wheat, vegetable food and algae in further discussions of risk management [41]. Besides, the wheat test material used in IMEP-112 was also analyzed as external QC [32]

IMEP-116/39: Total Cd, Pb, As, Hg and inorganic As in mushrooms

Since mushroom consumption has increased considerably in recent years due to promotion of their nutritional properties, two PT programs were organized using the same test item (shiitake mushroom) [42]: IMEP-116 (for NRLs) [250] and IMEP-39 (for OCLs and other laboratories) [251]. Reference values were satisfactory assigned by five expert laboratories which analyzed the test item. In general, the performance of the participating labs was satisfactory for iAs: in IMEP-116 (NRLs), a high percentage of satisfactory results was obtained (z = 81%, n = 13) which is considerably higher than in IMEP-107 (rice). The organizers also pointed out that in IMEP-39, five out of the seven laboratories which obtained a satisfactory z-score for iAs used AAS-based techniques, showing that sound determinations of iAs can be made without the need for expensive sophisticated instrumentation [42]. Furthermore, the IMEP-116/39 PT item, shiitake mushroom, has also been used as external QC for iAs analysis [29].

IMEP-118: Determination of total As, Cd, Pb, Hg, Sn and iAs in canned food

In 2014, a PT program was produced focused on the determination of total As, Cd, Pb, Hg, Sn and iAs in canned food (peas in brine) (IMEP-118) [44]. Participation in the PT was mandatory for nominated NRLs, and open to other OCLs and interested laboratories. Unlike other IMEPs, the test material was spiked with arsenic during preparation. Expert and participant laboratories were asked to analyze total As and iAs in the canned vegetables, in both the drained product and the solid/liquid composite. Good agreement between the theoretical and the assigned value for total As in the solid/liquid composite was obtained; but not in the case of iAs. The brine was spiked with arsenate and the iAs mass fraction in the solid/liquid composite was found to be lower than the respective total As mass fraction: 35% lower than the theoretical one. Some possible causes are discussed and summarized in the IRMM report [44]. In spite this, the results from the two expert laboratories were in agreement and a reference value for the iAs mass fraction was assigned. From the PT results, it was concluded that the performance of the participating laboratories at determining iAs was satisfactory for both sample preparation approaches. However, few laboratories carried out analysis for iAs determination (only 33% reported values). Furthermore, the outcome of the PT clearly indicated that guidelines are needed on the sample preparation protocol to be used when analyzing canned food drained products and solid/liquid composites.

IMEP-41: Determination of inorganic arsenic in food

An inter-laboratory comparison was performed on a method evaluation by means of a collaborative trial for the determination of iAs in seven food products (IMEP-41) [43]. The method under evaluation was previously developed and in-house validated and final measurement was performed by FI-HG-AAS [126]. The organizers clearly stated that the standard operating procedure (SOP) was to be strictly followed and any deviation from the method should be reported. The seven test food items used in this exercise were RMs covering a broad range of matrices and concentrations (Table III). Five experts analyzed the test items using a method of their choice, different from the one being assayed. From the results, the organizers concluded that the method evaluated is robust and does not require any adaptation according to the matrix to be analyzed. Furthermore, the proposed method is considered fit-for-purpose, i.e., determination of iAs in different food products [43].

4.2.2 Other inter-laboratory comparisons

Other inter-laboratory comparisons focused on the determination of iAs in food have been organized in recent years. Institutions, organizations and laboratories regularly organize PTs to evaluate competency in the analysis of iAs species in food matrices. The Food Analysis Performance Assessment Scheme (FAPAS) of the Food and Environment Research Agency (FERA) has organized PT for several years, focused on several analytes in foodstuffs, with a wide range of tests available throughout the year. PTs on the determination of total and iAs in several food matrices is regularly organized [255]. A rice test material from the FAPAS interlaboratory tests [256] was analyzed in several studies as QC for iAs [32,33,207]. Brooks Rand Labs organized an inter-laboratory comparison study for arsenic speciation in white rice flour, brown rice flour, kelp powder, and apple juice in 2013. A large group of participating laboratories from around the world, forty-six laboratories from fifteen countries, registered to participate [257].

Specific PTs focused on iAs in rice has recently been organized. The Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan organized a collaborative study of speciation and determination of iAs in rice using HPLC-ICPMS. For it, an SOP of the method was developed and the proposed method was validated through the collaborative study of eastern and southeastern Asian countries [258]. Further PT based on the iAs content of rice was organized by the Inorganic Analysis Working Group (IAWG) of the Consultative Committee for Amount of Substance (CCQM). The CCQM-K108 key comparison was organized to test the capacities of the national metrology institutes or the designated institutes to measure the mass fractions of arsenic species and tAs in brown rice flour; while the National Metrology Institute of Japan (NMIJ) acted as the coordinating laboratory. The participants used different measurement methods to determine the iAs content of a rice sample [259].

4. CONCLUSIONS AND FUTURE TRENDS

Food control laboratories, consumers, authorities, institutions, health agencies and legislators have recently become more interested in iAs contents in food. This has led to several initiatives that move towards the development of robust and reliable analytical methods for selective determination of iAs in a range of food products. Although several techniques have been used in iAs determination, spectroscopic methods are the most commonly applied. Several such methods and techniques have been developed, but mild chemical extraction of iAs species and further determination by HPLC-ICPMS is undoubtedly the most popular approach used in iAs analysis in food. However, some non-chromatographic approaches that determine iAs accurately even in presence of other organoarsenic compounds have been reported as being less time-consuming and more cost-effective alternatives than those based on HPLC-ICPMS.

Although numerous CRMs have been analyzed to evaluate the accuracy of the methods for total arsenic, few of them are certified for iAs content. The differences found in the literature between the concentration of iAs in seafood CRMs illustrates that it is difficult to obtain a consistent value and reinforce the need to develop reliable methods for its determination, especially when matrices with a complex distribution of arsenic species are analyzed, as in the case of food of a marine origin. Further production of seafood CRMs would help in the validation of iAs methods and in providing reliable iAs data. Furthermore, more PTs for iAs determination in seafood are needed to assess the reliability of the proposed methods, since to date, they have shown unsatisfactory performance.

Concerning food safety, the distinction between iAs and total As content or other species in foodstuffs should be addressed in future maximum levels of arsenic in food. Moreover, more reliable data on iAs content in foodstuffs, especially less studied food products, are needed for reliable risk assessment and to estimate the health risk associated with dietary As exposure.

Finally, more efforts should be made to transfer the knowledge obtained by the analytical community concerning the development of selective methodologies for the determination of iAs to the future implementation of that knowledge as routine methods in food control laboratories. To this end, the validation of methods as well as participation in PT and the analysis of CRMs should be performed, as mandated by the ISO/IEC 17025 standard for laboratory accreditation purposes.

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PART II: OBJECTIVES

Chapter 3

Objectives

Interest in the determination of inorganic arsenic (iAs) in food reflects the wide recognition of its toxic effects on humans, even at low concentrations. According to the literature there are several arsenic speciation methods in use, however their suitability for a range of food samples and/or arsenic species needs to be established. Furthermore, there is an urgent need for robust validated analytical methods suitable for the determination of inorganic arsenic in a range of food items. This need has been emphasized by various international safety and health agencies, and by organizations in charge of establishing maximum levels of iAs in foodstuffs.

In view of the above, the main goal of this thesis was to develop a robust analytical methodology for the determination of inorganic arsenic as well as other arsenic species in foodstuffs. The proposed methodology was applied to several food commodities, providing reliable results in response to demands made by international safety agencies.

This overall aim can be divided into specific objectives, which are detailed below:

- Establishment and validation of methods for the determination of total arsenic and arsenic species in foodstuffs.

- As external quality control of the validated methods, participation in feasibility studies for the preparation of Certified Reference Materials with certified arsenic species and in proficiency tests for inorganic arsenic determination.

- Application of the proposed methodology in several foods of both terrestrial and marine origin: rice, cereal-based food, infant food products, mushrooms, seaweed, fish, crustaceans and bivalves.

- Estimation of daily dietary exposure to total and inorganic arsenic intake to assess the toxicological implications of the ingestion of the selected foodstuffs.

Publications

Publications included in the present thesis are summarized below:

- **Article I**
  Recent developments and quality assessment of inorganic arsenic determination in food: a review
  Llorente-Mirandes, T., Rubio, R., López-Sánchez, J. F.
  Article under revision

- **Article II**
  A fully validated method for the determination of arsenic species in rice and infant cereal products.
  Llorente-Mirandes, T., Calderón, J., López-Sánchez, J. F., Centrich, F., Rubio, R
  Pure Applied Chemistry, 2012, 84(2), 169–333.

- **Article III**
  A need for determination of arsenic species at low levels in cereal-based food and infant cereals. Validation of a method by IC-ICPMS.
  Llorente-Mirandes, T., Calderón, J., Centrich, F., Rubio, R., López-Sánchez, J.F.
  Food Chemistry, 2014, 147, 377–385.

- **Article IV**
  Occurrence of inorganic arsenic in edible Shiitake (Lentinula edodes) products.
  Llorente-Mirandes, T., Barbero, M., Rubio, R., López-Sánchez, J.F.
  Food Chemistry, 2014, 158, 207–215.

- **Article V**
  Arsenic speciation in commercial edible mushrooms and mushroom supplements by HPLC-ICPMS. Assessment of inorganic arsenic exposure.
  Llorente-Mirandes, T., Barbero, M., Rubio, R., López-Sánchez, J.F.
  Article sent for publication

- **Article VI**
  Measurement of arsenic compounds in littoral zone algae from the Western Mediterranean Sea. Occurrence of arsenobetaine.
  Llorente-Mirandes, T., Ruiz-Chancho, M.J., Barbero, M., Rubio, R., López-Sánchez, J. F.
  Chemosphere, 2010, 81, 867–875.

- **Article VII**

Determination of Water-Soluble Arsenic Compounds in Commercial Edible Seaweed by LC-ICPMS.

Llorente-Mirandes, T., Ruiz-Chancho, M.J., Barbero, M., Rubio, R., López-Sánchez, J. F

Journal of Agricultural and Food Chemistry, 2011, 59, 12963–12968.

- **Article VIII**

  Establishment of a method for determination of arsenic species in seafood by LC-ICP-MS.

  Zmozinski, A.V., **Llorente-Mirandes, T**., López-Sánchez, J.F., da Silva, M.M.

  Food Chemistry, 2015, 173, 1073–1082.

- **Article IX**

  Assessment of arsenic bioaccessibility in raw and cooked edible mushrooms by a PBET method.

  Llorente-Mirandes, T., Llorens-Muñoz, M., Funes-Collado, V., Sahuquillo, A., López-Sánchez, J. F.

  Food Chemistry, 2016, 194, 849–856

- **Article X**

  Performance of laboratories in speciation analysis in seafood. Case of methylmercury and inorganic arsenic.

  Baer, I., Baxter, M., Devesa, V., Vélez, D., Raber, G., Rubio, R., **Llorente-Mirandes, T**., Sloth, J.J., Robouch, P., de la Calle, B.

  Food Control, 2011, 22, 1928-1934.

- **Article XI**

  Is it possible to agree on a value for inorganic arsenic in food? The outcome of IMEP-112.

  de la Calle, M. B., Baer, I., Robouch, P., Cordeiro, F., Emteborg, H., Baxter, M. J., Brereton, N., Raber, G., Velez, D., Devesa, V., Rubio, R., **Llorente-Mirandes, T**., Raab, A., Feldmann, J., Sloth, J. J., Rasmussen, R. R., D'Amato, M., Cubadda, F.

  Analytical and Bioanalytical Chemistry, 2012, 404, 2475–2488.

- **Article XII**

  Determination of total cadmium, lead, arsenic, mercury, and inorganic arsenic in mushrooms: the outcome of IMEP-116 and IMEP-39.

  Cordeiro, F., **Llorente-Mirandes**, T., López-Sánchez, J.F., Rubio, R., Sánchez Agullo, A., Raber, G., Scharf, H., Vélez, D., Devesa, V., Fiamegos, Y., Emteborg, H., Seghers, J., Robouch, P., de la Calle., M.B.

  Food Additives & Contaminants: Part A, 2015, 32(1), 54–67.

- **Article XIII**

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

  Fiamegkos, I., Cordeiro, F., Robouch, P., Vélez, D., Devesa, V., Raber, G., Sloth, J.J., Rasmussen, R.R., **Llorente-Mirandes**, **T**., Lopez-Sanchez, J.F., Rubio, R., Cubadda, F., D'Amato, M., Feldmann, J., Raab, A., Emteborg, H., de la Calle, M.B.

  Article sent for publication

PART III: RESULTS

Chapter 4

Development and validation of methods for the determination of arsenic species in foodstuffs

In 2009, the EFSA Panel on Contaminants in the Food Chain (CONTAM Panel) assessed the risks to human health related to the presence of arsenic in food in European population [5]. As a general recommendation, dietary exposure to iAs should be reduced. Among the conclusions from this report, rice, cereal and cereal-based products were identified as the mainly contributors to daily iAs exposure in the general European population. Moreover, children aged less than three years were the most exposed to iAs, which was directly related to the intake of rice-based products. Furthermore, EFSA report [5] emphasized that there is a need for robust validated analytical methods for determining iAs in a range of food items. Recently, the European Union published Regulation (EU) 2015/1006 [107] amending Annex to Regulation (EC) No 1881/2006 [108] regarding the maximum levels of iAs in rice and rice-based products. The new MLs of iAs range from 0.10 to 0.3 mg As kg^{-1} depending of the rice product. Additionally, the European Commission has recently published a recommendation on the monitoring of arsenic in food by Member states during the years 2016, 2017 and 2018 [109]. The monitoring should include a wide variety of foodstuffs and Member States should carry out the analysis of arsenic, preferably by determining the content of iAs and tAs and, if possible, other relevant arsenic species.

Therefore, analytical laboratories of food control have to be ready to determine iAs in rice and also should now be prepared to analyse tAs and iAs in other foodstuffs, so they will need suitable and robust validated methods as a requirement of the ISO-UNE-EN 17025 [138] standard which is mandatory for analytical laboratories working on food control. A comprehensive scheme of quality assurance in analytical chemistry laboratories would include the following elements: validation of analytical methods; participation in proficiency tests (PTs); use of CRMs and routine application of internal quality control (QC). Proficiency testing is the means of ensuring that method validation and internal QC procedures are working satisfactorily. The participation in PTs could be useful to detect method validation interferences and initiate the solution of problems related unrecognized sources of error. Its main virtue is that it provides a means by which participants can obtain an external and independent assessment of the accuracy of their results. Hence, the participation in PT is a valuable tool to test the reliability of a method by comparing the obtained results with an assigned reference value.

In view of all this, we aimed to develop and validate analytical methods for the determination of arsenic species in food commodities with especial emphasis in rice and cereal-based foods that would be applied in routine analysis by food control laboratories. The analytical process for development and validation of a speciation method in these matrices is

complex. Different aspects have to be considered, including, sample-treatment, preparation procedures, detection method, calibration strategy as well as quality assurance evaluation including internal quality control assessment, establishment and validation of method parameters and external quality control evaluation. All the research work related to development and validation of speciation methods is presented in **Chapter 4.**

As a summary, publications included in **Chapter 4** are presented below:

- ✓ **Article II**
  A fully validated method for the determination of arsenic species in rice and infant cereal products.
  Llorente-Mirandes, T., Calderón, J., López-Sánchez, J. F., Centrich, F., Rubio, R
  Pure Applied Chemistry, 2012, 84(2), 169–333.

- ✓ **Article III**
  A need for determination of arsenic species at low levels in cereal-based food and infant cereals. Validation of a method by IC-ICPMS.
  Llorente-Mirandes, T., Calderón, J., Centrich, F., Rubio, R., López-Sánchez, J.F.
  Food Chemistry, 2014, 147, 377–385.

- ✓ **Article X**
  Performance of laboratories in speciation analysis in seafood. Case of methylmercury and inorganic arsenic.
  Baer, I., Baxter, M., Devesa, V., Vélez, D., Raber, G., Rubio, R., **Llorente-Mirandes, T**., Sloth, J.J., Robouch, P., de la Calle, B.
  Food Control, 2011, 22, 1928-1934.

- ✓ **Article XI**
  Is it possible to agree on a value for inorganic arsenic in food? The outcome of IMEP-112.
  de la Calle, M. B., Baer, I., Robouch, P., Cordeiro, F., Emteborg, H., Baxter, M. J., Brereton, N., Raber, G., Velez, D., Devesa, V., Rubio, R., **Llorente-Mirandes, T**., Raab, A., Feldmann, J., Sloth, J. J., Rasmussen, R. R., D'Amato, M., Cubadda, F.
  Analytical and Bioanalytical Chemistry, 2012, 404, 2475–2488.

- ✓ **Article XII**

  Determination of total cadmium, lead, arsenic, mercury, and inorganic arsenic in mushrooms: the outcome of IMEP-116 and IMEP-39.

  Cordeiro, F., **Llorente-Mirandes, T**., López-Sánchez, J.F., Rubio, R., Sánchez Agullo, A., Raber, G., Scharf, H., Vélez, D., Devesa, V., Fiamegos, Y., Emteborg, H., Seghers, J., Robouch, P., de la Calle., M.B.

  Food Additives & Contaminants: Part A, 2015, 32(1), 54–67.

- ✓ **Article XIII**

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

  Fiamegkos, I., Cordeiro, F., Robouch, P., Vélez, D., Devesa, V., Raber, G., Sloth, J.J., Rasmussen, R.R., **Llorente-Mirandes**, **T**., Lopez-Sanchez, J.F., Rubio, R., Cubadda, F., D'Amato, M., Feldmann, J., Raab, A., Emteborg, H., de la Calle, M.B.

  Article sent to publication

4.1 A fully validated method for the determination of arsenic species in rice and infant cereal products

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A fully validated method for the determination of arsenic species in rice and infant cereal products

Toni Llorente-Mirandes, Josep Calderón, José Fermín López-Sánchez, Francesc Centrich, and Roser Rubio

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A fully validated method for the determination of arsenic species in rice and infant cereal products*

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Abstract: A full validation of inorganic arsenic (iAs), methylarsonic acid (MA), and dimethylarsinic acid (DMA) in several types of rice and rice-based infant cereals is reported. The analytical method was developed and validated in two laboratories. The extraction of the As species was performed using nitric acid 0.2 % and hydrogen peroxide 1 %, and the coupled system liquid chromatography-inductively coupled plasma-mass spectrometry (LC-ICP-MS) was used for speciation measurements. Detection limit (DL), quantification limit, linearity, precision, trueness, accuracy, selectivity, as well as expanded uncertainty for iAs, MA, and DMA were established. The certified reference materials (CRMs) (NMIJ 7503a, NCS ZC73008, NIST SRM 1568a) were used to check the accuracy. The method was shown to be satisfactory in two proficiency tests (PTs). The broad applicability of the method is shown from the results of analysis of 29 samples including several types of rice, rice products, and infant cereal products. Total As ranged from 40.1 to 323.7 μg As kg^{-1}. From the speciation results, iAs was predominant, and DMA was detected in some samples while MA was not detected in any sample.

Keywords: arsenic speciation; chemical speciation; food chemistry; infant cereals; inorganic arsenic; liquid chromatography-inductively coupled plasma-mass spectrometry (LC-ICP-MS); method validation; rice.

INTRODUCTION

Rice is the main food for over half of the world's population owing to its nutritive properties and its relatively low cost. It is estimated that in many countries rice may contribute up to 50 % of the daily intake of protein, and in Asian countries it is a staple food. Moreover, rice is also extensively produced and consumed in Europe and in the United States [1,2]. From the point of view of health, rice is of interest because many types may contain higher contents of As than other food of terrestrial origin. Thus, it could be considered an important contributor to total As intake in many parts of the world where the diet is mainly rice-based [3–7]. Furthermore, it is estimated that the As content of rice is over 10 times

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greater than that found in other cereals [8,9]. Rice may thus be an important exposure route for As, a non-threshold class1 human carcinogen [10], which underlines the importance of fundamental studies on rice consumption data and calculation of estimated daily intake (EDI) of inorganic arsenic (iAs), to protect consumer health [11,12].

The origin and forms of As present in rice have been studied in depth, and it is concluded that the inorganic forms, arsenite and arsenate, along with dimethylarsinic acid (DMA) are the main species, among which the inorganic forms predominate. But the relative amount of iAs compared with the total As content varies substantially from one cultivation zone to another [4,13–15]. The species-dependent differences in toxicity must be considered when establishing maximum tolerated levels in food directives. Currently, no such levels have been fixed for iAs in European legislation, probably due to a lack of fully validated, standardized analytical methods and reference materials for this measurand [16]. Aware of this situation, the EFSA (European Food Safety Authority) included rice among the foods that most contribute to iAs exposure and pointed out the need to produce speciation data for different food commodities to estimate the health risk associated with dietary As exposure [17]. Moreover, it has recently been reported that rice-based food products intended for infants contain concentrations of iAs that are above the Chinese regulatory limit (0.15 mg As kg^{-1}) [18]. Several analytical methods have been proposed for the determination of As species in rice [2,19–22], and current interest is focused on the availability of robust methodologies that allow us to distinguish between total As and iAs in rice samples [4,6,23,24]. The establishment of such methods is of paramount importance, in order to press for legislation to establish guideline levels for iAs for food in general, as has recently been stressed [25]. The European Union Reference Laboratory for Heavy Metals in Feed and Food (EU-RL-HM) promotes the evaluation of the performance of European laboratories in relation to analytical methods for iAs, through proficiency tests (PTs) in the International Measurement Evaluation Program (IMEP). After evaluation of the results from the PT IMEP-107 it was shown that the determination of iAs in rice is not method-dependent, since good agreement was obtained from different laboratories participating with their own analytical method. It was concluded that the introduction of a maximum level for iAs in rice should not be postponed for analytical reasons [26]. Thus, analytical laboratories of food control should now be ready to determine iAs in food (mainly rice), so they will need suitable and robust methods for oncoming legislation. The use of validated methods, a requirement of the ISO-UNE-EN 17025 [27] standard, is mandatory for analytical laboratories working on food control.

The present study reports the full validation of an analytical method for the determination of iAs, MA, and DMA in rice and rice products, performed in two laboratories: (A) the Department of Analytical Chemistry of the University of Barcelona and (B) the Public Health Agency of Barcelona, under different instrumental and operating conditions. The applicability was also assessed by applying the validated method to 29 samples of rice and rice-based baby cereals.

MATERIALS AND METHODS

Reagents and standards

Analytical-grade reagents were used throughout the study.

All solutions in both laboratories were prepared with doubly deionized water obtained from Millipore water purification systems (Elix & Rios) (18.2 $M\Omega\ cm^{-1}$ resistivity and total organic carbon $<30\ \mu g\ L^{-1}$). All the stock solutions were kept at 4 °C, and further diluted solutions for the analysis were prepared daily.

Details of specific reagents and standards of both laboratories can be found in the Supplementary Material.

Instruments and apparatus

A microwave digestion system, Milestone Ethos Touch Control, with a microwave power of 1000 W and temperature control was used. Table SM-1 summarizes the chromatographic systems and operating conditions used in the study. In both cases, the outlet of the liquid chromatography (LC) column was connected via polyetherether ketone (PEEK) capillary tubing to the nebulizer of the inductively coupled plasma-mass spectrometry (ICP-MS) system, which was the As-selective detector. The ion intensity at *m/z* 75 (^{75}As) was monitored using time-resolved analysis software. Additionally, the ion intensities at *m/z* 77 (^{40}Ar^{37}Cl and ^{77}Se) were monitored to detect possible argon chloride (^{40}Ar^{35}Cl) interference at *m/z* 75.

Samples and sample pretreatment

For the applicability study, 29 rice products, which are representative of all types of rice and rice-based baby food consumed in Spain, were purchased from local supermarkets and health food shops in Barcelona, Spain, during February 2011. All samples were of different brands and origin. Some of them were typical rice products that are widely available in supermarkets (e.g., rice crackers, white rice, or rice-based infant cereals) whilst others are more specialized foods (e.g., rice noodles, basmati rice, sushi rice, or jasmine rice). Rice samples were ground to a fine powder in a commercial coffee mill (Moulinex, Vidrafoc). Powdered samples were placed in plastic containers and stored in the refrigerator at –4 °C until analysis. Rice certified reference materials (CRMs) were SRM 1568a Rice Flour, obtained by NIST (Gaithersburg, MD, USA); NMIJ CRM 7503a White Rice Flour, obtained by NMIJ (Japan), and NCS ZC73008 Rice, obtained by NCS (Beijing, China).

Procedures

Moisture determination

Moisture was determined gravimetrically. Aliquots of 0.5 g samples were dried, in triplicate, at 102 °C to constant weight in an oven with natural convection. Moisture ranged from 4 to 14 %, and all further results refer to dry mass.

Total arsenic analysis

The total As content of the samples and the CRMs was determined in triplicate by ICP-MS measurement after microwave digestion, as follows: 0.5 g aliquots of the samples or the CRMs were weighed in the digestion vessels, and 8 mL of nitric acid solution (diluted 1:1 with doubly deionized water) and 2 mL of hydrogen peroxide was added. The mixtures were digested from room temperature ramped to 190 °C in 45 min. After cooling to room temperature, the digested samples were diluted in water up to 20 mL. For the final measurements, further dilution was carried out when necessary. He gas was used in the collision cell to remove interferences in the ICP-MS measurements. ^{103}Rh was used as the internal standard. The samples were quantified by means of an external calibration curve from arsenate standards. For quality control purposes, the standards of the calibration curve were run before and after each sample series. The corresponding digestion blanks (one for each sample digestion series) were also measured. Quality control standard solutions at two concentrations levels were measured after every 10 samples.

Arsenic speciation analysis

As speciation was carried out on the extracts by LC-ICP-MS. The extraction procedure of As species is based on the study of [28] with slight modifications. For speciation analysis, 0.25 g aliquots of the powdered rice products were weighed in the digestion vessels and then extracted by adding 10 mL of 0.2 % (w/v) nitric acid and 1 % (w/v) hydrogen peroxide solution in a microwave digestion system. The temperature was raised to 95 °C in 45 min. Samples were cooled to room temperature and centrifuged

at 3000 rpm for 12 min. The supernatant was filtered through PET filters (pore size 0.45 μm). The extracts were kept at 4 °C until analysis (up to 24 h). Total As was determined in the extracts by ICP-MS (as described above) and As speciation was carried out on the extracts by LC-ICP-MS using a method previously applied to marine algae [29] (see Table SM-1) for operating conditions. As species in the chromatograms were identified by comparison of the retention times with those of the standards. External calibration curves were used to quantify MA, DMA, arsenite, and arsenate against the corresponding standards. Extraction blanks were also analyzed by LC-ICP-MS in each session. Quality control standard solutions at two concentrations levels were measured in each speciation run.

VALIDATION STUDY FOR iAs, DMA, AND MA DETERMINATION

The parameters of the method developed for the determination of As species in rice were calculated as specified elsewhere [30]. The following parameters were established to evaluate the method: detection limit (DL), quantification limit, linearity, precision (repeatability and intermediate precision), accuracy, trueness, selectivity, expanded uncertainty and applicability were assessed for iAs, MA, and DMA using spiked samples of rice and infant cereals at various concentrations.

Assessment of the quantification of inorganic arsenic

One of the goals of this study is to validate a method for the quantification of iAs in samples of rice foodstuffs. When using a LC as chromatographic system for As speciation with a strong anionic exchange column (Hamilton PRP-X100) and a mobile phase of ammonium phosphate, As(III) could elute near the void volume, and it could co-elute with other cationic species potentially present in rice [31]. So one possible strategy for a routine analytical approach is to quantify the iAs as arsenate, by using an oxidizing agent as extractant [25]. The extraction method used in the present study caused complete oxidation of As(III) to As(V), so we quantified iAs as As(V) and it was not necessary to quantify two peaks, so errors were minimized. During the recovery study, which was performed by spiking experiments with standards [As(III), DMA, MA, and As(V)] the recovery of DMA and MA was satisfactory (see Table SM-2). As(V) appeared as the only inorganic species showing the quantitative oxidation of As(III), and good recoveries of iAs were found. This behavior is illustrated in Fig. 1, which shows differences in the chromatograms with and without addition of H_2O_2 in the extracting agent. As in the spiked samples, As(III) was also quantitatively oxidized to As(V) in the rice-based CRMs (see Table 1). NMIJ 7503a rice is certified in As species: As(III) = 71.1 μg As kg^{-1}, As(V) = 13.0 μg As $kg^{-}1$, and DMA = 13.3 μg As kg^{-1}. The value found was 84.9 μg As $kg^{-1}$of iAs, as As(V) form, which is in agreement with the sum of arsenite and arsenate in the CRM (84.1 μg As kg^{-1}). For the NIST SRM, 104 μg As kg^{-1} of iAs, quantified as As(V), were obtained, which is consistent with the literature data [28,32–35]. According to [32], in a similar extraction method (diluted HNO_3 extraction) applied to similar matrices only 0.5 % of added As(III) remained unchanged. It has also been reported that preservation of As(III) and As(V) speciation during HNO_3 extraction of rice grains occurs at a narrow range of acid concentrations, i.e., 0.28–0.70 M [19].

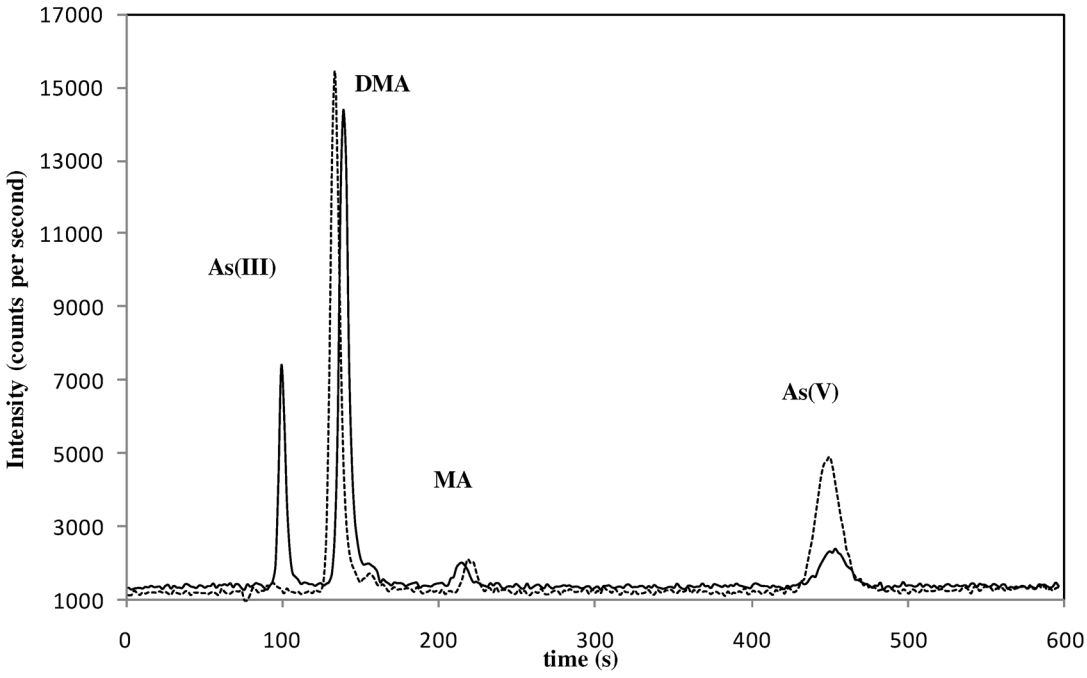

Fig. 1 Chromatograms of NIST SRM 1568a extracts from anion exchange by LC–ICP–MS, continuous line: extraction with 1 % HNO_3 and dotted line: extraction of 0.2 % HNO_3 and 1 % H_2O_2.

Validation parameters

Linearity

Linearity of the method was evaluated by analyzing six working standard solutions in triplicate for each As species [As(III), As(V), DMA, and MA]. Linearity was determined by the calculation of the regression line using the method of least squares, and it is expressed by the correlation coefficient (R^2). Linearity was validated through three analytical runs on three different days. The acceptance criterion was $R^2 \geq 0.9990$ for every calibration. As recommended [30], the residuals errors (difference between nominal and observed concentration) at each calibration point were checked, accepting a residual error ≤15 % for the lowest calibration level and ≤10 % for the higher ones. External calibration range for DMA and MA was (0.25–10.0 µg As L^{-1}). External calibration range for iAs was (0.50–10.0 µg As L^{-1}). These standards concentration ranges covered the usual concentrations of the studied As species in all analyzed food samples.

Detection limit and quantification limit

For most modern analytical methods, the DL may be divided into two components, instrumental detection limit (IDL) and method detection limit (MDL) [36].

In the validation study, IDL and instrumental quantification limit (IQL) were calculated for iAs, DMA, MA, on the SD of *y*-intercepts of regression analysis (σ) and the slope (S) of the standard curves, using the following equation IDL = 3 σ/S. IQLs were calculated from the equation IQL = 10 σ/S. Similar IDLs and IQLs were obtained for both laboratories. The IDLs for DMA, MA, and iAs were 0.03, 0.04, and 0.06 µg As L^{-1}, respectively. The IQLs for DMA, MA, and iAs were 0.12, 0.14, and 0.20 µg As L^{-1}, respectively.

According to [37], method quantification limit (MQL) is the lowest concentration that can be reliable achieved within specified limits of precision and accuracy during routine laboratory operating con-

Table 1 Accuracy assessment. Concentrations expressed as µg As kg^{-1} on dry mass (mean ± SD, $n = 3$) for total As and As species in rice CRMs.

Certified reference material		Total As	Total extracted As	DMA	MA	iAs	iAs (%)[a]	Sum of As species	Extraction efficiency (%)	Column recovery (%)
NCS ZC73008 Rice										
	Laboratory A	105.1 ± 5.8	100.3 ± 6.2	19.0 ± 0.3	<MDL	79.8 ± 3.4	80.7	98.8	95.4	98.5
	Laboratory B	–	–	19.2 ± 0.7	<MQL	83.6 ± 1.2	81.3	102.8	–	–
	Certified value	102 ± 8								
NIST SRM 1568a Rice										
	Laboratory A	287.8 ± 4.5	286.1 ± 3.8	168.0 ± 2.2	13.6 ± 2.5	104.3 ± 4.4	36.5	285.9	99.4	99.9
	Laboratory B	275 ± 15	–	179.5 ± 5.2	13.2 ± 0.2	105.2 ± 1.6	35.3	297.9	–	–
	Certified value	290 ± 30								
NMIJ CRM 7503-a Rice										
	Laboratory A	97.6 ± 4.7	96.8 ± 4.1	11.7 ± 1.9	<MDL	84.9 ± 0.7	87.8	96.6	99.2	99.8
	Laboratory B	–	–	–	–	–	–	–	–	–
	Certified value	98 ± 7		13.3 ± 0.9		84.10[b]				

[a]iAs divided by sum of As species.

[b]NMIJ 7503a rice is certified in As species: As(III) = 71.1 µg As kg^{-1} and As(V) = 13.0 µg As kg^{-1} so the value for iAs is 84.10 µg As kg^{-1}.

ditions. In order to assess the MQL of As species in the samples we fortified three different samples at a concentration close to the lowest concentration of the standard calibration curves (10 μg As kg^{-1} for DMA and MA and 20 μg As kg^{-1} for iAs), on three different days and processed through the entire analytical method. Below such concentrations, the values obtained for precision and accuracy could not reach the specified limits established for routine laboratory operating conditions. We assume that the lowest limit validated could be equivalent as the MQL for the three As species. MDLs were calculated from the equation MDL = 3 MQL/10. The MDLs for DMA, MA, and iAs were 3, 3, and 6 μg As kg^{-1}, respectively. The values obtained for MQL and MDL were suitable for determining the As species at the low levels found in the samples studied.

Precision

Precision was assessed as within-day repeatability and as between-day intermediate precision [38]. In both cases, spiking experiments were carried out by adding As(III), As(V), DMA, and MA standards to solid samples and homogenized. The mixtures were then left to stand for 30 min before extraction. Unspiked samples were also analyzed in triplicate in order to calculate the spike recovery. Precision, expressed in terms of relative standard deviation (% RSD) of As recovery, was assessed by analyzing spiked rice samples at three concentration levels in triplicate: low (10 μg kg^{-1} corresponding to MQL), medium (50 μg kg^{-1}), and high (200 μg kg^{-1}) (Table SM-2). To evaluate the between-day precision (intermediate precision) various factors were changed: three different analysis days over three weeks, different analysts and different standards for spiking. For within-day repeatability, six samples for each spiking level were analyzed within a day. The precision acceptance criterion [39,40] matches the 2/3 Horwitz function [41], which was: 14.7 % for values ≤ 100 μg kg^{-1}, 13.6 % RSD for 200 μg kg^{-1}, and 12.2 % RSD for 400 μg kg^{-1}. The between-day (intermediate precision) and within-day (repeatability) precisions (expressed in terms of % RSD) calculated for both laboratories ranged from 1.7 to 7.0 % and from 0.8 to 5.0 %, respectively. Good precision was obtained in all cases, and the results obtained are consistent with the precision acceptance criteria. All details of precision of both laboratories can be found in the Supplementary Material, Table SM-2.

Trueness

The same spiked samples analyzed to evaluate precision of the method were also used for evaluation of the trueness, which is expressed in terms of recovery, according to [30]. As commented above, no added As(III) was found in spiked extracts, so we calculated iAs recoveries assuming that all of the As(III) was oxidized to As(V). Recoveries were calculated as follows: recovery (%) = $(a\text{-}b)*100/c$, where a is the As concentration measured in the extracts of samples which were spiked with standards solutions; b is the As concentration measured in the unspiked sample and c was the known concentration added to the sample. The between-day and within-day recoveries for both laboratories were in the range 97.0–104.2 % and 98.0–103.0 %, respectively. More information about recoveries of both laboratories can be found in Table SM-2. For assessing trueness, our acceptance criteria for recovery validation is 85–115 %. The recoveries for both laboratories were satisfactory compared to acceptance criteria set by CODEX [42]: (60–115 % for 10 μg kg^{-1} and 80–110 % for 0.1–10 mg kg^{-1}). The results of two laboratories indicated excellent trueness of the proposed method.

Accuracy

To evaluate the accuracy of the procedure applied, CRMs were analyzed. Rice CRMs (NMIJ 7503a, NCS ZC73008, and NIST SRM 1568a) were used throughout the study (Table 1). NMIJ 7503a rice is certified in total As and also in As species: the present results showed good agreement with the certified values. SRM NIST 1568a rice is certified for total As: 290 μg As kg^{-1}. Although the concentration of As species is not certified, our results showed good agreement with other reported results on As species on this material [28,32–35]. NCS ZC73008 rice is certified for total As but not for As species. As speciation results on this CRM are not found in the literature. In the present study, the sum of the As species compared well with the certified total As value (102 μg As kg^{-1}) (Table 1).

Expanded uncertainty

The relative expanded uncertainty was calculated in order to complete the validation study. Although this parameter is important in the evaluation of the results of toxic substances in food control, it is rarely described in the literature [26]. Relative expanded uncertainty was estimated by a top-down method, adapted from [43].

$$U(\%) = \frac{1}{\mathrm{Rec}}\left(\left(k\sqrt{\left(\mathrm{RSD}_{\mathrm{Rec}}\right)^2 + \left(\frac{\mathrm{RSD}_{\mathrm{Rec}}}{\sqrt{n}}\right)^2}\right) + \left(\left|1 - \mathrm{Rec}\right|\right)\right)$$

in which Rec is the average recovery of all spiked concentration levels, $\mathrm{RSD}_{\mathrm{Rec}}$ is the relative standard deviation of Rec values, n is the number of replicates made in the validation and k is the coverage factor 2. The results for each species and each spiked level are shown in Table SM-2. The results of the relative expanded uncertainty range from 3.6 to 14.8 % for both laboratories for all species in the three spiked levels. The acceptable criteria are: $U_{\max} < 2$ * RSD (according to [41,44]). The results obtained of $U(\%)$ agree with this criterion.

Selectivity

A blank sample (0.2 % HNO_3 and 1 % H_2O_2 solution) was analyzed by LC-ICP-MS in each session, and no signal was observed at the retention times of the As species studied. Therefore, reagents in the blank did not induce interferences in the chromatograms. The presence of a high content of chloride (Cl^-) in the matrices could lead to the misidentification of As with ICP-MS detection [45]. As commented above, the ion intensity at *m/z* 75 (^{75}As) was monitored and additionally, the ion intensities at *m/z* 77 ($^{40}Ar^{37}Cl$ and ^{77}Se) and *m/z* 35 (^{35}Cl) were monitored to detect possible argon chloride ($^{40}Ar^{35}Cl$) interference at *m/z* 75. A blank sample (MilliQ-water) spiked at 50 mg L^{-1} with Cl standard solution was analyzed to check the possible interference with As(V), and no signal was observed at the retention time of As(V). The ($^{40}Ar^{35}Cl$) peak eluted at 7.92 min, whereas As(V) eluted at 6.0 min. The selectivity of the method regarding the ($^{40}Ar^{35}Cl$) interference for the As species studied was verified.

External quality control

The method accuracy was assessed with participations in PTs. Laboratory A participated as an expert laboratory in the IMEP-107: Total and iAs in rice [26,46]. Laboratory B participated in a PT of the Central Science Laboratory-Food Analysis Performance Assessment Scheme (CSL-FAPAS), in the determination of total and inorganic As in rice, with good results.

Applicability

In order to assess the wide applicability of the method, it was applied to 29 samples.

TOTAL ARSENIC

The results are shown in Table 2, and total As in all the samples ranged from 40.1 to 323.7 μg As kg^{-1}. The mean As concentration from all rice and rice products (n = 20, excluding baby food) was 169.5 μg As kg^{-1}. The results for total As are similar to others reported in the literature [20,28,33,47] and show that, compared with other cereals (wheat, barley, and maize), rice accumulates much higher levels of As [8,9]. With respect to infant products, some studies reported that rice-based baby food contains high concentrations of total As [18,48]; our results are in agreement, and ranged from 40.1 to 309.5 μg As kg^{-1}. As shown in Table 2, among the products labeled as "infant cereals", the rice-based products contained higher As concentrations than other infant cereals (multicereals). Total As was determined in three CRMs to assess the accuracy and for mass balance purposes. The results are shown in Table 1. The instrumental detection and quantification limits were calculated as 3 times the signal (3σ) and 10 times the signal (10σ) of 10 digestion blanks, respectively, and the results obtained are: 7.3 and 24.2 μg As kg^{-1}, respectively. The intermediate precision (three times within a day during three dif-

Table 2 Concentrations of total As and As species in rice and infant cereals expressed as µg As kg^{-1} on dry mass (mean ± SD, n = 3).

Rice product	Color	Grain type	Total As	Total extracted As	DMA	MA	iAs	iAs (%)[a]	Sum of As species	Extraction efficiency (%)	Column recovery (%)
Rice	White	Medium	173.2 ± 9.9	166.2 ± 8.0[b]	57.3 ± 0.7	<MDL	112.9 ± 8.2	66.3	170.1	96.0	102.4
Rice	White	Long	156.5 ± 7.1	156.2 ± 4.4[b]	45.1 ± 3.2	<MDL	108 ± 0.5	70.5	153.1	99.9	98.0
Rice	White	Medium	184.9 ± 8.7	165.3 ± 2.0[b]	58.6 ± 1.9	<MDL	109.2 ± 2.7	65.1	167.7	89.4	101.5
Rice	White	Medium	186.1 ± 3.8	173.2 ± 0.7[b]	55.1 ± 0.4	<MDL	117.6 ± 3.3	68.1	172.7	93.0	99.8
Rice	White	Medium	193.4 ± 3.8	193.6 ± 8.0[b]	68.4 ± 1.6	<MDL	116.8 ± 5.4	63.1	185.3	100.1	95.7
Rice	White	Basmati	90.9 ± 2.3	86.6 ± 6.3	25.7 ± 1.3	<MDL	53.1 ± 1.8	67.4	78.8	95.2	91.0
Rice	White	Sushi	143.4 ± 2.7	129.2 ± 11.8	41.0 ± 1.0	<MDL	85.6 ± 3.2	67.6	126.6	90.1	97.9
Rice	White	Bomba	174.0 ± 2.6	161.4 ± 8.4	80.6 ± 4.9	<MDL	71.9 ± 2.8	47.2	152.5	92.8	94.5
Rice crackers	White	Original crackers	86.3 ± 4.8	80.7 ± 4.5	36.6 ± 2.3	<MDL	42.5 ± 3.2	53.7	79.1	93.5	98.0
Rice crackers	White	Sesame crackers	79.7 ± 5.4	77.9 ± 3.4	44.4 ± 3.9	<MDL	34.4 ± 5.8	43.7	78.9	97.7	101.2
Rice noodles	n.s.[c]	Rice noodles	104.6 ± 2.7	102.1 ± 2.6	21.4 ± 2.3	<MDL	82.5 ± 3.5	79.4	103.8	97.7	101.7
Rice	Brown	Whole long grain	151.1 ± 5.3	129.1 ± 18.6	17.6 ± 0.8	<MDL	109.0 ± 5.1	86.1	126.6	85.5	98.1
Rice	Brown	Basmati	170.5 ± 5.6	147.0 ± 12.7	16.4 ± 1.6	<MDL	129.0 ± 2.5	88.7	145.3	86.2	98.9
Rice	Brown	Jasmine	234.4 ± 7.2	223.9 ± 11.3	39.3 ± 2.9	<MDL	182.8 ± 9.4	82.3	222.0	95.5	99.2
Rice	Brown	Whole medium grain	323.7 ± 25.2	281.6 ± 6.2	49.7 ± 0.9	<MDL	238.9 ± 8.5	82.8	288.6	87.0	102.5
Rice crackers	Brown	Crackers	261.7 ± 11.1	213.0 ± 7.8	51.2 ± 3.4	<MDL	160.1 ± 5.0	75.8	211.2	81.4	99.2
Rice crackers	Brown	Crackers	252.0 ± 2.8	242.5 ± 4.7	52.0 ± 5.2	<MDL	189.8 ± 6.6	78.5	241.7	96.2	99.7
Rice	Red	Long	160.6 ± 6.8	160.6 ± 6.8	19.1 ± 1.9	<MDL	125.4 ± 8.2	86.8	144.5	99.8	90.2
Mixture of wild rice	Mixture	Mixture of whole-grain basmati, red and black rice	180.0 ± 19.4	153.9 ± 11.1	22.8 ± 0.2	<MDL	126.5 ± 9.4	84.7	149.4	85.5	97.1
Rice	Black	Medium	83.3 ± 9.4	73.9 ± 15.4	22.1 ± 0.5	<MDL	49.9 ± 1.4	69.3	72.1	88.7	97.5
Infant cereal	n.s.[c]	Multigrain	46.3 ± 3.4	46.6 ± 7.5	21.0 ± 2.0	<MDL	24.2 ± 0.6	53.5	45.2	100.5	97.0
Infant cereal	n.s.[c]	Multigrain with fruits	40.1 ± 5.4	38.3 ± 3.8 [b]	11.9 ± 1.4	<MDL	26.3 ± 1.2	68.9	38.2	95.4	99.9

(*continues on next page*)

Table 2 (*Continued*).

Rice product	Color	Grain type	Total As	Total extracted As	DMA	MA	iAs	iAs (%)[a]	Sum of As species	Extraction efficiency (%)	Column recovery (%)
Infant cereal	Brown	Organic whole-wheat rice	231.7 ± 9.5	237.9 ± 1.0	30.0 ± 1.3	<MDL	200.0 ± 5.0	87.0	230.0	102.7	96.7
Infant cereal	n.s.[c]	100 % rice	309.5 ± 11.2	312.2 ± 2.0	182.6 ± 5.0	<MQL	128.0 ± 3.4	41.2	310.6	100.9	99.5
Infant cereal	n.s.[c]	Rice based	94.5 ± 2.0	91.7 ± 7.0	19.0 ± 1.1	<MDL	67.7 ± 1.0	78.0	86.7	97.0	94.6
Infant cereal	n.s.[c]	Rice based	80.5 ± 10.5	78.1 ± 2.9	12.8 ± 1.0	<MDL	63.6 ± 1.6	83.3	76.3	97.0	97.8
Infant cereal	n.s.[c]	Rice based with fruits	42.6 ± 6.5	39.8 ± 4.8	11.1 ± 1.2	<MDL	29.6 ± 0.4	72.7	40.7	93.5	102.2
Infant cereal	n.s.[c]	Cereals without gluten	64.2 ± 3.9	55.4 ± 6.8 [b]	12.8 ± 0.6	<MDL	43.7 ± 0.3	77.3	56.5	86.2	102.0
Infant cereal	n.s.[c]	Corn based	<MQL	<MQL	<MDL	<MDL	<MQL				

[a]Inorganic As divided by sum of As species.
[b]Analyzed in duplicate ($n = 2$).
[c]No specific information on the type of the rice grain is available.

ferent days, $n = 9$) and the repeatability (six times within a day, $n = 6$) were assessed for the results obtained by analyzing different replicates on CRMs (Table 1). The results of RSD % are: 6.7 and 5.7 %, respectively, for NCS ZC73008 Rice; 3.0 and 2.4 %, respectively, for NIST SRM 1568a Rice Flour, and 5.2 and 4.1 %, respectively, for NMIJ CRM 7503a White Rice Flour.

ARSENIC SPECIATION

Table 2 summarizes the results of As speciation, total extracted As, total As, column recovery, extraction efficiency, and the percentage of the species detected in inorganic form. In the present study, extraction efficiencies (calculated as the ratio of total As in the extract to total As in the sample) are comparable with others reported in the literature [6]. The values ranged from 81.4 to 102.7 % and extracted on average 93.7 %, which indicates that the HNO_3/H_2O_2 solution could be a suitable solvent for the extraction of As species in this type of matrix.

For quality assessment, column recovery must also be established, to guarantee the correctness of the chromatographic separation. With this aim, we calculated the ratio of the sum of the species eluted from the chromatographic columns to the total As in the extract injected into the column. This parameter, assessed in replicates with good reproducibility, allowed us to evaluate the quantification of the As species. The values obtained for column recoveries (Table 2), ranged between 90.2 and 102.5 % and showed average recoveries of 98.3 %.

Data of As speciation concentrations for rice and infant food samples are summarized in Table 2. In this study, we only found two As species, As(V) [as commented above, As(III) is oxidized to As(V) under the extraction conditions] and DMA, which are known to be the main As components of both white and brown rice [15,20,49,50]. iAs was the predominant form, ranging from 41.2 to 88.7 %, and DMA (11.3–58.85 %) was also detected. For iAs and DMA, the average percentages found in all samples were 71.0 and 29.0 %, respectively. MA was below MQL (10 μg As kg^{-1}) in all the samples analyzed. The present results are in agreement with the literature [4,20,47] but differ from the data on U.S. rice, in which DMA is reported to dominate [4,13,20]. iAs levels in all samples ranged from 24.2 to 238.9, with an average value of 101.0 μg As kg^{-1}. It has been shown that iAs is elevated in the bran layer of rice, resulting in brown rice having a higher content than corresponding white rice [15]. The present results are consistent with these reports, and showed that brown rice has more iAs (75.8–88.7 %) than white rice (43.7–70.5 %), whereas in rice noodles the percentage of iAs was higher (79.4 %). No specific information on the type of the rice grain was found on the packaging of rice products for babies, so we could not establish a relationship between rice type and iAs content. From the results shown in Table 2, iAs concentration in infant cereals products ranged from 24.2 to 200.0 μg As kg^{-1}, with an average value of 72.9 μg As kg^{-1}. Other authors reported a similar range of iAs levels in rice for babies: 60 to 160 μg As kg^{-1} [18]. In one sample of whole-grain rice, 200 μg As kg^{-1} was measured.

From all these results, the iAs levels in rice-based infant cereals should not be ignored and should be of concern. There are currently no EU regulations regarding As levels in foods [26]. The Chinese standard for iAs in rice is probably the strictest in the world, with a standard limit of 150 μg As kg^{-1} iAs [51]. Four samples of rice and one sample of rice-based infant products examined in this study (see Table 2) exceed this limit.

Correlations have been reported between As species and total As [4,52], and it is proposed that rice may be classified into two populations, depending on the form of As in the grain: iAs-type and DMA-type [13]. According to our results, if we consider all the products listed in Table 2, iAs and also DMA concentrations increase with total As, the slope corresponding to iAs being steeper (Fig. 2).

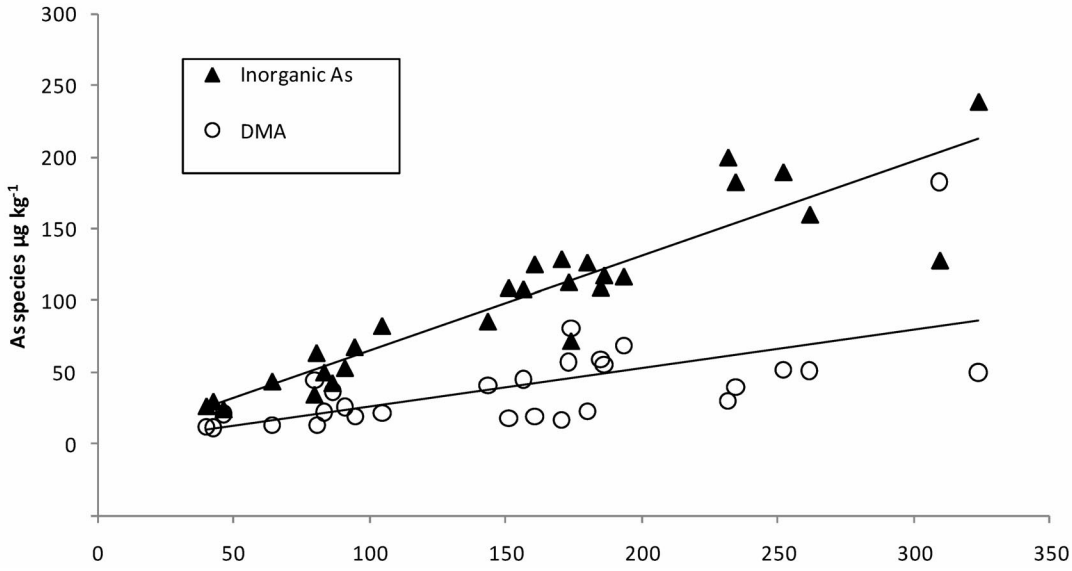

Fig. 2 Relationship between As species and total As for rice products.

CONCLUSIONS

From the validation study, it can be concluded that excellent trueness (% recovery) and good precision (as intermediate precision and repeatability) were obtained for both laboratories. The MQLs achieved were low enough and suitable for determining the As species at the low levels found in the samples. The results on CRMs show good agreement with the certified values, as well as with the results on As species reported in the literature. The validated method was applied successfully to 29 samples of rice and rice-based infant products, and it is currently accredited under the ISO/IEC 17025 and used for routine analysis in Laboratory B, for food control purposes. From the speciation results in the samples studied, iAs was the major As compound, highlighting the importance of rice as a possible source of iAs in the diet, which is especially important in rice-based infant products.

The present validated method could be a valuable tool for assessing the iAs in rice. The method can be considered straightforward enough to be applied in routine analysis, as required in food control laboratories according to the ISO/IEC 17025:2005 standard.

SUPPLEMENTARY MATERIAL

Details of specific reagents and standards of both laboratories are included in the Supplementary Material.

Table SM-1 provides LC-ICP-MS operating conditions used by both laboratories.

Table SM-2 provides validation results about precision, trueness, and expanded uncertainty ($k = 2$) data for As species in spiked rice and infant cereal (rice-based).

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A fully validated method for the determination of arsenic species in rice and infant cereal products

SUPPLEMENTARY MATERIAL

Reagents and standards

Details of specific reagents and standards of both laboratories.

Laboratory A: Nitric acid (69%) (Panreac, Hiperpur), ammonium dihydrogen phosphate (Panreac, p.a.), 25% aqueous ammonia solution (Panreac, p.a.), and 31% hydrogen peroxide (Merck, Selectipur) were used. Stock standard solutions (1000 mg L^{-1}) were prepared as follows: arsenite, from As_2O_3 (NIST, USA, Oxidimetric Primary Standard 83d, 99.99%) dissolved in 4 g L^{-1} NaOH (Merck, Suprapure); arsenate, from $Na_2HAsO_4 \cdot 7H_2O$ (Carlo Erba) dissolved in water; MA, prepared from $(CH_3)AsO(ONa)_2 \cdot 6H_2O$ (Carlo Erba) dissolved in water; and DMA, prepared from $(CH_3)_2AsNaO_2 \cdot 3H_2O$ (Fluka) dissolved in water. Arsenate, arsenite, DMA and MA were standardized against As_2O_3 (NIST Oxidimetric Primary Standard 83d) as our internal control. Arsenic standard solution from NIST High-Purity Standards with a certified concentration of 1000 ± 2 mg As L^{-1} was used as the calibrant in the determination of total arsenic content using ICPMS.

Laboratory B: Nitric acid (67-69%) (Carlo Erba, Superpure), ammonium dihydrogen phosphate (Merck, p.a.), aqueous ammonia solution (Panreac, p.a.), and 30% hydrogen peroxide (J.T.Baker, p.a.) were used. Stock standard solutions (1000 mg L^{-1}) were prepared as follows: DMA, prepared from cacodylic acid $C_2H_7AsO_2$ (Aldrich, >99.0%) dissolved in water, MA, prepared from $Na_2CH_3AsO_3$ (Supelco, 98%) dissolved in water, arsenite was supplied by Fluka, As(III), standard solution (1000 ± 2 mg As L^{-1}), and arsenate was supplied by Merck, As(V), standard solution (1000 mg As L^{-1}).

Table SM-1. LC-ICP/MS operating conditions used by both laboratories.

	Laboratory A	**Laboratory B**
Instrumentation		
ICPMS	Agilent 7500ce	Agilent 7500cx
Chromatographic conditions		
LC	Quaternary pump, Agilent 1200	Dionex ICS-2500 Ion Chromatograph
Column	Hamilton PRP-X100 (250mm x 4.1 mm, 10µm)	Hamilton PRP-X100 (150mm x 4.1 mm, 5µm)
Pre Column	Hamilton PRP-X100 (20 x 2.0 mm, 10µm)	none
Mobile phase	20 mM $NH_4H_2PO_4$ pH= 5.8 (adjusted with aqueous ammonia)	26 mM $NH_4H_2PO_4$ pH= 6.2 (adjusted with aqueous ammonia)
Flow rate (mL min^{-1})	1.5	1.0
Injection volume (µL)	100	125
Column temperature (ºC)	Room temperature	30
Pressure (bar)	130	83
Arsenic species	As(III), As(V), MA and DMA	As(III), As(V), MA and DMA
ICP-MS Parameters		
RF power (W)	1550	1500
Make up Gas flow, Ar (L min^{-1})	0.32	0.15
Carrier Gas Flow, Ar (L min^{-1})	0.85	0.95
Nebuliser	BURGENER Ari Mist HP	Microconcentric
Collision Cell	OFF	ON (He= 4 mL min^{-1})
QP/OctP Bias difference (V)	3	2

Table SM-2. Precision, trueness and expanded uncertainty (k=2) data for arsenic species in spiked rice and infant cereal (rice-based).

Analyte	Sample	Laboratory	Added (µg As kg^{-1})	RSD (%)	Recovery (%)	U (%)	RSD (%)	Recovery (%)
				Between-day (intermediate precision, n=9)			Within-day (repeatability, n=6)[a]	
DMA	Infant cereal (rice-based)	**Lab A**	10	4.7	101.4	9.8	3.3	100.2
DMA	Long-grain rice	**Lab A**	50	2.7	98.1	5.6	2.9	99.4
DMA	Long-grain rice	**Lab A**	200	2.3	101.6	4.8	1.5	101.5
MA	Infant cereal (rice-based)	**Lab A**	10	5.7	100.2	12.1	5.0	98.0
MA	Long-grain rice	**Lab A**	50	2.2	101.6	4.6	4.0	101.8
MA	Long-grain rice	**Lab A**	200	1.7	102.4	3.6	1.4	102.4
iAs	Infant cereal (rice-based)	**Lab A**	20	4.2	100.7	8.8	3.7	99.3
iAs	Long-grain rice	**Lab A**	50	3.6	99.0	7.6	3.3	99.4
iAs	Long-grain rice	**Lab A**	200	3.4	99.3	7.2	3.8	99.5
DMA	Infant cereal (rice-based)	**Lab B**	10	7.0	97.4	14.8	2.2	99.2
DMA	Long-grain rice	**Lab B**	50	4.9	103.7	10.4	3.0	99.8
DMA	Long-grain rice	**Lab B**	200	1.9	104.2	3.9	0.8	103.0
MA	Infant cereal (rice-based)	**Lab B**	10	4.3	101.9	9.0	3.8	102.8
MA	Long-grain rice	**Lab B**	50	2.4	101.2	5.2	1.6	99.8
MA	Long-grain rice	**Lab B**	200	3.6	103.6	7.6	1.7	101.5
iAs	Infant cereal (rice-based)	**Lab B**	20	3.9	100.2	8.2	2.8	101.4
iAs	Long-grain rice	**Lab B**	50	4.5	97.0	9.4	1.1	101.3
iAs	Long-grain rice	**Lab B**	200	1.8	100.6	3.9	1.4	101.5

[a] For laboratory B, n=3 (three times within a day). A: Laboratory of Analytical Chemistry Department, (UB). B: Laboratory of the Public Health Agency of Barcelona

4.2 A need for determination of arsenic species at low levels in cereal-based food and infant cereals. Validation of a method by IC-ICPMS

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Analytical Methods

A need for determination of arsenic species at low levels in cereal-based food and infant cereals. Validation of a method by IC–ICPMS

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ABSTRACT

The present study arose from the need to determine inorganic arsenic (iAs) at low levels in cereal-based food. Validated methods with a low limit of detection (LOD) are required to analyse these kinds of food. An analytical method for the determination of iAs, methylarsonic acid (MA) and dimethylarsinic acid (DMA) in cereal-based food and infant cereals is reported. The method was optimised and validated to achieve low LODs. Ion chromatography-inductively coupled plasma mass spectrometry (IC–ICPMS) was used for arsenic speciation. The main quality parameters were established. To expand the applicability of the method, different cereal products were analysed: bread, biscuits, breakfast cereals, wheat flour, corn snacks, pasta and infant cereals. The total and inorganic arsenic content of 29 cereal-based food samples ranged between 3.7–35.6 and 3.1–26.0 μg As kg^{-1}, respectively. The present method could be considered a valuable tool for assessing inorganic arsenic contents in cereal-based foods.

1. Introduction

Humans are exposed to arsenic (As) in the environment primarily through the ingestion of food and water (Abernathy et al., 2001; EFSA Panel on Contaminants in the Food Chain (CONTAM), 2009). Speciation of As in food products is necessary because of the varying toxicity of different As compounds. Inorganic arsenic (iAs) (arsenite or As(III) and arsenate or As(V)) is considered the most dangerous form due to its biological availability, as well as physiological and toxicological effects (iAs is classified as a non-threshold, class 1 human carcinogen) (ATSDR Toxicological profile for arsenic, 2007). Children are particularly vulnerable to the toxic effects of iAs. Other arsenic compounds, such as arsenobetaine (AB), commonly present in seafood, is non-toxic and can be consumed without health concern, while arsenosugars, usually found in edible algae, are potentially toxic (Feldmann & Krupp, 2011). Therefore, species-dependent differences in toxicity must be considered when establishing the maximum tolerated levels in food directives. Currently, no such levels have been fixed for iAs in European legislation, probably due to the lack of fully validated, standardised analytical methods and the unavailability of certified reference materials (CRMs) for this measurand in food matrices (Baer et al., 2011). Only a regulatory limit of 0.15 mg iAs kg^{-1} is currently applied in China (USDA Maximum Levels of Contaminants in Foods, 2006). In 2009, the European Food Safety Authority (EFSA) (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2009) reviewed the diet of the European Union population and pointed out the need to produce speciation data, particularly inorganic arsenic data, for different food commodities to estimate the health risk associated with dietary As exposure. As a general recommendation, dietary exposure to iAs should be reduced (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2009). Among the conclusions from this report, cereal and cereal-based products were identified as contributors to daily iAs exposure in the general European population. Moreover, children aged less than three years were the most exposed to iAs, which was directly related to the intake of rice-based products. Several authors (Carbonell-Barrachina et al., 2012; Llorente-Mirandes, Calderon, Lopez-Sanchez, Centrich, & Rubio, 2012; Meharg et al., 2008) have recently reported that some rice-based infant products have elevated levels of iAs that exceed the Chinese regulatory limit aforementioned. Therefore, iAs levels in rice-based baby food should be of concern. In addition, infants with coeliac disease, who are forced to consume gluten-free products, with high percentages of rice, should be paid special attention due to the most elevated intakes of iAs.

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However, other infant cereals are prepared using mixtures of cereals (wheat, barley, oat and mixed cereals, among others) and their iAs contents are lower compared to rice products. The available results on arsenic speciation in infant food products are limited and confused. Thus, more studies are required to provide information that can be useful in the risk assessment of an infant's diet.

Wheat is the most widely consumed grain in Europe and in most other countries where the diet is not rice-based. For example, in Catalonia (Spain), the majority of the cereals consumed by the average adult are wheat-based (Serra-Majem et al., 2007). Although it is also true that the total As content of wheat is very low compared to that of other foods, arsenic is present almost exclusively as iAs (D'Amato, Aureli, Ciardullo, Raggi, & Cubadda, 2011). Therefore, wheat should not be ignored as a potential contributor to the dietary iAs intake (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2009) and validated methods with low limits of detection (LODs) are needed to analyse these kinds of food due to the high consumption of wheat-based products such as bread and pasta in populations with a predominantly wheat-based diet. To this end, the European Union Reference Laboratory for Heavy Metals in Feed and Food (EU-RL-HM) organised a proficiency test (PT) in 2012 for measuring total and inorganic arsenic in wheat, vegetable food and algae (de la Calle et al., 2012). The main conclusion derived from this exercise was that the concentration of iAs determined in any of the matrices covered was not method-dependent. Moreover, there was a need to consider the option of introducing possible maximum levels for iAs in wheat for risk management. Thus, analytical laboratories of food control should now be ready to determine iAs levels in food (mainly rice and cereals). They will therefore need suitable and robust methods for oncoming legislation. The use of validated methods, a requirement of the ISO-UNE-EN 17025 standard, is mandatory for analytical laboratories working on food control.

In summary, infant cereals and cereal-based food deserve special attention with respect to iAs content, and validated methods with a low limit of detection (LOD) are required to analyse these kinds of food. Therefore, the main objective of this study was to validate an analytical method for the determination of iAs, methylarsonic acid (MA) and dimethylarsinic acid (DMA) levels in cereal-based products that could be used in routine analysis for food control purposes. First, instrumental conditions for the determination of arsenic species were optimised, with the aim of improving the limits of detection (LODs). Second, the validation parameters of the method were evaluated. Finally, several samples were analysed to establish wide applicability and provide iAs occurrence data on cereal-based food.

2. Experimental procedures

2.1. Chemicals and reagents

Deionised water (18.2 M Ω cm^{-1}) was used to prepare the reagents and standards. All glassware was treated with 10% (v/v) nitric acid (HNO_3) for 24 h and then rinsed three times with deionised water before use to reduce background As levels. Concentrated super-pure HNO_3 (Carlo Erba, Rodano, Italy) and 30% (w/w) hydrogen peroxide (H_2O_2) (Merck, Darmstadt, Germany) were used. Isopropyl alcohol (Merck) was used within the inductively coupled plasma mass spectrometry (ICPMS) method. A commercial solution (Agilent Technologies, Barcelona, Spain) containing 10 μg L^{-1} of lithium, yttrium, cerium, thallium and cobalt in 2% (v/v) nitric acid was used to tune the ICPMS instrument. Ammonium dihydrogen phosphate (Merck, p.a.) and aqueous ammonia solution (Panreac, p.a.) were used for speciation analysis. External calibration standards for total arsenic were prepared weekly by diluting a multi-element plasma stock solution, traceable to the National Institute of Standards and Technology, with 100 mg L^{-1} of As (J.T. Baker, Phillipsburg, NJ) in 5% (v/v) HNO_3 (Carlo Erba). A diluted solution (0.2 mg L^{-1} in 40% (v/v) of isopropyl alcohol) of a 100 mg L^{-1} multi-element internal standard stock solution (Agilent Technologies, Barcelona, Spain) containing Ge was used as an internal standard to correct possible instrumental drifts and matrix effects.

Stock standard solutions (1000 mg As L^{-1}) for arsenic speciation were prepared as follows: DMA, prepared from cacodylic acid $C_2H_7AsO_2$ (Aldrich, >99.0%) dissolved in water; MA, prepared from $Na_2CH_3AsO_3$ (Supelco, 98%) dissolved in water; arsenite was supplied by Fluka, As(III), as a standard solution (1000 ± 2 mg As L^{-1}); and arsenate was supplied by Merck, As(V), as a standard solution (1000 mg As L^{-1}). Arsenate, arsenite, DMA and MA, were standardised against As_2O_3 (NIST Oxidimetric Primary Standard 83d) for our internal quality control. All the stock solutions were kept at 4 °C, and further diluted solutions for the speciation analysis were prepared daily.

2.2. Samples and sample pretreatment

For the applicability study, 30 cereal-based foods, which are representative of all the types of cereal products consumed in Spain, were purchased from local supermarkets and retail stores in Barcelona, Spain, during 2011. A selection of cereal products representing different types, such as bread, biscuits, breakfast cereals, corn snacks, wheat flour, pasta and infant cereals, were analysed for As speciation and total As. All samples were of different brands and origin, but no specific information on the origin of the cereal grain was found on the packaging and product labels. Samples were brought to the laboratory the same day of purchase and kept for not more than one day in the refrigerator until sample preparation. Samples were ground into a fine powder in a commercial coffee mill (Moulinex, Vidrafoc). Powdered samples were placed in plastic containers and stored at 4 °C until analysis.

2.3. Certified reference materials

Two certified reference materials (CRMs) were analysed throughout the study. NIST SRM 1568a Rice Flour was purchased from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) and is certified for total arsenic. NMIJ CRM 7503-a White Rice Flour was purchased from the National Metrology Institute of Japan (NMIJ, Japan) and is certified for As (III), As (V), DMA and total arsenic. All samples were used as provided, without further grinding.

2.4. Moisture determination

Aliquots of 0.5 g samples were dried, in triplicate, at 102 °C to constant weight in an oven with natural convection (Binder Inc., Bohemia, NY). Moisture ranged from 5% to 11%, and all the results are expressed as dry mass.

2.5. Total arsenic determination

Samples were processed as described before (Fontcuberta et al., 2011). Briefly, a total of 0.5 g from every sample was weighed and 9 mL of 16% HNO_3 and 1 mL of 30% H_2O_2 were added to perform a microwave digestion using an Ethos 1 microwave system (Milestone, Gomensoro, Barcelona, Spain). The digestion method was as follows: 15 min up to 200 °C and held for 15 min, working with a maximum power of 800 W. Finally, the digested sample was made up to 30 g with deionised water. Arsenic was measured on an Agilent quadrupole inductively coupled plasma mass spectrometer (ICPMS) 7500 cx (Agilent Technologies, Barcelona, Spain) at 1500 kW, measuring mass at m/z 75 and using helium as a collision gas to remove $^{40}Ar^{35}Cl$ interference.

The results were quantified using external calibration standards of 0.125, 0.25, 0.5, 1 and 5 μg As L^{-1} prepared in 5% HNO_3 for total As. A solution of 5 μg L^{-1} of germanium was used as an internal standard and measured at *m*/*z* 72. The final solutions (standards and samples) were prepared with 2% isopropyl alcohol (or 40% if introduced within the online internal standard) to minimise the effects of the dissolved carbon on arsenic response (Pettine, Casentini, Mastroianni, & Capri, 2007). Each sample was digested and analysed in triplicate. Digestion blanks were analysed together with samples. Quality control standard solutions at two concentrations levels were measured after every 10 samples. To assess the accuracy of total As measurements, two certified reference materials were analysed throughout the routine sample analyses: NIST SRM 1568a Rice with a certified value of 290 ± 30 μg As kg^{-1} for total As, our method obtaining 292 ± 9 μg As kg^{-1} (n = 3, all data are expressed as mean ± standard error), and NMIJ CRM 7503-a Rice with a certified value of 98 ± 7 μg As kg^{-1} for total As, our method obtaining 95 ± 5 μg Askg^{-1} (n = 3). The instrumental detection limit was 0.03 μg As L^{-1} (calculated as three times the standard deviation of a blank sample). The lowest concentration level validated was 7.5 μg As kg^{-1} for total As.

2.6. Arsenic speciation analysis

The extraction procedure of arsenic species was based on our previous study in rice samples (Llorente-Mirandes et al., 2012). Briefly, 0.25 g aliquots of the cereal products were weighed in PTFE vessels and then extracted by adding 10 mL of 0.2% (w/v) HNO_3 and 1% (w/v) H_2O_2 solution in a microwave digestion system. This extraction method completely oxidises As(III) into As(V), without conversion of the methylated arsenic species into iAs, so we quantified iAs as As(V). Arsenic species were determined by ion chromatography IC–ICPMS. Speciation analysis by IC was performed using a Dionex ICS-3000 Ion Chromatograph. The outlet of the column was connected via polyether ether ketone (PEEK) capillary tubing to the nebuliser of the ICPMS system. Separation of As(III), As(V), DMA and MA was achieved with an anion exchange column (Hamilton PRP-X100, 150 × 4.1 mm, 5 μm, Hamilton, USA) and using the conditions shown in Table 1. The ion intensity at *m*/*z* 75 (^{75}As) was monitored using Agilent Chemstation ICPMS software rev. B.04.00. Additionally, the ion intensities at *m*/*z* 77 ($^{40}Ar^{37}Cl$) and *m*/*z* 35 (^{35}Cl) were monitored to detect possible argon chloride ($^{40}Ar^{35}Cl$) interference at *m*/*z* 75. Arsenic species in the chromatograms were identified by comparison of the retention times with those of the standards. External calibration curves were used to quantify MA, DMA and arsenate against the corresponding standards. Both water blanks and extraction blanks were also analysed by IC–ICPMS in each batch of samples. Each sample was extracted and analysed in triplicate. Sample solutions were analysed in batches including internal quality control, such as a standard solution and two certified reference materials every ten samples and also at the end of the sequence, to control the stability of the instrument sensitivity during the analytical run.

After full validation, the method was recently accredited by ENAC (Spanish National Accreditation Entity) under the ISO/IEC 17025 standard for its applicability in cereal-based food.

3. Results and discussion

3.1. Optimisation of the IC–ICPMS parameters

Some IC–ICPMS parameters were modified and optimised from our previous study to improve LODs. First, the injection volume was increased to 250 μL and an increase in arsenic sensitivity (by a factor of around two) in IC–ICPMS measurements were achieved without this affecting the good chromatographic resolution between the peaks. The ion intensities at *m*/*z* 77 ($^{40}Ar^{37}Cl$ and ^{77}Se) and *m*/*z* 35 (^{35}Cl) were monitored to detect possible argon chloride interference at *m*/*z* 75 on the IC–ICPMS measurements. Since no interferences were found, helium was not required, resulting in a noticeable increase in As sensitivity in IC–ICPMS measurements. The ionisation of arsenic may be significantly increased by the presence of carbon in the ICPMS plasma, according to the chemical ionisation process (Pettine et al., 2007). Hence, isopropyl alcohol (IPA) and methyl alcohol (MeOH) solutions containing different proportions of alcohol were examined to improve sensitivity to arsenic detection. The best signal-to-noise ratio was obtained with the IPA solution. Therefore, a 10% IPA solution was added through a T-piece after the column and before the nebuliser, using a peristaltic pump and thus, ensuring a compromise between increasing As sensitivity and maintaining suitable plasma conditions. The conditions for arsenic speciation analysis are reported in Table 1.

Table 1
Operating conditions of the IC–ICPMS system.

ICPMS Parameters	
RF power	1500 W
Make up gas flow, Ar	0.15 L min^{-1}
Carrier gas flow, Ar	0.95 L min^{-1}
Spray chamber (type and temperature)	Scott-type and 2 °C
Sampler and skimmer cones	Niquel
Nebuliser	Microconcentric
Sampling depth	8.0 mm
Cell exit	−70 V
Masses	*m*/*z* 75 (^{75}As), *m*/*z* 35 (^{35}Cl) and *m*/*z* 77 ($^{40}Ar^{37}Cl$)
Collision cell	OFF
Dwell time	2.0 s (*m*/*z* 75), 0.1 s (*m*/*z* 35 and 77)
QP/OctP Bias difference	2 V
Organic solvent	10% Isopropyl alcohol post-column
Chromatographic conditions	
Column	Hamilton PRP-X100 (150 × 4.1 mm, 5 μm)
Mobile phase	26 mM $NH_4H_2PO_4$, pH = 6.2 (adjusted with aqueous ammonia)
Flow rate	1 mL min^{-1}
Injection volume	250 μL
Column temperature	30 °C
Pressure	95 bar
Arsenic species	As(III), DMA, MA and As(V)
Elution	Isocratic, 10 min

3.2. Validation parameters

The validation parameters were established as specified elsewhere (Thompson, Ellison, & Wood, 2002).

3.2.1. Linearity, limit of detection and limit of quantification

Linearity was assessed by analyses of mixed standard solutions in triplicate from 0.05 to 5 μg As L^{-1} (six calibration points) in doubly deionised water (Table 2). It was then validated through three analytical runs on three different days.

Limits of detection (LODs) were estimated for iAs, DMA and MA with the standard error of y-intercepts of regression analysis (σ) and the slope (S) of the standard curves, using the following equation LOD = 3 σ/S (Table 2) (Miller & Miller, 2005). Compared to the previous method (Llorente-Mirandes et al., 2012), lower instrumental detection limits for As species were obtained (see Table 2). Limits of quantification (LOQs) were estimated in the same manner from the equation LOQ = 10 σ/S (Table 2) (Miller & Miller, 2005).

Table 2
Linearity, LOD, LOQ, accuracy and repeatability of the validated method.

Analyte	Linearity[a]	LOD	LOQ	Accuracy[c]					Repeatability[e]	
	Range (μg As L^{-1})	(μg As kg^{-1})	(μg As kg^{-1})	NIST SRM 1568a		NMIJ CRM 7503-a			NIST SRM 1568a (RSD%, $n = 6$)	NMIJ CRM 7503-a (RSD%, $n = 6$)
				Measured value ($n = 6$)	Literature value	Measured value ($n = 6$)	Certified value	(Recovery %)		
DMA	0.05–5.0	0.3	1.1	168.4 ± 8.2	160–174[b]	13.5 ± 0.7	13.3 ± 0.9	101.5	2.5	3.7
MA	0.05–5.0	0.3	0.9	12.8 ± 0.5	2–14[b]	<LOD			3.6	
iAs	0.05–5.0	0.4	1.2	103.3 ± 4.6	80–110[b]	83.7 ± 1.6	84.1 ± 3.0[d]	99.5	2.7	1.9

[a] Acceptance criteria: $R^2 \geqslant 0.9990$ and residual error $\leqslant 15\%$ for the lowest calibration level and $\leqslant 10\%$ for the others, as recommended (Horwitz, 1982).
[b] No certified values, values reported by other studies (Carbonell-Barrachina et al., 2012; D'Amato et al., 2011).
[c] Concentrations expressed as μg As kg^{-1} on dry mass (mean ± SD).
[d] As sum of certified values for As(III) and As(V) ± the square sum of their uncertainties.
[e] Acceptance criterion: %RSD (repeatability) $\leqslant$ 2/3 * %RSD (intermediate precision).

3.2.2. Accuracy and repeatability

To evaluate the accuracy of the speciation method, two rice CRMs were analysed throughout the study (Table 2). NMIJ CRM 7503-a rice has a certified value of 84.1 ± 3.0 μg As kg^{-1} for iAs (sum of the certified values for As(III) and As(V) (the square sum of their uncertainties)) and a certified value of 13.3 ± 0.9 μg As kg^{-1} for DMA. The results obtained were in agreement with the certified values. NIST SRM 1568a rice is certified only for total arsenic, but when performing speciation, our results were consistent with the literature on the presence of arsenic species in this material (Carbonell-Barrachina et al., 2012; D'Amato et al., 2011). Moreover, the sum of the As species (284.5 μg As kg^{-1}) compared well with the certified total As value of 290 μg As kg^{-1}. For within-day repeatability, six replicates of NMIJ CRM 7503-a White Rice Flour and NIST SRM 1568a Rice were analysed within a day and by the same analyst (Table 2).

3.2.3. Intermediate precision, trueness and expanded uncertainty

Intermediate precision, trueness and expanded uncertainty were assessed for iAs, MA and DMA using spiked cereal-based products at three concentrations in triplicate. Biscuit, breakfast cereal and white bread were chosen for the spiking experiments at low and medium concentrations, while black rice, long-grain rice and infant cereal (rice-based) were selected to evaluate high concentrations. Spiking experiments were performed by adding As(III), As(V) DMA and MA standards to solid samples and then homogenised. The mixtures were then left to stand for 30 min before microwave extraction. Unspiked samples were also analysed in triplicate to calculate spike recovery. The lowest concentration levels validated were 4 μg As kg^{-1} for iAs, DMA and MA. Below such concentration, the values obtained for precision and accuracy could not reach the specified limits established for further routine laboratory operating conditions.

Trueness was expressed in terms of recovery, according to the method of (Thompson et al., 2002). No As(III) was found in spiked extracts, so we calculated iAs recoveries assuming that all of the added As(III) was oxidised into As(V). Recoveries were calculated as follows: recovery (%) = $(a - b)*100/c$, where a is the As concentration measured in the extracts of samples which were spiked with standards solutions; b is the As concentration measured in the unspiked sample and c was the known concentration added to the sample. The values for DMA, MA and iAs are given in Table 3, and show that all species were recovered successfully.

To evaluate intermediate precision, various factors were changed: three different analysis days over three weeks, different analysts and different standards for spiking. Intermediate precision was expressed in terms of relative standard deviation (%RSD) of arsenic recovery and the results are shown in Table 3. They were consistent with the precision acceptance criterion.

The relative expanded uncertainty (k=2) was estimated by a top-down method, adapted from (Maroto, Boqué, Riu, Ruisánchez, & Òdena, 2005) and was calculated using a formula that combined the precision and trueness values of the spiking experiments (Llorente-Mirandes et al., 2012). The results for each species and each spiked level are shown in Table 3 and agree with the uncertainty acceptance criterion.

3.2.4. External quality control

The method was tested in two proficiency tests as external quality control. It was checked by an interlaboratory comparison of the European Union-Reference Laboratory for Heavy Metals in Feed and Food, IMEP-112, Total and inorganic arsenic in wheat, vegetable food and algae (de la Calle et al., 2012). The wheat test material was analysed during the validation process and accurate results were obtained: for an assigned value of 169 ± 25 μg As kg^{-1} for iAs, 170.0 ± 3.5 μg As kg^{-1} was obtained. Moreover, the laboratory had previously participated in a proficiency test of the Central Science Laboratory-Food Analysis Performance Assessment Scheme (CSL-FAPAS) to determine total and inorganic As levels in rice (FAPAS round 07151 (Food Analysis Performance Assessment Scheme (FAPAS) Report 07151, 2011). The result obtained was satisfactory: for an assigned value of 390 ± 72 μg As kg^{-1} for iAs, 424.3 ± 5.1 μg As kg^{-1} was obtained.

There are few certified reference materials (CRMs) for arsenic species in food matrices. Recently, the JRC-IRMM released a new certified reference material, ERM-BC211 (rice). The CRM was prepared from rice destined for human consumption and is certified for total arsenic, the sum of arsenite and arsenate, and dimethylarsinic acid. The present method was employed in the certification study of ERM-BC211 and accurate results were obtained compared to the final certified values, further demonstrating its validity and reliability (Boertz et al., 2012).

3.3. Method application

A selection of 30 cereal-based food samples representing different types were analysed for their contents of As species and total As. Table 4 summarises the As speciation results, total As and mass balance for all analysed samples. For quality assessment, mass balance (calculated as the ratio of the sum of As species in the extract to total As) was calculated and the results were comparable with others reported in the literature (Cubadda et al., 2010; D'Amato et al., 2011; Jackson, Taylor, Punshon, & Cottingham, 2012b; Zhao et al., 2010). Mass balance values were satisfactory notwithstanding the low arsenic concentration in cereal samples. Values ranged from 73% to 123%, averaging 96%, which indicated a full quantification of the As species that may exist in cereal-based samples. The extraction solution was suitable solvent for the extraction of As

Table 3
Precision, trueness and expanded uncertainty ($k = 2$) values of the validated method.

Analyte	Sample	Spiked levels (Added μg As kg^{-1})	Intermediate precision[a] (RSD in %, $n = 9$)	Trueness[b] (Recovery in %, $n = 9$)	Expanded uncertainty[c] (U in %, $n = 9$)
DMA	Biscuit, breakfast cereal and white bread	4	6.3	107.6	19.5
DMA	Biscuit, breakfast cereal and white bread	40	3.4	106.6	13.0
DMA	Black rice, long-grain rice, infant cereal (rice based)	125	4.2	98.2	8.9
MA	Biscuit, breakfast cereal and white bread	4	6.5	108.6	20.5
MA	Biscuit, breakfast cereal and white bread	40	1.7	101.5	5.0
MA	Black rice, long-grain rice, infant cereal (rice based)	125	2.6	101.2	5.5
iAs	Biscuit, breakfast cereal and white bread	4	5.6	95.9	11.8
iAs	Biscuit, breakfast cereal and white bread	40	1.8	100.3	3.8
iAs	Black rice, long-grain rice, infant cereal (rice based)	250	1.9	95.3	9.1

[a] Acceptance criterion: % RSD <2/3 Horwitz–Thomson function (Horwitz, 1982) and is (in % RSD): 14.7% for values ⩽100 μg kg^{-1}, 13.6% for 200 μg kg^{-1} and 12.2% for 400 μg kg^{-1} (Fryš, Bajerová, Eisner, Mudruňková, & Ventura, 2011).
[b] Acceptance criterion: Rec = 85–115%. CODEX criterion: 60–115% for 10 μg kg^{-1} and 80–110% for 0.1–10 mg kg^{-1} (Joint FAO/WHO Expert Committee on Food Additives, 2010).
[c] Acceptance criterion: U_{max} <2 * %RSD Horwitz function according to (Horwitz, 1982; Thompson et al., 2002).

Table 4
Concentrations of total As and As species in cereal-based products expressed as μg As kg^{-1} on dry mass (mean ± SD, $n = 3$).

Sample	Total As	Arsenic species			Mass balance (%)[b]
		DMA	MA	iAs	
Bread					
Loaf	7.2 ± 0.7	<LOD	<LOD	5.4 ± 0.3	74.7
White-1	4.9 ± 0.3[a]	<LOD	<LOD	5.1 ± 0.3	103.5
Whole grain	9.9 ± 0.1	<LOD	<LOD	7.2 ± 0.6	72.7
Toast	13.0 ± 0.3	<LOD	<LOD	10.9 ± 0.3	84.0
White-2	6.5 ± 0.5	<LOD	<LOD	5.7 ± 0.2	88.3
Biscuit					
Butter cookie	4.2 ± 0.2[a]	<LOD	<LOD	4.8 ± 0.6	115.2
Whole grain cookie	7.0 ± 0.7	<LOD	<LOD	7.1 ± 0.6	101.8
Chocolate cookie	3.7 ± 0.1[a]	<LOD	<LOD	3.8 ± 0.3	102.9
Breakfast cereal					
Multicereal	5.2 ± 1.1[a]	<LOD	<LOD	4.5 ± 0.4	87.3
Corn-based-1	10.5 ± 0.3	<LOD	<LOD	9.9 ± 0.9	94.1
Corn-based-2	<LOD	<LOD	<LOD	3.3 ± 1.1	n.c.[c]
Muesli	10.1 ± 2.7	<LOD	<LOD	8.0 ± 0.5	78.9
Flour					
Wheat-1	4.6 ± 0.3[a]	<LOD	<LOD	3.9 ± 0.2	84.7
Whole	5.3 ± 0.2[a]	<LOD	<LOD	5.3 ± 0.7	99.3
Wheat-2	10.5 ± 1.5	<LOQ	<LOQ	10.0 ± 0.3	95.7
Snack					
Corn-1	4.1 ± 0.2[a]	<LOD	<LOD	3.6 ± 0.2	87.5
Corn-2	4.1 ± 1.5[a]	<LOD	<LOD	3.1 ± 0.5	76.3
Corn-3	9.1 ± 0.5	<LOD	<LOD	6.7 ± 0.3	73.4
Pasta					
Noodle	7.7 ± 1.3	<LOQ	<LOD	8.7 ± 0.2	112.3
Spaghetti	4.9 ± 0.2[a]	<LOQ	<LOD	6.0 ± 0.4	122.9
Macaroni	23.3 ± 1.2	<LOQ	<LOD	23.4 ± 0.5	100.4
Infant cereal					
Multicereals (seven cereals with honey and fruits)	14.4 ± 0.2	2.5 ± 0.1	<LOD	14.0 ± 0.7	114.6
Organic spelt porridge	7.7 ± 0.3	<LOD	<LOD	8.1 ± 1.3	105.6
Multicereals (eight cereals with fruits)-1	15.9 ± 0.3	<LOD	<LOD	15.9 ± 0.2	99.8
Multicereals (five cereals)	21.8 ± 0.8	<LOD	<LOD	22.0 ± 0.6	100.7
Multicereals (eight cereals with honey)-1	9.8 ± 0.3	<LOD	<LOD	10.5 ± 1.0	106.5
Multicereals (eight cereals)	14.4 ± 0.6	<LOD	<LOD	13.5 ± 0.5	94.0
Multicereals (cereals with honey)-2	24.1 ± 0.7	3.6 ± 0.1	<LOD	22.5 ± 0.3	108.3
Multicereals (eight cereals with fruits)-2	35.6 ± 0.8	9.4 ± 0.2	<LOD	26.0 ± 1.9	99.6
Rice	267.4 ± 11.5	175.0 ± 3.7	6.3 ± 0.5	74.3 ± 0.6	95.6

[a] Values below the LOQ for total As (6.0 μg As kg^{-1}).
[b] Calculated as the ratio of the sum of As species in the extract to total As.
[c] No calculated.

species in this type of matrix. The total arsenic concentrations in some samples were below the LOQ (Table 4). Nevertheless, these values were estimated and used to calculate mass balance knowing that their precision and accuracy could not reach the specified limits established for routine laboratory operating conditions.

3.3.1. Cereal-based foods

Bread, biscuits, breakfast cereals, corn snacks, wheat flour and pasta were analysed and the results are shown in Table 4. Total As content ranged from 3.7 to 23.3 μg As kg^{-1} and the mean As concentration was 7.8 μg As kg^{-1}. Total As content was below the

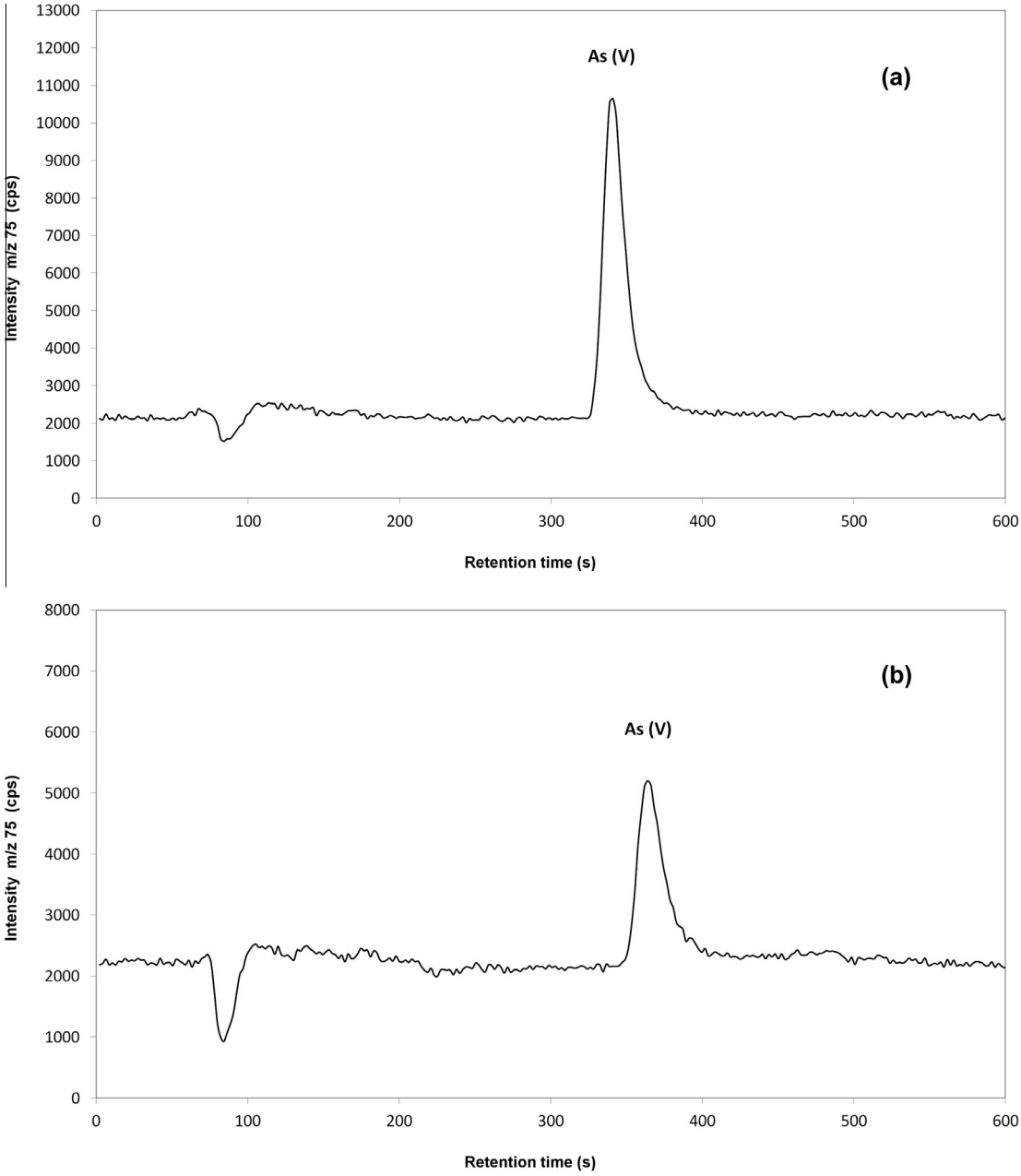

Fig. 1. Chromatograms of (a) macaroni and (b) wheat flour extracts from anion exchange by IC–ICPMS.

LOD in a breakfast cereal sample. The present results are similar to others reported in the literature for total As in cereal-based food (range from 4.6 to 128.0 μg As kg^{-1}) (Cubadda et al., 2010; D'Amato et al., 2011; Fontcuberta et al., 2011; Jackson, Taylor, Karagas, Punshon, & Cottingham, 2012a). A recent study on cereal bars showed that the bars not listing any rice product among the ingredients were among the lowest As-containing ones (range from 8 to 27 μg As kg^{-1}) (Jackson et al., 2012a). The As level in cereal grains (e.g., wheat, barley and maise) is typically about one order of magnitude lower than that in rice (Duxbury & Panaullah, 2007). Different factors such as soil physical conditions or water may affect As concentration in wheat grain. For example, high As content was found in wheat grown in an area with high water As concentrations in West Bengal (India) (Roychowdhury, Uchino, Tokunaga, & Ando, 2002). Furthermore, another study also reported high As levels in wheat from contaminated areas, with a mean of 69 μg As kg^{-1} (range = 41–101 μg As kg^{-1}), at an arsenic-rich site in France (Zhao et al., 2010). The authors also found that As concentration in wheat bran was higher than that in white flour, containing only iAs and no methylated As. Cubadda and colleagues (Cubadda et al., 2010) analysed 726 samples of wheat grains collected from 22 different locations in Italian agricultural areas over three consecutive years. They observed an average arsenic concentration of 9 μg As kg^{-1}, with a range of 2–55 μg As kg^{-1}. The authors concluded that iAs was the major As compound, highlighting the importance of wheat as a source of inorganic arsenic in the Italian diet.

Regarding the present As speciation results, only inorganic As was quantified in cereal-based food (Table 4). Inorganic arsenic ranged from 3.1 to 23.4 μg As kg^{-1} with a mean value of 7.0 μg As kg^{-1}. DMA was found below the LOQ in some samples, while MA was below LOD in all samples. The finding that almost all the arsenic in cereal-based food is present as iAs is in agreement with other studies showing very low levels of methylated As

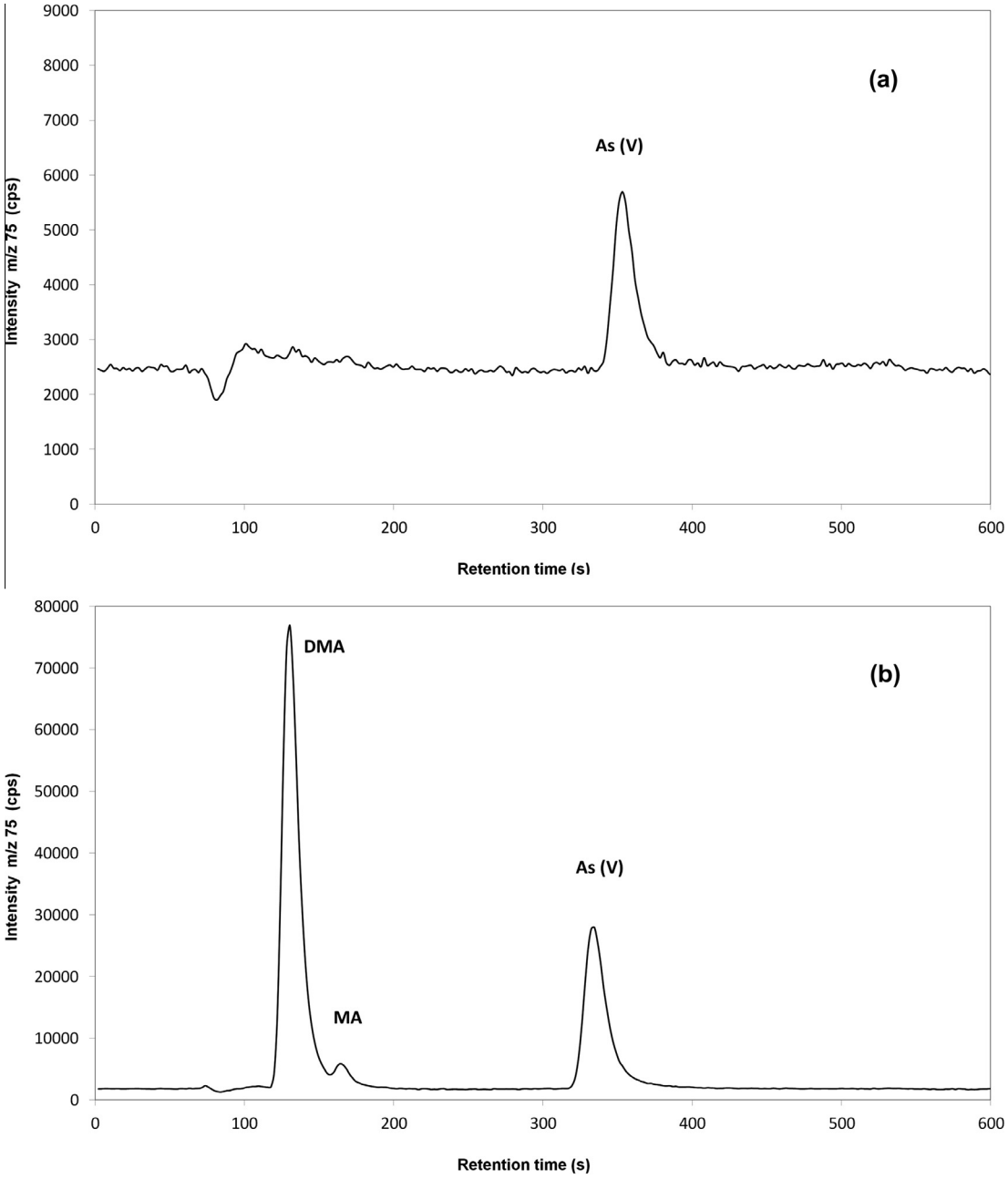

Fig. 2. Chromatograms of (a) organic spelt infant cereal and (b) rice-based infant cereal extracts from anion exchange by IC–ICPMS.

species (Cubadda et al., 2010; Zhao et al., 2010). This behaviour is illustrated in Fig. 1, which shows that all arsenic in the present study was in form of inorganic As in the chromatograms of macaroni (a) and wheat flour (b) extracts. Some As speciation studies have focused on wheat or wheat flour, but limited information is available in the literature about cereal-based products. Moreover, no studies on biscuits and snack products have been reported. Other study analysed several wheat-based food (whole grain, flour, bread and pasta) and observed that about 95% of the As in wheat-based food was in the inorganic form, whereas the remainder was mainly DMA (D'Amato et al., 2011).

There is little information of As speciation in cereal-based products in the literature, probably due to the low LOD that is required to analyse these kinds of food. Although the iAs content is much lower than that of rice, cereals and especially wheat should not be ignored as potential contributors to dietary iAs exposure in populations with a predominantly wheat-based diet. Further research on As speciation in cereal food products is required to estimate dietary exposure to inorganic As in such populations.

3.3.2. Infant cereals

Currently, there is a very broad range of infant products on the market such as infant cereals (rice-based or mixed cereals), pureed foods (meat and fish, etc.) and formulas (Carbonell-Barrachina et al., 2012; Hernández-Martínez & Navarro-Blasco, 2013; Ljung, Palm, Grandér & Vahter, 2011). Nine infant cereal samples marketed in Spain by different manufacturers were selected. Seven of them were made with a mixture of cereals (wheat, barley, oat, corn, rye, sorghum, millet and rice) combined with fruit or honey; the other two were an organic spelt porridge and a rice-based infant cereal. The results of total arsenic and arsenic species measurements are given in Table 4. For non-rice-based formulations

($n = 8$), total arsenic contents ranged from 7.7 to 35.6 μg As kg^{-1} with a mean value of 18 μg As kg^{-1}. These levels were comparable to other reported values in infant cereals and formulas, but lower than those in other studies of rice-based infant cereals displaying high As concentrations (Carbonell-Barrachina et al., 2012; Hernández-Martínez & Navarro-Blasco, 2013; Jackson et al., 2012b; Llorente-Mirandes et al., 2012; Meharg et al., 2008). The infant cereals analysed here had a very low rice percentage or did not contain rice (according to the labelled formulation), thus explaining the low arsenic contents found. The single rice-based infant cereal (above 90% of rice content) was analysed and as expected, the total As content increased by an order of magnitude (267.4 ± 11.5 μg As kg^{-1}) compared to the non-rice-based infant cereals. Moreover, not only did the percentage of rice contribute to arsenic content, but also the product brand and the mode of cereal production (conventional or organic). A recent study analysing 91 infant cereals marketed in Spain from eight different manufacturers concluded that infant cereals based on raw materials obtained in a conventional way displayed lower amounts of arsenic than those based on raw materials procured in an organic way. This study affirmed that the content of arsenic is affected by environmental conditions of the system (Hernández-Martínez & Navarro-Blasco, 2013).

We found that iAs was the major As species in all the non-rice-based infant cereals studied (mean of 93% of the extracted As), while DMA was only found in three samples as a minor species and MA was below the detection limit. Inorganic arsenic levels ranged from 8.1 to 26.0 μg As kg^{-1} with a mean value of 16.6 μg As kg^{-1}. Therefore, none of the samples exceeded the Chinese regulatory limit of 0.15 mg As kg^{-1} for iAs (USDA Maximum Levels of Contaminants in Foods, 2006). Few studies have reported As speciation results in infant cereals (non-rice-based), probably due to the low LODs required to analyse these kinds of food. A recent study reported that As in baby food was present mainly as iAs (Jackson et al., 2012b). Similar iAs results were reported in infant cereals with gluten (wheat, oat, barley, rye and sorghum), in which the iAs content was 26 μg As kg^{-1} (corresponding to 98% of the extracted As) (Carbonell-Barrachina et al., 2012).

Additionally, recent studies have shown that rice-based infant cereals contain elevated concentrations of the toxic iAs (Carbonell-Barrachina et al., 2012; Meharg et al., 2008). Our results of the rice-based infant sample showed that DMA was the major species (accounting for 68% of the extracted As), while iAs accounted for 29% and MA was a minor species. Fig. 2 shows, as an example, differences in the chromatograms of organic spelt infant cereal (a) and rice-based infant cereal (b) extracts.

In brief, inorganic arsenic contents were higher in products based on rice than in similar products prepared using mixtures of other cereals with gluten (wheat, barley and oat). Therefore, the potential of high iAs concentrations in rice-based products intended for infants requires special attention. A wide range of rice-based products are fed to babies, increasing the risk of dietary exposure to iAs. Thus, there is a fundamental need to reduce the rice content of baby products which would reduce the infant exposure to iAs. The elimination of rice from infant cereals or the diversification of diets by including other cereals could reduce the risk of iAs exposure. In addition, special attention should be paid to infants with coeliac disease who have to eat gluten-free food that is mainly based on rice.

4. Conclusions

In summary, a straightforward method for the determination of iAs, DMA and MA in cereal-based food and infant cereals was optimised and fully validated. The optimised IC–ICPMS operating parameters provided low LODs, suitable for determining the As species present in samples. The method was successfully applied to 30 cereal-based food. Inorganic arsenic was the major As compound found in the food products studied, highlighting the importance of cereal products as a possible source of iAs in cereal-based diets. The validated method is sensitive and selective for iAs and could be a valuable tool for assessing iAs in cereal-based food currently a subject of high interest in food control analysis. Moreover, the present results may contribute to the on-going discussions for establishing and implementing maximum levels on inorganic arsenic in food commodities, as it is stated within the European Union, and for further studies on risk assessment.

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4.3 Performance of laboratories in speciation analysis in seafood-Case of methylmercury and inorganic arsenic

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Performance of laboratories in speciation analysis in seafood-Case of methylmercury and inorganic arsenic

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Performance of laboratories in speciation analysis in seafood – Case of methylmercury and inorganic arsenic

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ABSTRACT

The international measurement evaluation program (IMEP) has together with the European Reference Laboratory for Heavy Metals in Feed and Food (EU-RL-HM) carried out two interlaboratory comparisons (ILC) in 2010 on the measurement of trace metals, as well as methylmercury and inorganic arsenic in seafood. In IMEP-109 only EU National Reference Laboratories (NRL) took part, while IMEP-30 was open to all laboratories. In this article only methylmercury and inorganic arsenic analysis will be discussed, as these appear generally to be more problematic measurands. They are also particularly interesting to legislators, as no maximum limits have been set yet for them in European legislation. The aim of the two ILCs was to produce more information to help tackling this issue. The results of the two exercises were pooled together, evaluated, and compared with former ILC projects for methylmercury and inorganic arsenic analysis. Results for inorganic arsenic were spread, but not method dependant. The measurand seems to be difficult to analyse in this matrix and possible method issues were identified. Methylmercury results were satisfactory, but not many laboratories perform this type of analysis because it is generally believed that specific instrumentation is needed. As an answer to this presumption, alternatives are suggested.

1. Introduction

From a toxicological point of view metal speciation is of paramount importance since in most cases different species have different toxicologies. For instance, methylmercury (MeHg) is more toxic than the inorganic mercury compounds (de la Calle et al., 2008) while inorganic arsenic (iAs) is more toxic than the organic species of arsenic, with arsenosugars and arsenobetaine not being toxic (Capar, Mindak, & Cheng, 2007; Léonard, 1991). On the other hand, the existence of toxic organic arsenic species cannot be excluded.

According to a Scientific Report published by the European Food Safety Authority (EFSA, 2009) the foods mostly contributing to iAs exposure are: cereal grains and cereal based products, followed by foods for special dietary uses, bottled water, coffee and beer, rice grains and rice based products, fish and vegetables. It was furthermore established that …

"the national inorganic arsenic exposures from food and water across nineteen European countries using lower bound and upper bound concentrations, are estimated to range from 0.13 to 0.56 μg kg^{-1} body weight (b.w.) per day for average consumers. Dietary exposure to inorganic arsenic for children under three years of age is in general estimated to be from 2 to 3-fold that of adults… A range of benchmark dose lower confidence limit ($BMDL_{01}$) values between 0.3 and 8 μg/kg b.w. per day was identified for cancers of the lung, skin and bladder, as well as skin lesions. The estimated dietary exposures to inorganic arsenic for average and high level consumers in Europe are within the range of the $BMDL_{01}$ values identified, and therefore there is little or no margin of exposure and the possibility of a risk to some consumers cannot be excluded".

A problem in the evaluation of iAs levels is that there is a lack of fully validated methods and reference materials. In Europe, one standard has been published for the measurement of iAs in seaweed (EN 15517:2008) and China has a standard method for the determination of total arsenic and abio-arsenic in foods

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Abbreviations

AMC	Analytical Methods Committee of the Royal Society of Chemistry
CRM	Certified Reference Material
EFSA	European Food Safety Authority
EN	European Standard
EU-RL-HM	European Union Reference Laboratory for Heavy Metals in Feed and Food
GC–MS	Gas chromatography – mass spectrometry
GLC-ECD	Gas-liquid chromatography - electron capture detection
HPLC-ICP-MS	High-performance liquid chromatography - inductively coupled plasma - mass spectrometry
iAs	inorganic arsenic
ILC	Interlaboratory Comparison
IMEP	International Measurement Evaluation Programme
IRMM	Institute for Reference Materials and Measurements
ISO	International Organisation for Standardisation
JRC	Joint Research Centre
LoQ	Limit of quantification
MeHg	Methylmercury
NRC	National Research Council of Canada
NRL	National Reference Laboratory
PT	Proficiency Test

(GB/T5009.11–2003). For years a debate has taken place in the respective scientific community on whether the measurement of the inorganic arsenic content in food commodities was method dependent or not (de la Calle et al., 2011). The outcome of IMEP-107, an interlaboratory comparison (ILC) on iAs in rice, has shown that the reported iAs mass fractions were not method dependent (de la Calle et al., 2011).

MeHg is largely responsible for the accumulation of mercury in marine organisms and the transfer of the element from one trophic level to another in the food chain. Foods with the highest level of Hg are seafood and most of this Hg is in the form of MeHg (Capar et al., 2007; Storelli, Busco, & Marcotrigiano, 2005). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has established a Provisional Tolerable Weekly Intake (PTWI) for MeHg of 0.0016 mg kg^{-1} of b.w. and 0.005 mg kg^{-1} of b.w. for total Hg (Internet-WHO, 1989; JECFA, 2006).

In general, exposure to organic mercury can cause brain damage to a developing foetus (Marsh, Myers, & Clarkson, 1981). Epidemiological studies also suggest that prenatal exposure to MeHg may affect the neurobehavioural development of young children. (Drabæk & Iverfeldt, 1995; Grandjean, 2007). For this reason the European Commission recommends pregnant women, breast feeding women and children to limit their consumption of big fish predators in which high contents of MeHg are known to be present (EFSA, 2004).

The species-dependent differences in toxicology should be taken into consideration when fixing maximum levels in legislation. No maximum levels have been established, so far, for iAs in European legislation, due to a lack of fully validated, standardised analytical methods for this measurand. Maximum levels for total mercury in foodstuffs are given in the EU legislation, varying from 0.5 to 1 mg kg^{-1} for different seafood (Commission Regulation (EC) No 1881/2006), but no maximum level exists for MeHg. The U.S. Food and Drug Administration established a guideline for MeHg in seafood proposing a maximum level of 1 mg kg^{-1} (Internet-FDA, 2001).

The International Measurement Evaluation Program (IMEP), owned by the Joint Research Centre – Institute for Reference Materials and Measurements (JRC – IRMM), tested laboratories' ability to measure MeHg for the first time in 2004 in the IMEP-20 exercise (Aregbe et al., 2004) in tuna. However, only 8 participants reported results. The European Union Reference Laboratory for Heavy Metals in Feed and Food (EU-RL-HM) started investigating laboratories' performance in the determination of MeHg and iAs in the IMEP-104 (de la Calle et al., 2008) in seafood and IMEP-107 in rice (de la Calle et al., 2011; de la Calle, Linsinger, Emteborg, Charoud-Got, & Verbist, 2010), respectively. All laboratories can participate in the regular IMEPs, while the IMEP-100 series are ILCs organised on behalf of the EU-RL-HM and are open for nominated National Reference Laboratories (NRL) only. This article will present the outcome of the IMEP-30 and IMEP-109 exercises which were carried out in parallel in 2010 using the same test material – seafood. The measurands were the trace elements lead, cadmium, total mercury and total arsenic, in addition to MeHg and iAs. However, only the two latter measurands will be discussed here.

2. Set-up of the exercises

2.1. Test material preparation

The commercially available certified reference material (CRM) DOLT-4 (Dogfish Liver for Trace Metals) was used for this proficiency test (PT). The material was purchased from the National Research Council of Canada (NRC). The samples were relabelled to avoid identification by the participants as an existing CRM. Comprehensive information on the preparation of the CRM can be found in the certification report on the NRC website (Internet-CNRC). The certificate was valid for the duration of both exercises (until April 2014).

2.2. Homogeneity and stability

Information on the homogeneity and stability of the test material was gathered from the DOLT-4 certificate. According to the latter, uncertainties related to possible between-bottle variation (u_{hom}) are included in the overall uncertainty of the certified value. In the experience of the CRM producer, uncertainty components for long and short term stability were considered negligible and are thus not included in the uncertainty budget. As total arsenic is homogenous and stable, it was assumed that this is also the case for iAs, on the basis of previous experience on the two measurands (de la Calle et al., 2010).

2.3. Reference values and target standard deviation $\hat{\sigma}$

The DOLT-4 certificate provided certified values for all the measurands in this study except for iAs. The certified values were used as assigned values (X_{ref}) for both intercomparisons. The uncertainties provided in the certificate represent the expanded uncertainties (U_{ref}) with a coverage factor $k = 2$, corresponding to a level of confidence of about 95%.

2.3.1. Reference value for iAs

Five laboratories (listed below), experts in the field of trace element speciation analysis, analysed the test material to establish the reference value for iAs. The expert laboratories involved in the establishment of the assigned values were:

- Institute of Agrochemistry and Food Technology (CSIC), Spain
- Institute of Chemistry, Karl-Franzens University Graz, Austria
- The Food and Environment Research Agency (FERA), United Kingdom

- National Food Institute, Technical University of Denmark (DTU), Denmark
- Department of Analytical Chemistry, University of Barcelona, Spain

They were asked to use the method of analysis of their choice without further requirements, and to report their results together with the measurement uncertainty and a description of the method they have used. The latter are described in detail in the final project reports (Baer, de la Calle, Verbist, Emteborg, & Robouch, 2010; de la Calle, Emteborg, Robouch, & Verbist, 2010).

The means reported by the expert laboratories and their associated standard uncertainties (u_{exp}) for iAs are shown in Table 1. The numbers for the certifiers in Table 1 are given randomly and do not correspond to the order of appearance in the list above.

Table 1 presents strong discrepancies among the results reported by the expert laboratories, contrary to what was observed in IMEP-107 (total As and iAs in rice). For this reason, it was not possible to establish an assigned value for this measurand and the laboratories' results for iAs could not be scored.

2.3.2. Reference value for MeHg

The assigned value for MeHg derived from the DOLT-4 certificate was 1.33 mg kg^{-1} with an associated expanded uncertainty of 0.12 mg kg^{-1}. The standard deviation for proficiency assessment (also called target standard deviation), $\hat{\sigma}$, was fixed at 15% by the advisory board of both ILCs, on the basis of the outcome of previous ILCs organised by IMEP and on the state-of-the-art in this field of analysis.

2.4. Scores and evaluation criteria

Individual laboratory performance is expressed in terms of z- and ζ-scores in accordance with ISO 13528 (ISO 13528:2005).

$$z = \frac{x_{lab} - X_{ref}}{\hat{\sigma}} \quad \text{and} \quad \zeta = \frac{x_{lab} - X_{ref}}{\sqrt{u_{ref}^2 + u_{lab}^2}}$$

Where x_{lab} is the measurement result reported by a participant, X_{ref} is the certified reference value (assigned value), u_{lab} the standard uncertainty reported by a participant, u_{ref} the standard uncertainty of the reference value, and $\hat{\sigma}$ the standard deviation for proficiency assessment. Both scores can be interpreted as:

- satisfactory result for $|score| \leq 2$,
- questionable result for $2 < |score| \leq 3$ and
- unsatisfactory result for $|score| > 3$,

with |score| being the absolute value of the score.

Table 1
Values for iAs and their associated uncertainties as reported by the expert laboratories.

Certifier	X_{exp} (mg kg^{-1})	u_{exp} (mg kg^{-1})	U_{exp} (mg kg^{-1})[a]
1	<0.040[b]		
2	n.d.[c]		
3	0.047	0.006	0.012
4	0.075	0.005	0.010
5	0.152	0.010	0.020

[a] $U_{exp} = k \cdot u_{exp}$ is the estimated expanded uncertainty; with a coverage factor $k = 2$ corresponding to a level of confidence of about 95%.
[b] this is the limit of quantification (LoQ) on dry matter content basis of the method used.
[c] not detected – the LoQ of the method used is 0.031 mg kg^{-1} for arsenite and 0.084 mg kg^{-1} for arsenate.

2.4.1. Z-score

The z-score compares the participant's deviation from the reference value with the standard deviation accepted for the PT, $\hat{\sigma}$. Therefore, $\hat{\sigma}$ is the maximum acceptable standard uncertainty as defined by the organiser of the PT.

2.4.2. ζ-Score

The ζ-score states if the laboratory result agrees with the assigned value within the respective uncertainties. The denominator describes the combined uncertainty of the assigned value and the measurement uncertainty as stated by the laboratory. The ζ-score is therefore the most relevant evaluation parameter, as it includes the measurement result, the expected value (assigned value), its uncertainty as well as the uncertainty of the reported values. An unsatisfactory ζ-score can either be caused by an inappropriate estimation of the expected value and/or of its measurement uncertainty.

3. Results

A first general observation that can be made from the results is that speciation analysis does not seem to be wide spread among control laboratories. When considering the total number of reported results in the two exercises for all measurands, 57 laboratories reported results in the IMEP-30 and 38 laboratories in the IMEP-109. Of this total number of 95 participants, only 22 reported results for iAs and 14 for MeHg.

3.1. Participants' results for iAs

Thirteen participants of the IMEP-30 reported iAs results and 9 in the IMEP-109. The results were pooled together to get a higher number of data and to detect potential tendencies. They are represented in Fig. 1 together with the results obtained by the expert laboratories and the Kernel density curve shown in the lower right hand corner. Kernel density plots are an alternative to histograms and a useful method to represent the overall structure of a data group. The software used to calculate the densities was provided by the Statistical Subcommittee of the Analytical Methods Committee (AMC) of the Royal Society of Chemistry (AMC/RSC, 2006). Furthermore shown in Fig. 1 are the "less than" values and the reported experience of the participants.

Of the total of 22 results reported for iAs in the two ILCs, 3 laboratories (2 in IMEP-30, 1 in IMEP-109) reported values higher than 1 mg kg^{-1} and 7 laboratories reported "less than" values (4 in IMEP-30 and 3 in IMEP-109). With such a scattering of results it was not possible to derive any conclusion about the concentration of iAs in this test material, which confirms what has been observed with the results from the expert laboratories beforehand.

It seems however, that 16 laboratories (73%) agree on the fact that the mass fraction of iAs in this seafood material is below 0.25 mg kg^{-1} (Fig. 1) as confirmed by the results reported by the expert laboratories.

The scattered results could not be explained by a method dependence, as 7 out of the 16 reported results were obtained using the European Standard EN 15517 – but no clusters were observed. One reason for the unexpected spread of results might be that iAs is more difficult to measure in a seafood matrix, as this spread was not observed in the IMEP-107 exercise, where rice was used as test material (de la Calle et al., 2008). It could also be argued that the low mass fraction range makes the determination of iAs difficult. However, the range is the same than in the IMEP-107 project ($X_{ref} = 0.107 \pm 0.014$ mg kg^{-1}) and thus seems unlikely to be a factor of influence.

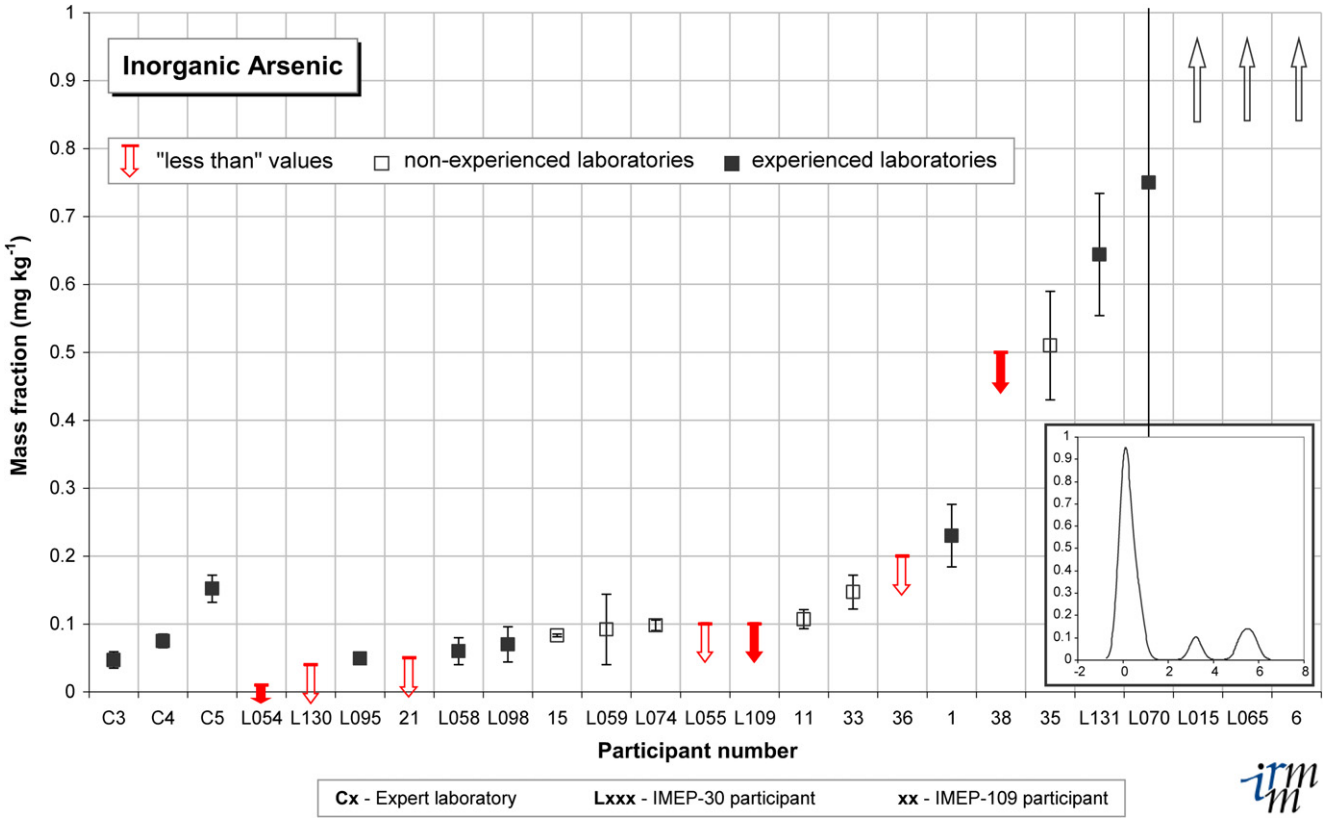

Fig. 1. Results reported for iAs by participants of the IMEP-30 & IMEP-109 exercises.

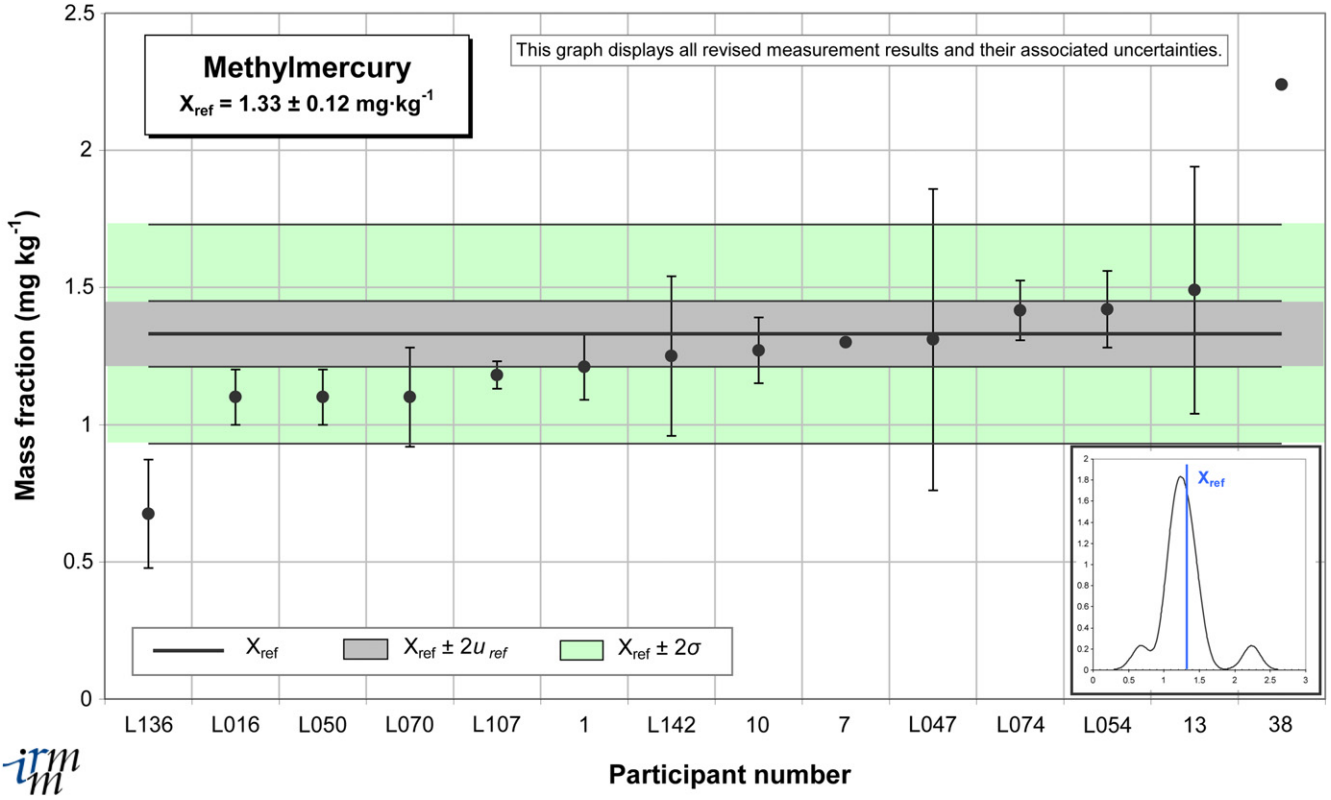

Fig. 2. Results for MeHg including the Kernel plot in the lower right hand corner.

Possible causes for the spread were most likely attributed to the extraction and/or detection steps and are listed hereafter. However, the following explanations strictly relate to the present context of the analysis of iAs in DOLT-4 and might not apply to other situations:

- The use of MeOH/water, diluted HCl, or of trifluoroacetic acid (TFA) as extracting reagents might not have provided quantitative recovery of iAs.
- The amount of oxidant added (H_2O_2) to oxidise As(III) to As(V), which is the species of As measured when using HPLC-based methods, might have been insufficient.
- Detection might be problematic when applying extraction of iAs with chloroform and concentrated HCl, as a cleaning step of the chloroform phase should be carried out to eliminate all traces of HCl and with it the arsenobetaine present, which is usually found at high concentrations in seafood. Remains of the concentrated HCl in the chloroform phase might introduce a high contamination of the sample with arsenic coming from the arsenobetaine.
- Finally, when analysing complex matrices by HPLC-ICP-MS, a shift in the retention time of the iAs species (As(III) and As (V)) might be observed, and consequently possible co-elution with minor organic arsenic species (other than arsenobetaine) may occur. This could potentially be remedied by introducing an extra step of hydride generation between the HPLC and the ICP-MS which would allow the selective determination of inorganic arsenic.

The evaluation of results in comparison with the participants' answers to the questionnaire, which they were asked to fill in when submitting results, reveals a better agreement among values from more experienced laboratories (Fig. 1). Results from non-experienced laboratories happen to contain all of the higher values and thus their spread is larger. Ten of the 24 participants declared analysing iAs on a routine basis, with 5 laboratories doing 0 to 50 samples *per* year and the other 5 between 50 and 250 samples *per* year.

Finally, it should be pointed out that EN 15517, the standard applied by many participants of these two PTs, was designed for the determination of iAs in seaweed and thus might not be suitable for the dogfish liver used here. A method was proposed in a recent publication by (Leufroy, Noël, Dufailly, Beauchemin, & Guérin, 2011) for the speciation of arsenic in seafood and thus might be an appropriate alternative. Generally speaking, there seems to be a lack of available validated methods for the determination of iAs in seafood and also of certified reference materials for iAs determination.

3.2. Participants' results for MeHg

Fourteen participants reported results for MeHg, of which 9 in the IMEP-30 and 5 in the IMEP-109. Again, the results were pooled in order to get a higher number of data and to detect potential tendencies. The results are presented in Fig. 2 with the Kernel density curve shown in the lower right hand corner.

Fig. 2 shows that the results are normally distributed around the assigned value (X_{ref}) within the $\pm 2\hat{\sigma}$ range. Most of the laboratories in these two ILCs were well able to analyse MeHg, as shown by the high number of satisfactory z-scores (Fig. 3). The lower share of satisfactory ζ-scores is attributed to unreliable measurement uncertainty statements by the laboratories.

The results were also compared to those of two previous ILCs analysing MeHg, IMEP-20 in tuna (Aregbe et al., 2004) and IMEP-104 in seafood (de la Calle et al., 2008), carried out in 2004 and 2008 respectively. Of the 235 participants in IMEP-20 only 3% (8 laboratories) reported results for MeHg. In the IMEP-104, it was 9% (4 participants out of 33), with one result reported as "less than" value. In the pooled IMEP-30 & 109 ILCs, 15% of participants have reported MeHg results. And thus, only a slight increase in the relative number of reported MeHg results took place since 2004.

Despite being only a limited number of laboratories, those who did measure MeHg in any of the four ILCs mostly achieved satisfactory z-scores, which means that laboratories are well able to measure MeHg with satisfactory results as can be seen in Fig. 4. Thus, the actual problem appears to be that there is only a small number of laboratories performing this type of analysis. The reason for that could be that laboratories do not have the required instrumentation (hyphenated techniques) to perform MeHg analysis, as was pointed out by some NRLs during discussions within their network on the MeHg issue.

However, information collected from the participants in the IMEP-30 & 109 exercises proved these assumptions being incorrect, as 6 out of the 14 participants did use other than hyphenated techniques and obtained good results. In particular, the authors would recommend laboratories to consider the following three methods:

(1) The method used by laboratory 7 (Fig. 2), where the samples are hydrolysed with hydrobromic acid followed by extraction with toluene and further separation of MeHg with cysteine. The detailed procedure can be found in the Final Report of the IMEP-109 exercise (Annex 14), which can be downloaded from the IRMM website (Internet-ILC) and is based on
(2) a procedure published by Scerbo and Barghigiani (1998). Both procedures do not require the use of any chromatographic set-up for the separation of MeHg from the other mercury species. Finally,
(3) a newly published method uses gas-liquid chromatography with electron capture detection (GLC-ECD) after dithizone extraction for the determination of MeHg in fish (Voegborlo, Matsuyama, Adimado, & Akagi, 2011) and according to the authors it was developed for the purpose of routine analysis.

These three methods are only a small selection and other alternatives can be found in relevant literature. There is a sufficient number of choices for laboratories to setup methods for MeHg analysis without the need of highly sophisticated instrumentation.

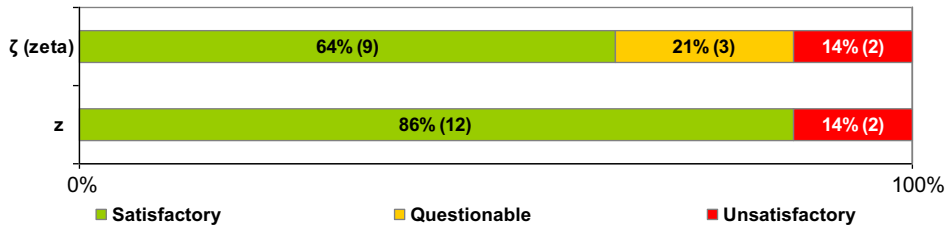

Fig. 3. Distribution of scores for the pooled results from IMEP-30 and IMEP-109.

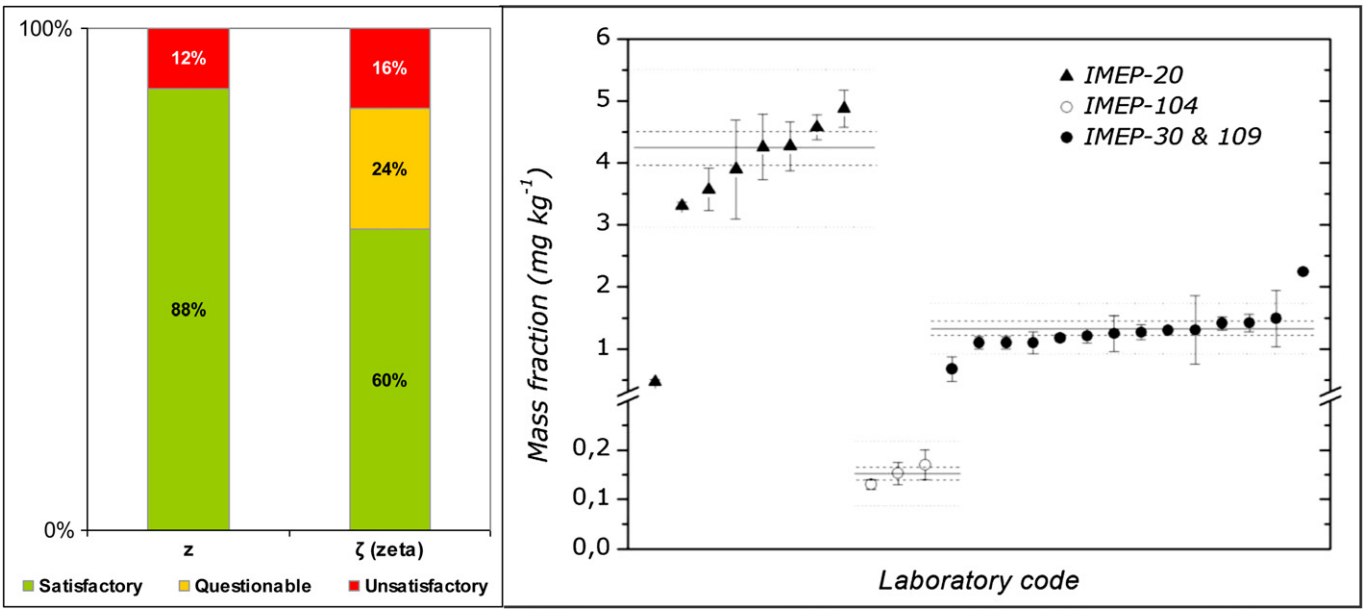

Fig. 4. Overview of all MeHg results reported since 2004.

4. Conclusion

By pooling IMEP-30 and IMEP-109 a total number of 95 participants reported results, considering all measurands of the two exercises. However, they were only 22 to report results for iAs (23%) and 14 for MeHg (15%). Although there were not many results, it was possible to make some observations which might help laboratories to improve their measurements or start measuring these compounds.

Considering iAs, the results were spread over a wide range, but 75% of the laboratories were agreeing that the iAs content does not exceed 0.25 mg kg^{-1} in the test material. Furthermore, there seems to be no clear clustering of results according to the methods used, despite the spread. This would confirm the results in IMEP-107 in that no method dependence was observed. However, the iAs was clearly more difficult to analyse in the seafood matrix than in rice, such as in the IMEP-107 exercise. This crucial information should be taken into account when fixing maximum levels in legislation.

Additionally, more research should be done in future to find appropriate and effective extraction procedures, as well as chromatographic conditions for reliable separation and quantification of iAs. Finally, the outcome should also be used as a trigger to obtain more data about measurements capabilities of iAs in other matrices.

With regard to the measurement of MeHg, the analytical performance as such does not seem to be a problematic issue, since the submitted results and resulting scores were satisfactory. It is rather the fact that only a limited number of laboratories carries out this type of analysis, despite recommendations on techniques given by IMEP in reports of former exercises. MeHg measurement can be done without sophisticated instrumentation, and laboratories are encouraged to verify the given references and test the proposed methods.

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4.4 Is it possible to agree on a value for inorganic arsenic in food? The outcome of IMEP-112

Is it possible to agree on a value for inorganic arsenic in food?
The outcome of IMEP-112

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ORIGINAL PAPER

Is it possible to agree on a value for inorganic arsenic in food? The outcome of IMEP-112

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Abstract Two of the core tasks of the European Union Reference Laboratory for Heavy Metals in Feed and Food (EU-RL-HM) are to provide advice to the Directorate General for Health and Consumers (DG SANCO) on scientific matters and to organise proficiency tests among appointed National Reference Laboratories. This article presents the results of the 12th proficiency test organised by the EU-RL-HM (IMEP-112) that focused on the determination of total and inorganic arsenic in wheat, vegetable food and algae. The test items used in this exercise were: wheat sampled in a field with a high concentration of arsenic in the soil, spinach (SRM 1570a from NIST) and an algae candidate reference material. Participation in this exercise was open to laboratories from all around the world to be able to judge the state of the art of the determination of total and, more in particular, inorganic arsenic in several food commodities. Seventy-four laboratories from 31 countries registered to the exercise; 30 of them were European National Reference Laboratories. The assigned values for IMEP-112 were provided by a group of seven laboratories expert in the field of arsenic speciation analysis in food. Laboratory results were rated with z and ζ scores (zeta scores) in accordance with ISO 13528. Around 85 % of the participants performed satisfactorily for inorganic arsenic in vegetable food and 60 % did for inorganic arsenic in wheat, but only 20 % of the laboratories taking part in the exercise were able to report satisfactory results in the algae test material.

Keywords Wheat · Vegetables · Algae · Inorganic arsenic

Introduction

From a toxicological point of view, speciation plays an important role in the case of arsenic: among the species

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found in food, inorganic As species (As(III) and As(V)) are more toxic to humans than the organic ones. The absorption and excretion of arsenic is affected by the type of arsenic compounds: Inorganic arsenic is more readily absorbed than organic arsenic, As (V) is excreted faster than As (III), and the organic As compounds ingested are excreted faster than the inorganic ones [1].

The highest total arsenic levels had been measured in the following food commodities: fish and seafood; products or supplements based on algae (especially hijiki, *Hizikia fusiformis*); and cereals and cereal products, with particularly high concentrations in rice grains, rice-based products, bran and germ. Nevertheless, in some of these food groups, the levels of inorganic arsenic (iAs) were low (e.g. fish and seafood) because in marine species, arsenic is found in the form of stable, non-toxic organic compounds such as arsenosugars and arsenobetaine.

According to the Scientific Opinion on As in food of the European Food Safety Authority Panel on Contaminants in the Food Chain [2], the following food subclasses were identified as the major contributors to the inorganic arsenic exposure in the general European population: cereal grains and cereal based products (approx. 50 % of total exposure), followed by foods for special dietary uses, bottled water, coffee and beer, rice grains and rice-based products, fish and vegetables.

In 2008, Meharg et al. [3] published data indicating that the intake of inorganic As via rice and rice-based baby food products by babies 4–12 months could be higher than the maximum exposures from drinking water predicted for adults, and that could negatively affect the health of these babies. For that reason, and because rice is a staple food and the only source of carbohydrate for many populations throughout the world, the European Union Reference Laboratory for Heavy Metals in Feed and Food (EU-RL-HM) organised in the second half of 2009 a proficiency test (PT) for the determination of total and inorganic arsenic in rice (IMEP-107). The main conclusion that could be derived from IMEP-107 was that the concentration of inorganic arsenic in rice determined is not dependent on the analytical method applied and that, purely from the analytical point of view, there was no reason not to consider the option of introducing possible maximum levels for inorganic arsenic in rice in further discussions on risk management.

At the European level, only one standard method has been published; it deals with the determination of inorganic arsenic in seaweed [4]. In China, a standard for the determination of total arsenic and abio-arsenic in foods exists since 2003 [5]. The Directorate General for Health and Consumers (DG SANCO) of the European Commission requested the EU-RL-HM to expand the study initiated with IMEP-107 to other food matrices and to evaluate the performance of European laboratories with regard to total and inorganic arsenic determinations in cereals (other than rice), vegetables and algae with a view to future discussions on the need for possible regulatory measures.

With that scope, the EU-RL-HM organised a PT (IMEP-112) on the determination of total and inorganic arsenic in wheat, vegetable food and algae that was open to all laboratories around the world with analytical capabilities in that field. As cereal, wheat was selected as being the most widely consumed grain in Europe and in most other countries where the diet is not rice-based. Arsenic is generally present at lower concentrations in wheat compared to rice, but it appears to be present almost exclusively as iAs [6].

This paper summarises and discusses the outcome of IMEP-112.

Test materials

The processing of the three test materials used in IMEP-112 was as follows:

- *Wheat*: Twenty-eight kilos of wheat, sampled in an area with a high content of arsenic in the soil, was provided by Dr. F. Cubadda (Istituto Superiore di Sanità in Rome, Italy). The material was dispatched by courier and, upon arrival at the Institute for Reference Materials and Measurements (IRMM), was stored at −20 °C until further processing. The material was sieved on a 5-mm sieve (model 17300, Russell Finex industrial sieve, London, UK) to remove the coarser pieces (mainly straw). The fine fraction was sieved on the same machine with a 2-mm sieve whilst sucking the lighter fraction away (chaff) with a vacuum cleaner. In that way, 27 kg of wheat grains were collected. The grains were divided over six plastic drums placed in liquid nitrogen for pre-cooling. They were fed using a vibrating feeder into a cryogenic vibrating mill (Palla VM-KT, Humboldt-Wedag, Köln, Germany) cooled down to −196 °C prior to milling. Each milling cycle lasted about 45 min from −196 °C to about −90 °C. Milling was then interrupted and the mill was cooled again. Nineteen kilograms of ground wheat grains were collected and stored at −20 °C. The material was then sieved on a 500-μm sieve. The fraction below 500 μm was kept and homogenized for 30 min in a three-dimensional mixer (WAB, Dynamix CM-200, Basel, Switzerland). The homogenised product was tested for its particle size distribution using laser diffraction, where it was confirmed that the top particle size was below 610 μm, X_{50} was about 70 μm and X_{90} approximately 200 μm. The water content was determined by heating at 105 °C (Sartorius MA150, Göttingen, Germany), and it was 12 % (*m*/

m). Such a high value is expected for materials like wheat and rice flours. Thereafter, 20-g powder portions were filled in 60-mL bottles with a PE insert and screw cap.

- *Vegetable food*: The commercially available SRM 1570a (spinach leaves) produced by the National Institute for Standards and Technology (NIST, Gaitherburg, MD, USA) was used for this PT. NIST dispatched 30 bottles of test materials at room temperature by courier to IRMM. The material was rebottled and relabelled to avoid identification by the participants. Comprehensive information on the preparation of this material can be found in the certification report on the NIST web site (https://www-s.nist.gov/srmors/view_detail.cfm?srm=1570A).
- *Algae*: Sixty kilograms of seaweed (*Fucus vesiculosus*) were placed on nylon sieves placed over polytetrafluoroethylene (PTFE)-coated trays in an Elbanton drying cabinet (Elbanton, Kerkdriel, the Netherlands) at a temperature of 26 °C and spread out. The material was manually moved in the tray now and then to achieve a more uniform drying of the material. After 2 weeks of drying, the material was taken out of the drying cabinet. Thereafter, the dry material (11.18 kg) was stored in a plastic drum. Once the seaweed had been dried and crushed, it was placed in stainless steel drums which were immersed in liquid nitrogen overnight. One by one, the drums were kept in Dewar vessels just prior to milling and then transferred to the cryogenic mill. Thereafter, the seaweed was scooped up and fed slowly into a Palla vibrating mill (KHD Humboldt Wedag, Köln, Germany), which previously had been cooled to −196 °C with liquid nitrogen. Since the machine parts in contact with the material are made of high-purity titanium, no contamination with other trace metals should occur. The obtained powder was sieved on a 125-μm nylon sieve using a Russel Finex Industrial sieve (model 17300, London, UK). The fraction <125 μm of the sieved powder was spread over ten Teflon-coated trays (600–800 g on each) and placed in a freeze drier (Epsilon 2-D 85 Martin-Christ, Osterode, Germany). After freeze drying, the homogenisation of the sample was carried out. The homogenisation of 6.3 kg of vacuum-dried material was performed with a three-dimensional mixer in one run of 0.5 h on a Dyna-MIX CM200 mixer (WAB). Filling the seaweed powder into 20-mL amber glass vials was performed using an All Fill automatic filling machine (All Fill, Sandy, UK). The hopper containing the material (a large funnel) inside the filling machine and the auger (feeding screw) were of stainless steel. A positive ion blower (Sartorius/Ion-care, Malmö, Sweden) was installed about 4 cm from the filling point. Under these conditions, each vial was reproducibly filled with slightly more than 5.0 g, as programmed. Once the vials were filled, lyo-inserts were automatically pressed down in the neck of the vials by the filling machine. The vials were flushed with nitrogen before and after filling; the hopper was also continuously flushed with nitrogen, providing an inert atmosphere above the material.

All vials were acid-washed (2 % HNO_3 solution in water, *m*/*m*) and rinsed with Milli-Q water before drying in a drying cabinet as to remove possible point contamination of the glassware.

Homogeneity and stability studies

In wheat, the measurements for the homogeneity and stability studies were performed by the Food and Environment Research Agency (Fera, York, UK). Homogeneity was evaluated according to ISO 13528 [7]. The material was proven to be homogeneous for total and inorganic arsenic. The methods used by Fera for the homogeneity and stability tests are those used by the same laboratory to assign a reference value for this material, described later on in "Reference values and their uncertainties". The contribution from homogeneity, u_{bb}, to the uncertainty of the reference value, u_{ref}, was calculated using SoftCRM (see www.softCRM.com).

In wheat, the stability study of the test item was conducted following the isochronous approach [8]. The evaluation of the stability of the test item was made using the software SoftCRM [9]. The material was proven to be stable, even at 60 °C for the 5 weeks that elapsed between the dispatch of the samples and the deadline for the submission of results, for both total and inorganic arsenic. The analytical results and statistical evaluation of the homogeneity and stability studies are provided in Annex 7 of the report to participants [10].

No homogeneity/stability tests were organised for the purpose of IMEP-112 for the vegetable food and algae because according to the producers of those test materials (NIST and IRMM), the materials are homogenous and stable.

Instructions to participants

Laboratories were asked to perform two or three independent measurements and to report the mean of the results and its associated uncertainty. The measurement results were to be corrected for moisture (following a procedure described in the accompanying letter which had been cross-checked by Karl-Fisher titration at IRMM) and for recovery. Participants were asked to follow their routine procedures for the analysis of total and inorganic arsenic, respectively. The

results were to be reported in the same manner (e.g. number of significant figures) as those normally reported to the customers.

The results were to be reported in a special on-line form for which each participant received an individual access code. A specific questionnaire was attached to this on-line form. The questionnaire was intended to provide further information on the measurements and the laboratories. The measurands and matrix were defined as the "total and inorganic As in wheat, vegetable food and algae".

Reference values and their uncertainties

The NIST certificate provided the assigned value for total As in the vegetable food. The assigned value for total As in the algae test material was provided by the Studiecentrum voor Kernenergie (SCK-CEN) using neutron activation analysis, using the method described in a previous publication by de la Calle et al. [11].

The remaining assigned values for total As and iAs (total As and iAs in wheat, iAs in vegetable food and in algae) were provided by a group of laboratories expert in the field of arsenic speciation analysis in food. The experts were asked to use the method of their choice and no further requirements were imposed regarding methodology. The experts were also asked to report their results together with the measurement uncertainty and with a clear and detailed description on how uncertainty was calculated. The mean of the independent means provided by the expert laboratories for total and inorganic arsenic was used as the assigned values (X_{ref}) for this PT according to ISO Guide 35. The standard uncertainties (u_{ref}) associated with the assigned values were calculated using Eqs. 1 and 2.

$$u_{ref} = \sqrt{u_{char}^2 + u_{bb}^2 + u_{sts}^2} \quad (1)$$

where u_{ref} is the standard uncertainty associated with the assigned value, u_{char} is the standard uncertainty of characterisation by expert laboratories, u_{bb} is the standard uncertainty contribution for the between-bottle homogeneity and u_{st} is the standard uncertainty contribution derived from the stability study.

u_{char} is calculated according to ISO Guide 35.

$$u_{char} = \frac{SD_{ExpertMeans}}{\sqrt{n}} \quad (2)$$

where $SD_{ExpertMeans}$ is the standard deviation of the means reported by the expert laboratories and n is the number of expert laboratories.

u_{bb} and u_{st} were set to zero for the vegetable food on the basis of the information given in the certificate by NIST. The u_{bb} and u_{st} for the algae test material were provided by IRMM. The means reported by the expert laboratories (certifiers) and their associated uncertainties (u_{char}) for total and inorganic arsenic are listed in Tables 1 and 2 together with the assigned values and their respective uncertainties.

The results reported by Cert. 5 for iAs in wheat and algae did not overlap with the results reported by the other certifiers, within their respective uncertainties. Concerning wheat, Cert. 5 reported a recovery factor for iAs of 65 %. When looking at the provided chromatogram, only one peak corresponding to As(V) and some traces of dimethylarsinate (DMA) could be observed. Cert. 5 also reported having had problems with carbon interferences in the determination of iAs.

As for algae, it was not possible to explain the discrepancy between Cert. 5 and the other certifiers by the mass balance due to the large amount of arsenic species (mainly organic) present in the algae test material. An alternative attempt was made to identify which step in the method of analysis used by Cert. 5 when analysing iAs could explain the bias observed for the wheat and algae results. The methods used by the certifiers in the determination of total and inorganic arsenic are summarised in Tables 3 and 4, respectively. Whilst most of the certifiers using HPLC-based methods added H_2O_2 before the microwave extraction to enhance the efficiency of the digestion, Cert. 5 added H_2O_2 after the microwave extraction, with the only purpose of

Table 1 Assigned vales for total As and their associated expanded uncertainties (in milligrams per kilogram)

Certifier	Wheat $X_n \pm U_n$	Vegetable food $X_n \pm U_n$	Algae $X_n \pm U_n$
Certifier 1	0.188±0.024		
Certifier 2	0.178±0.008		
Certifier 3	0.195±0.037		
Certifier 4	0.157±0.005		
Certifier 5	0.175±0.003		
Certifier 6	0.179±0.011		
Certifier 7	0.166±0.009		
X_{ref}	0.177	0.068	58.3
u_{char}	0.005	0.006	1.4
u_{bb}	0.003	0	0.9
u_{st}	0.002	0	3.1
u_{ref}	0.006	0.006	3.5
U_{ref} (k=2)[a]	0.012	0.012	7.0
$X_{ref} \pm U_{ref}$[a]	0.177±0.012	0.068±0.012	58.3±7.0

[a] U_{ref} is the estimated associated expanded uncertainty with a coverage factor k, corresponding to a level of confidence of about 95 %

Table 2 Assigned vales for iAs and their associated expanded uncertainties (in milligrams per kilogram)

Certifier	Wheat $X_n \pm U_n$	Vegetable food $X_n \pm U_n$	Algae $X_n \pm U_n$
Certifier 1	0.183±0.024	0.038±0.005	0.161±0.021
Certifier 2	0.176±0.010	0.075±0.004	0.205±0.035
Certifier 3	0.194±0.025	0.074±0.010	0.194±0.025
Certifier 4	0.154±0.003	0.060±0.002	0.190±0.010
Certifier 5		0.055±0.003	
Certifier 6	0.156±0.022	0.034±0.005	<0.100
Certifier 7	0.152±0.010	0.045±0.003	0.188±0.029
X_{ref}	0.169	0.054	0.188
u_{char}	0.007	0.006	0.007
u_{bb}	0.006	0	0.003
u_{st}	0.008	0	0.010
u_{ref}	0.012	0.006	0.013
U_{ref} (k=2)[a]	0.025	0.012	0.025
$X_{ref} \pm U_{ref}$[a]	0.169±0.025	0.054±0.012	0.188±0.025

[a] U_{ref} is the estimated associated expanded uncertainty with a coverage factor k, corresponding to a level of confidence of about 95 %

oxidising As(III) to As(V). This could explain the bias of the results reported by Cert. 5 for iAs in wheat and algae. It could be argued that the addition of H_2O_2 before the microwave digestion could induce the degradation of the organic arsenic species present in the algae. According to Cert. 7 which performed a thorough investigation on the effect of several extraction conditions on the different species of arsenic, organic species could degrade down to DMA in the presence of H_2O_2, but no further degradation into iAs would occur.

It was therefore decided not to use the results reported by Cert. 5 for iAs in wheat and algae when establishing X_{ref}. To avoid confusion, the results reported by Cert. 5 for wheat and algae are not shown in this manuscript. Since the bias of Cert. 5 in wheat and algae were matrix-related and no bias was detected in the results reported by that certifier for iAs in vegetable food, its results were included in the calculation of X_{ref} in that matrix.

Cert. 6 reported "less than 0.100 mg kg^{-1}" for iAs in algae; the same laboratory could not detect iAs in seafood in a previous exercise (IMEP-109) [12], and so its results for iAs in algae were not taken into consideration since the problem occurring in IMEP-109 seemed to persist.

The results reported by the certifiers are shown in Figs. 1, 2 and 3 together with the results reported by the other participants. The results of the certifiers are placed on the left-hand side of the graphs. In the case of iAs in vegetable food, two certifiers are outside the range $X_{ref} \pm U_{ref}$, which, from a pure metrologic point of view, is not fully orthodox. The higher scatter of results in this matrix should be interpreted whilst keeping in mind that the certified value for total As as given in the certificate SRM 1570a has an associated uncertainty of 17 %. The uncertainty for iAs is unlikely to be lower, and both uncertainties are probably related to the low mass fractions in that material.

For years, a debate has taken place within the scientific community on whether the iAs fraction in food commodities was dependent or not on the method used to perform the analysis. The expert laboratories that participated in the establishment of the assigned values in IMEP-112 used various methods of analysis (with the exception of Cert. 1 and Cert. 3 which applied the same approach for the sample pretreatment, although with a different instrumental setup, for the determination of inorganic As; Table 4). Nevertheless, all the results agree within a range of about 14 % for wheat, 22 % for vegetable food and 10 % for algae (95 % confidence interval). This indicates that the concentration of iAs is not method-dependent in those matrices.

Evaluation of the results reported by the laboratories taking part in IMEP-112

Seventy-four laboratories from 31 countries registered to the exercise, as shown in the report to participants [10], of which 65 reported results. The precise number of results sets per measurand and matrix can be seen in Table 5. The table also lists the number and percentages of participants having reported "less than" values. Those were not included in further data evaluations.

Scoring and evaluation criteria

Individual laboratory performance is expressed in terms of z and ζ scores in accordance with ISO 13528 [7].

Table 3 Methods used by the expert laboratories for sample pretreatment in the determination of total As

ID	Sample treatment	Technique
1	0.25–1 g of the sample was weighed into a tall heat-resistant glass beaker (250 mL), treated with 2.5 mL of ashing aid suspension (20 %, *w/v*, $MgNO_3$+2 %, *w/v*, MgO) and 5 mL of nitric acid (7 molL^{-1}). The mixture was evaporated to dryness in a sand bath and placed in the muffle furnace at an initial temperature not higher than 150 °C. The temperature was increased to 425±25 °C at a maximum rate of 50 °C/h and maintained for 12 h. The mineralization procedure was repeated until the sample was completely incinerated. For this purpose, 5 mL nitric acid (7 molL^{-1}) was added, the mixture was evaporated in the sand bath, and the ashes were again placed in the muffle furnace, i.e. the ashes had to be white/grey or slightly coloured. The white ash obtained was dissolved in 6 molL^{-1} HCl and reduced with a pre-reducing solution (5 %, *w/v*, KI and 5 %, *w/v*, ascorbic acid). After 30 min, this solution was filtered through Whatman no. 1 filter paper into a volumetric flask and diluted to volume with 6 molL^{-1} HCl. The arsenic was quantified by FI-HG-AAS using the following instrumental conditions: loop sample, 0.5 mL; reducing agent, 0.2 % (*w/v*) $NaBH_4$ in 0.05 % (*w/v*) NaOH, 5-mLmin^{-1} flow rate; HCl solution, 10 % (*v/v*), 10-mLmin^{-1} flow rate; carrier gas, argon, 100-mLmin^{-1} flow rate; wavelength, 193.7 nm; spectral band-pass, 0.7 nm; electrodeless discharge lamp system 2, lamp current setting 400 mA; cell temperature, 900 °C	FI-HG-AAS
2	0.35 g of sample (0.2 g for the algae) was placed in the digestion vessels with a mixture of 2.5 mL of concentrated HNO_3 and 0.5 mL of H_2O_2, covered and let stand overnight in a clean air hood at ambient temperature (pre-digestion). The next day, the samples were placed in the digestion system, the temperature was raised to 180 °C within 37 min and held for 15 min. The digested samples were cooled to room temperature, transferred to polypropylene test tubes and diluted to 10,000 g with water. The total As concentration was determined by ICP-MS in the DRC mode using H_2 as the reaction gas (10 % in Ar, flow 0.35 mLmin^{-1}, RPq 0.4) and ^{75}As as the analytical mass. It enabled overcoming any bias arising from the $^{40}Ar^{35}Cl$ interference. Quantification was performed by the method of standard additions using rhodium (1 μgL^{-1}) as the internal standard.	ICP-MS
3	Aliquots of the test sample and certified reference material were digested in 5 mL nitric acid using quartz high-pressure vessels and microwave heating; then, the resulting solution was diluted to 10 mL with pure water. A further tenfold dilution with dilute nitric acid containing rhodium was completed just prior to measurement by ICP-MS using collision cell technology (helium mode).	ICP-MS
4	All the samples were digested with HNO_3 solution (diluted 1:1 with doubly deionised water) and H_2O_2, under microwaves closed system. For the digestion, 0.5-g aliquots of the wheat and vegetable samples (0.25 of algae samples) were weighed in the digestion vessels, and 8 mL of 1:1 nitric acid solution and 2 mL of hydrogen peroxide were added. Mixtures were digested according to the following programme: 10 min from room temperature to 90 °C, maintained for 5 min at 90 °C, 10 min from 90 to 120 °C, 10 min from 120 to 190 °C and 20 min maintained at 190 °C. After cooling to room temperature, the digested samples were filtered through ash-free filter papers 7 (Whatman 40) and diluted in water up to 20 mL. For the final measurements, further dilution was carried out if it necessary. Measurements were carried out by ICP-MS with He as the gas in the collision cell to remove interferences. ^{103}Rh was used as the internal standard.	ICP-MS
5	Approximately 0.1 g of the sample is weighed out into a 50-mL polypropylene digest tube and 2 mL of conc. HNO_3 is added; the mixture is left to steep overnight. 2 mL conc. hydrogenperoxide is added before the samples are digested in a microwave oven. The temperature program is: first to 55 °C (and held for 5 min) then to 75 °C (and held for 5 min). Finally, the digest is taken up to 95 °C and maintained for 30 min. Samples are cooled to room temperature and diluted to a mass of 20 g (for wheat and vegetable matter) or 50 g (for algae, further diluted 1:10) with ultrapure deionised water. Quality controls of CRM and blanks are run with each digest set. Samples from the extraction are treated the same way, with the difference that 1 mL extract is mixed with 1 mL conc. HNO_3 and the sample is filled up to 5 g (wheat and vegetable matter, algae: 0.5 mL to 15 g).	ICP-MS
6	0.500 g was weighed into high-pressure (70 bar) quartz containers and 5 mL of concentrated nitric acid was added. The solutions were then digested in a microwave oven, left to cool and diluted with ultrapure Milli-Q water to approximately 20 g. The density of the solution was calculated by weighing of 1 mL solution. The solution was further diluted with Milli-Q water prior to analysis (dilution factor, 1.6) by ICP-MS. To all samples and blanks, rhodium (^{103}Rh) was added as an internal standard (at 1 μg/L).	ICP-MS
7	0.250 g of powder (wheat and vegetable food) was weighed with a precision of 0.1 mg and mineralised in an ultraclave microwave digestion system. The powders were transferred to 12-mL quartz tubes and were mineralised with 2 mL nitric acid and 2 mL H_2O. The tubes were transferred to a Teflon® rack, covered with Teflon® caps, and then the rack was mounted in the microwave system. An Ar pressure of 4×10^6Pa was applied and the mixture was heated to 250 °C for 30 min. After mineralisation, the samples were diluted with water to 9.0 mL (based on weight) in polypropylene tubes. Finally 1 mL of a solution containing 50 % methanol (to enhance the arsenic response) and 100 μgL^{-1} each of Ge and In as internal standards were added to all digested samples, giving a final concentration of 5 % methanol and 10 μgL^{-1} of Ge and In. For the determination of total arsenic in algae samples, the procedure was similar, with the exception that 0.250 mg of algae was digested with 5 mL nitric acid. The digested solutions were diluted with water to 45.0 mL (based on weight). Finally, 5 mL of a solution containing 50 % methanol (to enhance the arsenic response) and 100 μgL^{-1} each of Ge and In as internal standards were added to all digested samples, giving a final concentration of 5 % methanol and 10 μgL^{-1} of Ge and In. All standards for total arsenic, determinations were prepared with 20 % nitric acid and also 5 % methanol for matrix matching with the digested samples.	ICP-MS

$z = \frac{x_{lab} - X_{ref}}{\hat{\sigma}}$ and $\zeta = \frac{x_{lab} - X_{ref}}{\sqrt{u_{ref}^2 + u_{lab}^2}}$ where x_{lab} is the measurement result reported by a participant, X_{ref} is the reference value (assigned value), u_{ref} is the standard uncertainty of the reference value, u_{lab} is the standard uncertainty reported by a participant and $\hat{\sigma}$ is the standard deviation for proficiency assessment.

Table 4 Methods used by the expert laboratories for sample pretreatment in the determination of iAs

ID	Sample treatment	Technique
1	Lyophilized sample (0.5–1 g) was weighed into a screw-top centrifuge tube. Then, 4.1 mL of water was added and agitated until the sample was completely moistened. After that, 18.4 mL of concentrated HCl was added and the sample was agitated again for 5 min. It was left to stand for 12–15 h (overnight). The reducing agent (2 mL of HBr and 1 mL of hydrazine sulphate) was added and the sample was agitated for 30 s. Then, 10 mL of $CHCl_3$ was added and the sample agitated for 5 min. The phases were separated by centrifuging at 2,000 rpm for 5 min. The chloroform phase was separated by aspiration and then poured into another tube. The extraction process was repeated two more times. The chloroform phases were combined and centrifuged again. The remnants of the acid phase were eliminated by aspiration. Possible remnants of organic material in the chloroform phase were eliminated by passing it through Whatman GD/X syringe filters with a 25-mm PTFE membrane. The inorganic arsenic in the chloroform phase was back-extracted by agitating for 5 min with 10 mL of HCl (1 mol L^{-1}). The phases were separated by centrifuging (2,000 rpm for 5 min), and the aqueous phase was aspirated and poured into a beaker. This step was repeated once more and the back-extraction phases obtained were combined. For the determination of inorganic arsenic, 2.5 mL of ashing aid suspension (20 %, *w/v*, $Mg(NO_3)\square 6H_2O$ and 2 %, *w/v*, MgO) and 10 mL of nitric acid (14 mol L^{-1}) were added to the combined back-extraction phases. This was evaporated to dryness in a sand bath and placed in the muffle furnace at an initial temperature not higher than 150 °C. The temperature was increased to 425±25 °C at a maximum rate of 50 °C/h and maintained for 12 h. The white ash obtained was dissolved in 6 mol L^{-1} HCl and reduced with a pre-reducing solution (5 %, *w/v*, KI and 5 %, *w/v*, ascorbic acid). After 30 min, this solution was filtered through Whatman no. 1 filter paper into a volumetric flask and diluted to volume with 6 mol L^{-1} HCl.	FI-HG-AAS
2	0.35 g of the sample was added with 10 mL of 1 % (*v/v*) HNO_3 and 1 % H_2O_2 and left to stand overnight. Microwave irradiation was performed 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 75 °C, 2 min ramp to 95 °C, 30 min at 95 °C. The extracts were centrifuged (10 min, 8,000 rpm, 7 °C) and the supernatants filtered through a 0.22-mm filter	HPLC-ICP-MS
3	Aliquots of test sample and "in-house" certified reference material were solubilised overnight with concentrated HCl then HBr and hydrazine sulphate added prior to chloroform extraction. The chloroform extract was back-extracted into 1 mol L^{-1} HCl and this solution was directly measured by ICP-MS.	ICP-MS
4	For speciation analysis, 0.4 g aliquots of the wheat and vegetable food samples and 0.25 g of the algae sample were weighed in the PTFE vessels and were extracted adding10 mL of 0.2 % (*w/v*) nitric acid and 1 % (*w/v*) hydrogen peroxide solution using 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 was taken up to 95 °C and maintained for 30 min. The samples were cooled down to room temperature and centrifuged at 3,000 rpm for 12 min. The supernatant was filtered through PET filters (pore size, 0.45 μm).	HPLC-ICP-MS
5	Milled subsamples (0.5 and 0.25 g for algae) are weighed into polypropylene vials and mixed with 10 mL 1 % HNO_3. The mixture is allowed to stand overnight. The mixture is heated 10 min at 50 °C, 10 min at 75 °C and 20 min at 95 °C in a temperature-controlled microwave. After cooling, the mixture will be centrifuged, 1 % (*v/v*) hydrogen peroxide will be added and the sample stored at −20 °C before analysis for speciation or total arsenic content.	HPLC-ICP-MS
6	For the determination of inorganic arsenic, subsamples of approximate 0.25 g were weighed into microwave quartz containers and 10.00 mL of 0.07 M hydrochloric acid (Merck) in 3 % hydrogen peroxide was added. The solutions were placed in the microwave oven and the power was programmed to keep the solutions at 90 °C for 20 min. By this procedure, the inorganic arsenic is extracted from the sample matrix and furthermore As(III) is oxidized to As(V), thus allowing for the determination of total inorganic arsenic as As(V). Then, the solutions were allowed to cool to room temperature and the supernatant transferred to 15-mL plastic tubes and centrifuged at approximately 4,000 rpm for 10 min and subsequently filtered (0.45 μm) prior to analysis.	HPLC-ICP-MS
7	For the extraction, about 250 mg of powder (50, 100 and 200 mg for micro-homogeneity studies) was weighed with a precision of 0.1 mg into 12-mL quartz tubes, 5 mL of 0.02 mol L^{-1} trifluoracetic acid containing 1 % (*v/v*) of a 30 % H2O2 solution was added, and the suspension was sonicated for 15 min. Samples were microwave-extracted with an ultraclave microwave digestion system. The tubes were transferred to a Teflon® rack, covered with Teflon® caps, and then the rack was mounted in the microwave system. After closing the system, an argon pressure of 4×10^6 Pa was applied. Extraction was done using a one-stage temperature ramping program ramping to 95 °C over 10 min and maintaining the temperature for 60 min. After cooling to room temperature, the extracts were transferred to polypropylene tubes and centrifuged for 15 min at 8,900 rcf.	HPLC-ICP-MS

The assigned reference values (X_{ref}) and their respective uncertainties are summarised in Tables 1 and 2. The interpretation of the z and ζ scores is done as follows:

|score|≤2	Satisfactory result
2<|score|≤3	Questionable result
|score|>3	Unsatisfactory result

The ζ score denotes whether the laboratory result agrees with the assigned value within the respective uncertainty. The denominator is the combined uncertainty of the assigned value and the measurement uncertainty as stated by the laboratory. The ζ score is therefore the most relevant evaluation parameter as it includes all parts of a measurement result, namely the expected value (assigned value), its uncertainty and the unit of the result as well as the uncertainty of the reported

values. An unsatisfactory ζ score can either be caused by an inappropriate estimation of the concentration or of its uncertainty or both.

The standard uncertainty of the laboratory (u_{lab}) was estimated by dividing the reported expanded uncertainty by the reported coverage factor, k. When no uncertainty was reported, it was set to zero ($u_{lab}=0$). When k was not specified, the reported expanded uncertainty was considered as the half-width of a rectangular distribution; u_{lab} was then calculated by dividing this half-width by $\sqrt{3}$, as recommended by Eurachem and CITAC [13].

Uncertainty estimation is not trivial; therefore, an additional assessment was provided to each laboratory reporting uncertainty, indicating how reasonable their uncertainty estimate is. The standard uncertainty from the laboratory (u_{lab}) is most likely to fall in a range between a minimum uncertainty (u_{min}) and a maximum allowed (u_{max}). u_{min} is set to the standard uncertainty of the reference value. It is unlikely that a laboratory carrying out the analysis on a routine basis would measure the measurand with a smaller uncertainty than the expert laboratories chosen to establish the assigned value. u_{max} is set to the target standard deviation ($\widehat{\sigma}$) accepted for the PT. If u_{lab} is smaller than u_{min}, the laboratory may have underestimated its uncertainty. Such a statement has to be taken with care as each laboratory reported only the measurement uncertainty, whereas the uncertainty of the reference value also includes the contributions of homogeneity and stability. If those are large, measurement uncertainties smaller than u_{min} are possible and plausible. If $u_{lab}>u_{max}$, the laboratory may have overestimated the uncertainty. An evaluation of this statement can be made when looking at the difference of the reported value and the assigned value: If the difference is small and the uncertainty is large, then overestimation is likely. If, however, the deviation is large but is covered by the uncertainty, then the uncertainty is properly assessed, but large. It should be pointed out that u_{max} is not a normative criterion: It is up to the customer of the respective result to decide which uncertainty is acceptable for a certain measurement.

The z score compares the participant's deviation from the reference value with the target standard deviation for proficiency assessment ($\widehat{\sigma}$) used as a common quality criterion. $\widehat{\sigma}$ is defined by the PT organiser as the maximum acceptable standard uncertainty. Values for $\widehat{\sigma}$ in IMEP-112 were set to:

- 15 % for the total and inorganic arsenic in wheat. Fifteen percent was proven to be a sound target standard deviation in IMEP-107 on total and inorganic arsenic in rice, a matrix similar to wheat.
- 22 % for the total As and 25 % for iAs in vegetable food. The uncertainty associated with the certified value (total As) as provided by NIST was 17 %. The standard deviation of the means provided by the experts (u_{char}) was 23 % (iAs). Such a high $\widehat{\sigma}$ reflects the difficulty in analysing relatively low concentrations of total As and iAs.
- 15 % for the total As and 22 % for iAs in algae, to account for the high complexity of the determination of iAs in this type of samples due to the complex distribution of species in marine matrices.

Laboratory results and scorings

The results as reported by the participants for total and inorganic arsenic in wheat, vegetable food and algae are summarised in Figs. 1, 2 and 3. These figures also include the individual mean values, associated expanded uncertainties and the Kernel distribution plots, obtained using a software tool developed by AMC [14]. National Reference Laboratories are marked with an asterisk in Figs. 1, 2 and 3.

Regarding the z and ζ scores, the results for total and inorganic arsenic in wheat, vegetable food and algae are summarised in Fig. 4. Considering the z score, between 75 and 85 % of the participants performed satisfactorily for total As. For iAs, about 60 and 75 % of the participants reported satisfactory results for wheat and vegetable food, respectively, despite the relatively low concentration of iAs in vegetable food. However, <20 % of the participants scored satisfactorily for iAs in algae. The distribution of satisfactory results reported for the three test materials included in this exercise could reflect the difficulty introduced by the different matrices.

The percentage of satisfactory ζ scores is even lower than for the z scores, which points to the fact that laboratories presumably have problems in estimating the correct uncertainty of their results.

Wheat

Although it could be thought that wheat would behave in a similar way to rice, this exercise provided evidence that the determination of iAs in this matrix may require some extra care in the extraction step. For instance, H_2O_2 needs to be added before the microwave extraction of the sample to improve the efficiency of the treatment, whilst for rice, H_2O_2 is only needed to oxidise As(III) to As(V). Several of the laboratories that underestimated iAs in wheat did not add H_2O_2 during

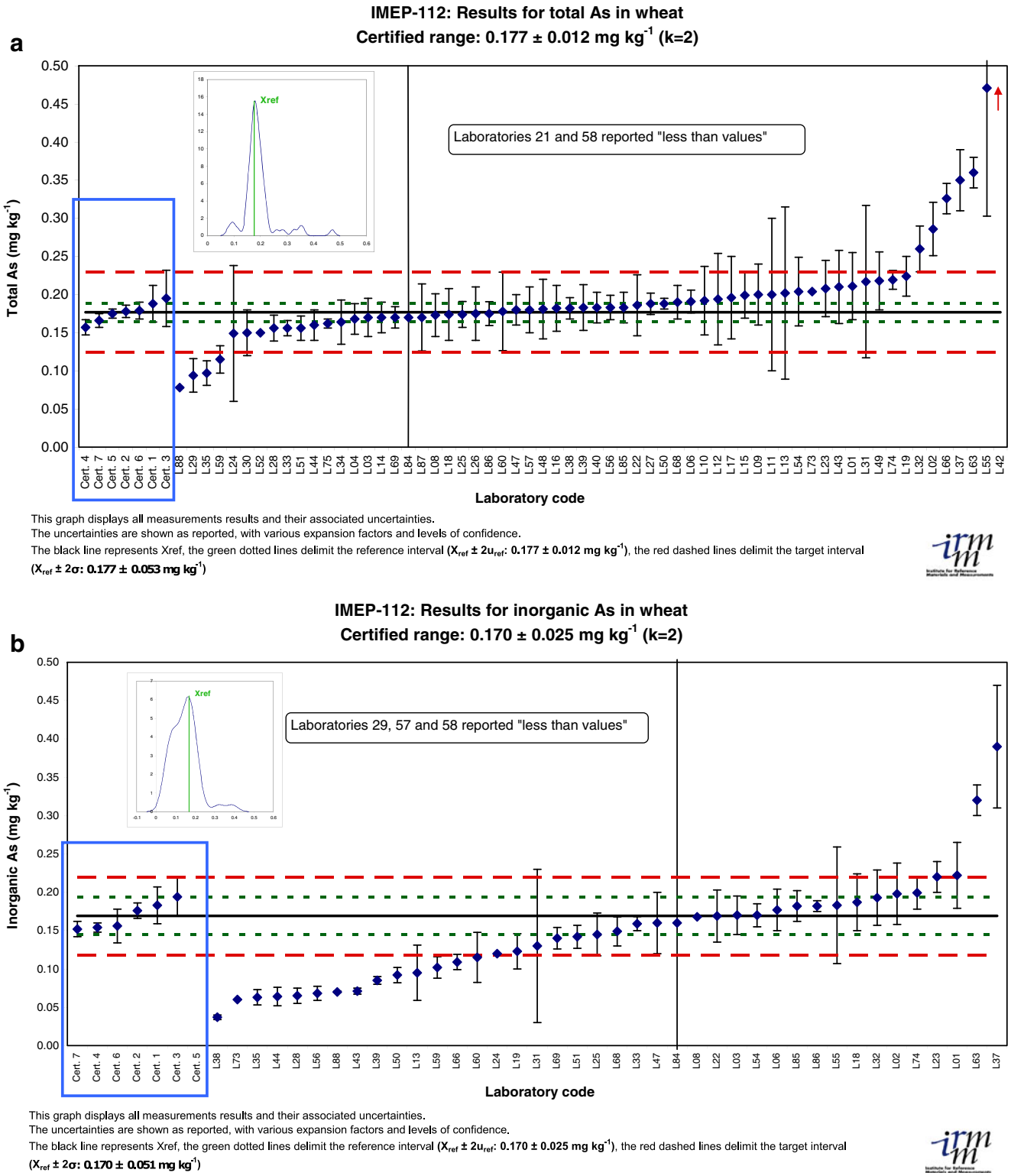
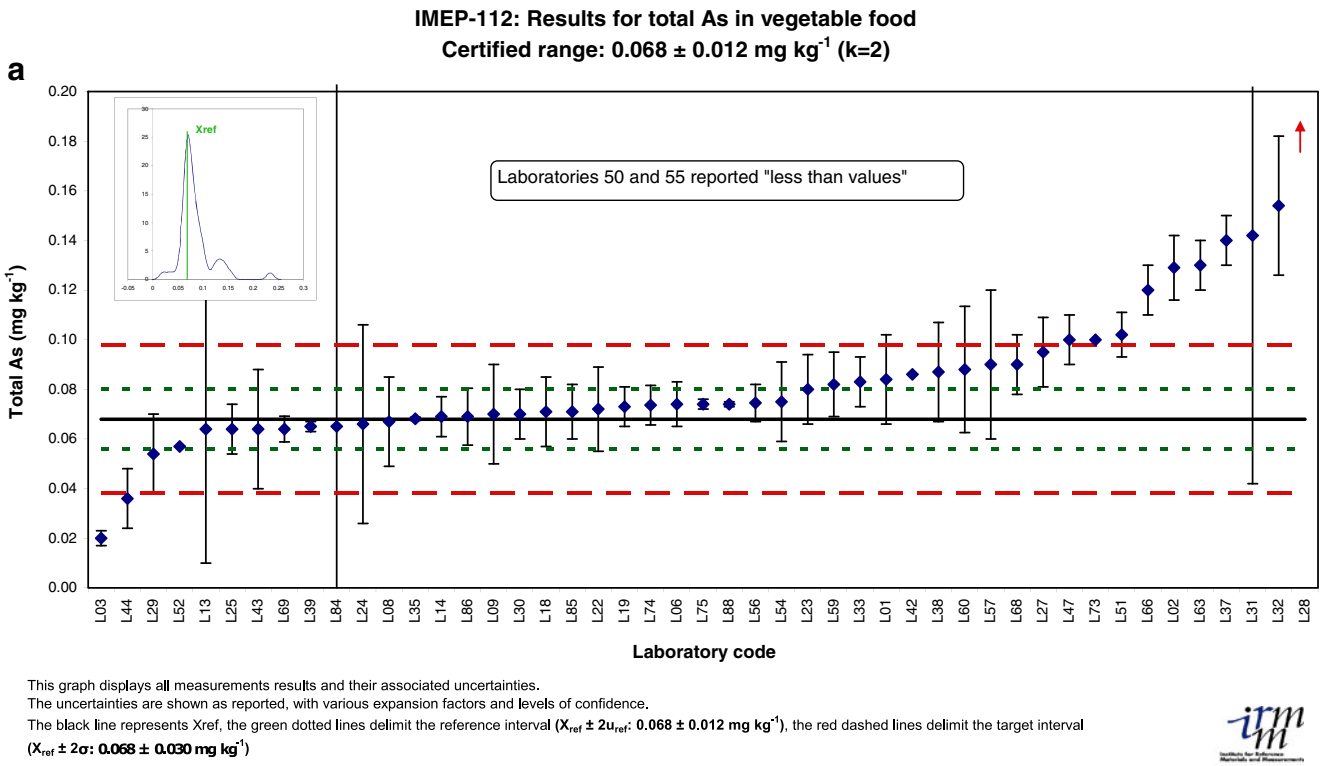


Fig. 1 Results reported by certifiers and participants in IMEP-112 for the wheat: total As (**a**) and iAs (**b**)

the digestion of the matrix (see L35, L38, L39, L50, L73 and L88). Of course, this is not the only parameter that may play a role in the quantitative determination of iAs, and some laboratories that did not add H_2O_2



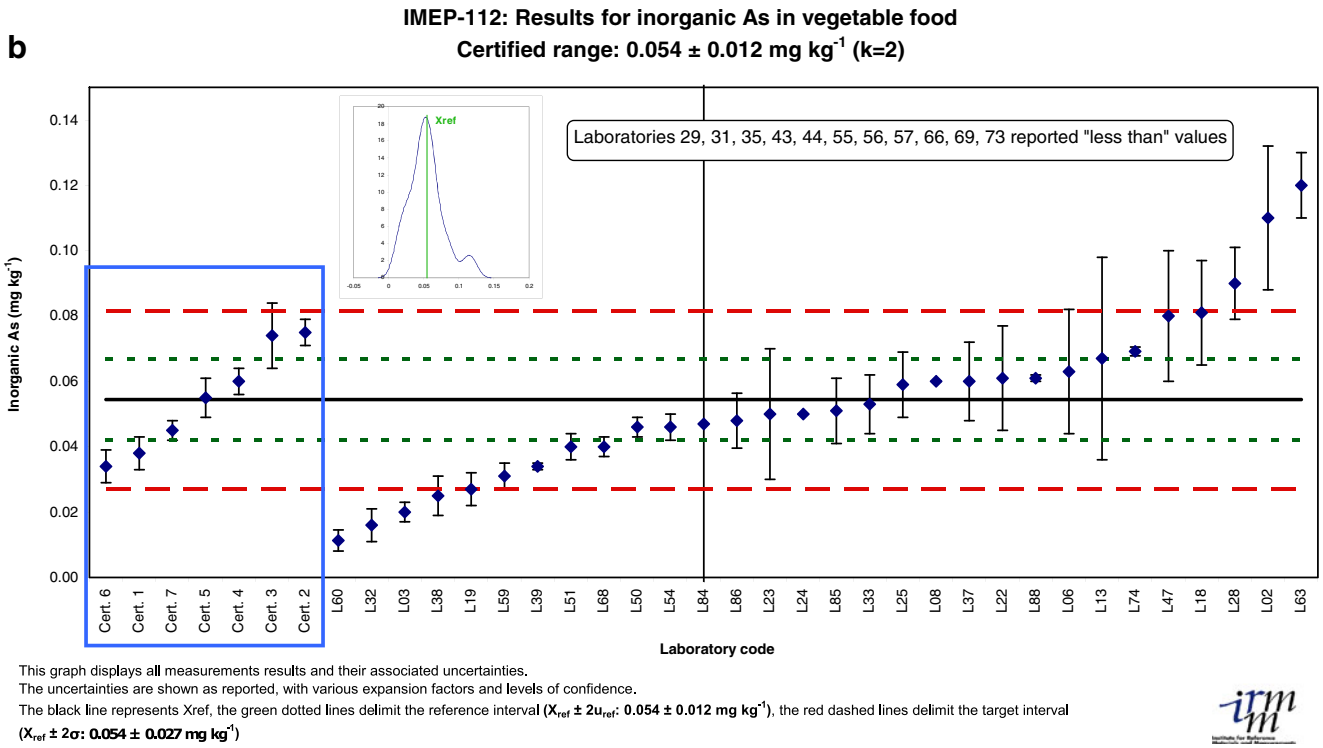


Fig. 2 Results reported by certifiers and participants in IMEP-112 for the vegetable food: total As (**a**) and iAs (**b**)

obtained satisfactory results. Other parameters such as the extraction reagent concentration and the extraction temperature may contribute to improve the efficiency of the iAs extraction even in the absence of H_2O_2. In terms of the arsenic species present, a major difference between wheat and rice is that in the first cereal, arsenic

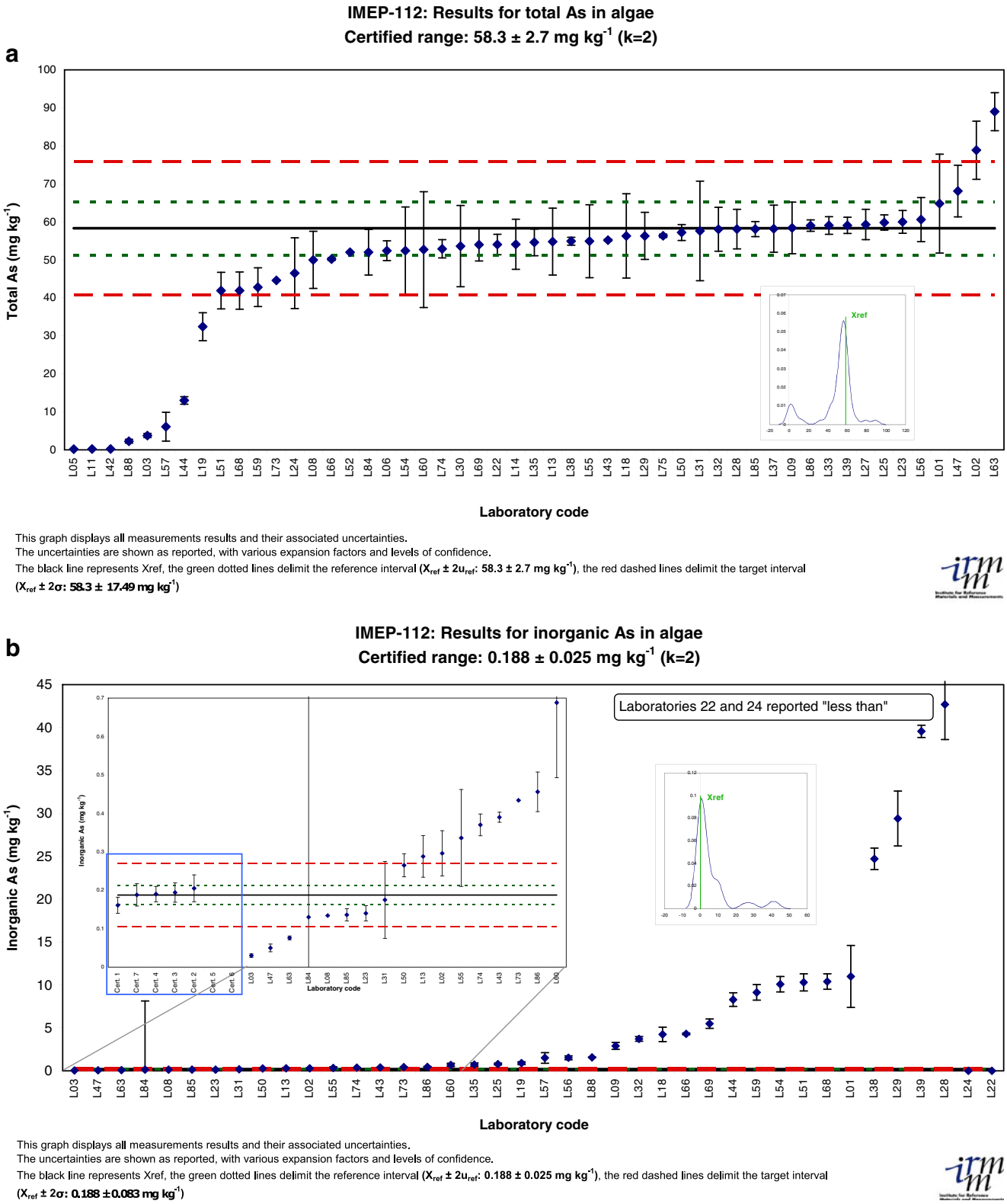


Fig. 3 Results reported by certifiers and participants in IMEP-112 for the algae: total As (**a**) and iAs (**b**)

is present almost exclusively as iAs 6, whereas in rice arsenic and iAs levels are normally higher, but substantial concentrations of other species, particularly DMA, are found.

Table 5 Number of reported results per measurand and matrix

	Wheat			Vegetable food			Algae		
	No.	"Less than"	%	No.	"Less than"	%	No.	"Less than"	%
totAs	62	2	3	47	2	4	51	–	–
iAs	40	3	7	30	11	27	38	2	5 %

No. number of participants having reported evaluable results

Vegetable food

It seems that the determination of iAs in vegetable food (spinach) presents less difficulties than in the other two matrices despite the low concentration of iAs in the test material. However, it must be kept in mind that the $\hat{\sigma}$ for this matrix was 25 % (as opposed to 15 % for wheat and 22 % for algae), and this could explain to a certain level the higher percentage of satisfactory results reported for iAs in this test material, certainly when compared to those reported for wheat.

Algae

As expected, algae seem to be a particularly difficult matrix, very likely due to the coexistence of a variety of organic arsenic species and the relatively high concentration in which they are present compared to the concentration of

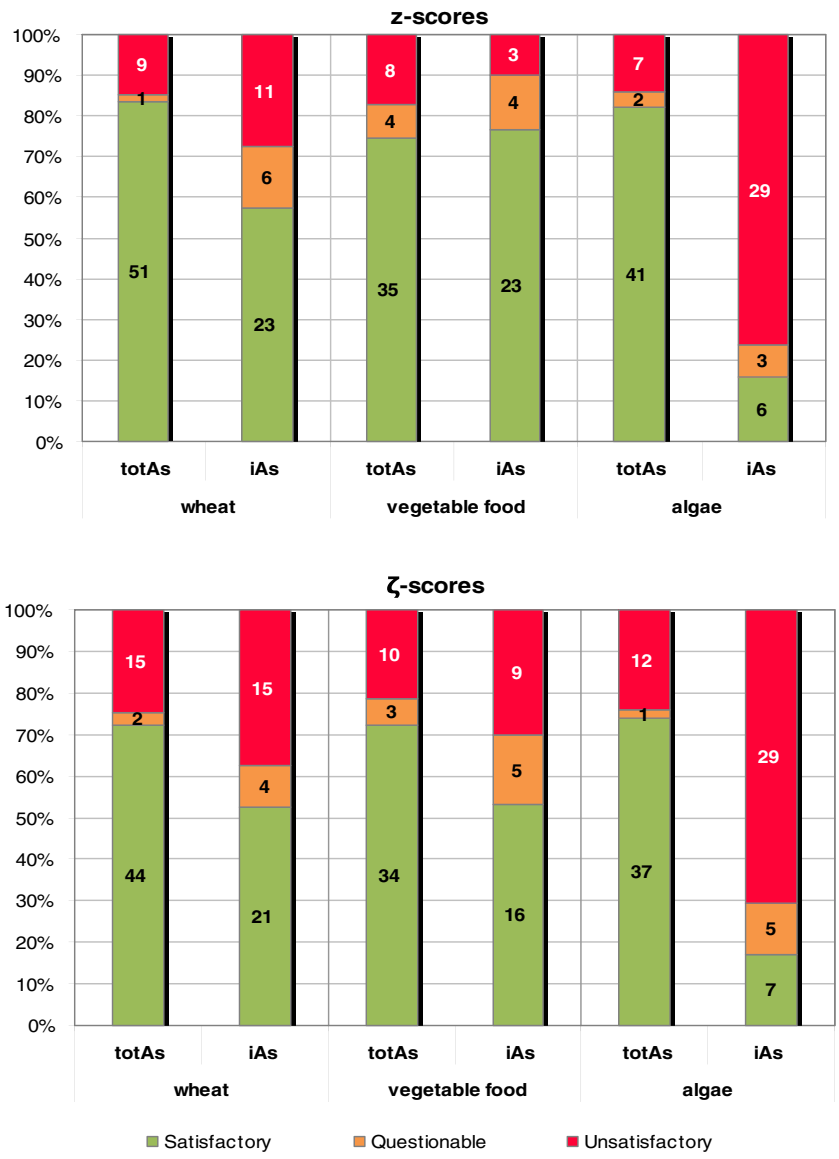


Fig. 4 Overview of scores

iAs, which would be particularly important for methods based on the use of HPLC to separate the species (Fig. 5). The identification of organic species of arsenic in algae as shown in Fig. 5 is done on the basis of work previously performed and published by the expert laboratory which provided the chromatogram. No studies on the identification of organic species of arsenic were performed in the frame of IMEP-112 because they are of little concern from a toxicological point of view. Concerning total As, a number of laboratories have significantly underestimated the mass fraction in this test material probably because the digestion of some organic compound of arsenic is difficult and requires temperatures of at least 280 °C when microwave digestion is used [15].

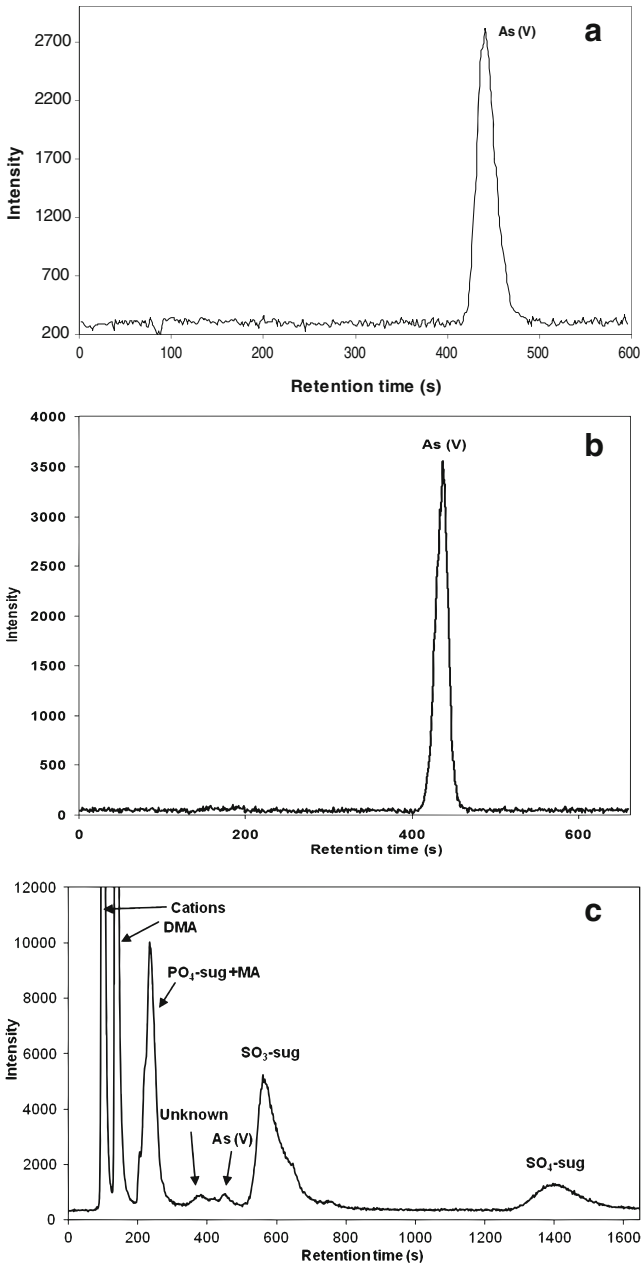


Fig. 5 Chromatograms obtained for wheat (**a**), spinach (**b**) and algae (**c**). These are typical chromatograms obtained by different certifiers

On the other hand, for iAs, there is a tendency to overestimate the concentration since 26 of the participants reported results for this measurand above the accepted range ($X_{ref}+2\hat{\sigma}$), even when taking into consideration their associated uncertainties. Only three participants (L3, L47 and L63) have underestimated the concentration of iAs in algae. A thorough discussion about the problems associated to the determination of iAs in marine samples is presented in the IMEP-30 report for participants [12] dealing with the determination of iAs in seafood, where it is indicated that "less than" values could be explained by an insufficient amount of oxidant added, H_2O_2 to oxidize As(III) to As(V), which is the species of As measured when using HPLC-based methods. Furthermore, the use of MeOH/water and diluted HCl as extracting reagents might not have provided quantitative extraction of iAs. On the other hand, when applying the extraction of iAs with chloroform and concentrated HCl, a cleaning step of the chloroform phase should be carried out to eliminate all traces of HCl and, with it, the present arsenobetaine. Remnants of the concentrated HCl in the chloroform phase might introduce a high contamination of the sample in organic species. Finally, it appears that when analysing complex matrices by HPLC-ICP-MS, the retention time of the iAs shifts and cannot be detected because of a possible co-elution with minor organic species. In this case, careful optimisation of the chromatographic separation of the species would help to solve the problem.

In IMEP-112, three laboratories (L24, L28 and L29) used the EN 15517:2008 standard [16] and six participants (L44, L51, L59, L63, L68 and L69) used the standard GB/T 5009.11-2003 [17] for the determination of iAs. None of them obtained satisfactory scores for iAs. With the exception of L24, which reported <0.025 mg kg^{-1}, the remaining laboratories having used the mentioned standards reported largely overestimated values ranging from 5 to 46 mg kg^{-1}. The results obtained with GB/T 5009.11-2003 range from 5.0 to 11 mg kg^{-1}, with the exception of the result reported by L63 (0.076±0.005 mg kg^{-1}).

Nevertheless, IMEP-112 has shown that when the analytical methods are properly optimised, it is possible to agree on a value for iAs in algae. This has been proven by five of the expert laboratories and by 20 % of the participants. The methods summarised in Table 4 for certifiers 1, 2, 3, 4 and 7 can serve as a basis for laboratories that want to develop a method for the determination of iAs in this type of matrix.

An important outcome of IMEP-112 is that neither for total nor for iAs has a clustering of results been observed for any of the test items on the basis of the method of analysis used.

Conclusions

The main conclusion derived from this exercise is that the concentration of iAs determined in any of the matrices covered does not depend on the analytical method applied, as has been proven by the results submitted by the seven expert laboratories and by the participants in IMEP-112.

In IMEP-112, a wide range of sample pretreatment methods (extraction into water, acid extraction with different acids, enzymatic digestion, etc) and instrumental setups (HGAAS, HPLC-ICP-MS, ETAAS) have been applied by participants and by the expert laboratories that provided the assigned values for iAs. Despite the use of these different methods, clustering of results related to the analytical approach was not observed.

The participating laboratories performed, in general, satisfactorily for the determination of iAs in wheat and vegetable food. Laboratories should remember that not all cereals behave analytically in the same way for the determination of iAs and that methods which perform satisfactorily in rice can provide biased results when applied to wheat. The low number of laboratories obtaining a satisfactory score for iAs in algae indicates that this matrix poses special problems for this type of analysis and that the methods need to be carefully validated for different matrices. Unfortunately, two existing standards for the determination of iAs (EN 15517:2008 and GB/T 5009.11-2003) did provide biased results when applied to algae. The number of laboratories that used these standards in this exercise is rather limited, and so further studies should be carried out before making more definitive statements.

The results show that, purely from the analytical point of view, there is no reason not to consider the option of introducing possible maximum levels for iAs in wheat, vegetable food and algae in further discussions on risk management. Furthermore, attention should also be paid to the determination of total As in algae since underestimations due to the incomplete digestion of some organic compounds of arsenic can occur.

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4.5 Determination of total cadmium, lead, arsenic, mercury and inorganic arsenic in mushrooms: outcome of IMEP-116 and IMEP-39

Determination of total cadmium, lead, arsenic, mercury and inorganic arsenic in mushrooms: outcome of IMEP-116 and IMEP-39

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Determination of total cadmium, lead, arsenic, mercury and inorganic arsenic in mushrooms: outcome of IMEP-116 and IMEP-39

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The Institute for Reference Materials and Measurements (IRMM) of the Joint Research Centre (JRC), a Directorate General of the European Commission, operates the International Measurement Evaluation Program (IMEP). IMEP organises inter-laboratory comparisons in support of European Union policies. This paper presents the results of two proficiency tests (PTs): IMEP-116 and IMEP-39, organised for the determination of total Cd, Pb, As, Hg and inorganic As (iAs) in mushrooms. Participation in IMEP-116 was restricted to National Reference Laboratories (NRLs) officially appointed by national authorities in European Union member states. IMEP-39 was open to all other laboratories wishing to participate. Thirty-seven participants from 25 countries reported results in IMEP-116, and 62 laboratories from 36 countries reported for the IMEP-39 study. Both PTs were organised in support to Regulation (EC) No. 1881/2006, which sets the maximum levels for certain contaminants in food. The test item used in both PTs was a blend of mushrooms of the variety shiitake (*Lentinula edodes*). Five laboratories, with demonstrated measurement capability in the field, provided results to establish the assigned values (X_{ref}). The standard uncertainties associated to the assigned values (u_{ref}) were calculated by combining the uncertainty of the characterisation (u_{char}) with a contribution for homogeneity (u_{bb}) and for stability (u_{st}), whilst u_{char} was calculated following ISO 13528. Laboratory results were rated with *z*- and zeta (ζ)-scores in accordance with ISO 13528. The standard deviation for proficiency assessment, σ_p, ranged from 10% to 20% depending on the analyte. The percentage of satisfactory *z*-scores ranged from 81% (iAs) to 97% (total Cd) in IMEP-116 and from 64% (iAs) to 84% (total Hg) in IMEP-39.

Keywords: inorganic arsenic; trace elements; mushrooms; proficiency test

Introduction

Asian countries have a long tradition of using mushrooms for their therapeutic properties, for instance to prevent hypertension, hypercholesterolemia and cancer (Bobek & Galbavy 1999; Borchers et al. 1999). From a nutritional point of view mushrooms are low in energy and fat but high in protein, carbohydrate and dietary fibre, vitamins and minerals (Cheung 2010). However, edible mushrooms, especially those wildly grown, may contain metals such as Cd, Pb and Hg at levels considerably higher than those in other food commodities (Kalač & Svoboda 2000). The levels of heavy metals in cultivated mushrooms are normally lower than in wild ones most likely due to the soil composition and contamination and to the age of the mycelium (part of the mushroom that grows under the ground surface) which may be several years in nature in a wild mushroom compared with a few months in the cultivated ones (Kalač & Svoboda 2000). The usual content, expressed as mg kg^{-1} in dry matter of heavy metals in mushrooms from unpolluted areas and accumulating species are: 0.5–5 mg kg^{-1} for As, 1–5 mg kg^{-1} for Cd, below 5 mg kg^{-1} for Pb, and below 0.5–5 mg kg^{-1} for Hg (Kalač 2010).

Not much information is available in the literature for metal speciation in mushrooms. The review published by Falandysz and Borovička (2013) indicates that bioaccumulation of methylmercury by mushrooms varies between studies and that in both wild and cultivated mushrooms methylmercury is less abundant than the inorganic Hg (between 2% and 60% of total Hg), although the proportions vary depending on the concentration and the analytical method used. Regarding As, the main species found in many mushrooms are arsenobetaine, arsenate and arsenite, although the type of mushroom has a strong influence (Kalač & Svoboda 2000). Arsenocholine, trimethylarsonium ion and some unidentified As compounds have also been detected (Vetter 2004). Llorente-Mirandes et al. (2014) carried out As speciation studies in shiitake mushrooms (both fresh and dehydrated)

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and in shiitake products (food supplements and canned shiitake), showing that inorganic As (iAs) is the predominant As species. To avoid health problems, maximum levels for heavy metals in mushrooms based on wet weight are set by the latest consolidated version of Regulation (EC) No. 1881/2006 (European Commission 2006). For common mushroom, oyster mushroom and shiitake mushroom the maximum levels are: 0.20 mg kg^{-1} Cd and 0.30 mg kg^{-1} for Pb. For other species the maximum level for Cd of 1 mg kg^{-1} applies. No maximum levels have been set yet for iAs and methylmercury, although they are the most toxic species of As and Hg, respectively. Both, European Food Safety Authority (2009, 2014) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (2011) have recently shown their interest in the content of iAs in food.

Since mushroom consumption has increased considerably in the last years due to their nutritional properties, the Directorate for Health and Consumers (DG SANCO) of the European Commission requested that the EURL-HM test the analytical capabilities of National Reference Laboratories (NRLs) to determine heavy metals in mushrooms. Two proficiency tests (PTs) were organised by IMEP on behalf of the EURL-HM using the same test item: IMEP-116 (for NRLs) and IMEP-39 (for official control laboratories (OCLs) and other laboratories), as defined in Commission Regulation (EC) No. 882/2004 (2004).

This paper discusses and compares the outcome of both PTs.

Test material

A preliminary screening of Cd, Pb, As, Hg and iAs in several fresh mushrooms was performed by the University of Barcelona (UB). For this, fresh mushrooms were hand-cleaned for soil and moss. The end of the stalk that had been in contact with the soil was cut off using a stainless steel knife. Mushrooms were cut into pieces, air dried in a batch-type drying chamber at RT for 24 h and dried in an oven at 40°C for 24–48 h. The dried mushrooms were minced using a commercial stainless steel mincer (Multiquick 5 Hand Processor, Braun), completely homogenised and analysed. From the results, shiitake mushroom was selected as the test material. Then, 5 kg of the selected fresh shiitake mushrooms were sent to IRMM under refrigerated conditions.

Upon arrival, the material was stored at –20°C until processing. At the time of processing the mushrooms were cut frozen into smaller pieces using an UMC-12 model cutter/mixer (Stephan Machinery GmbH, Hameln, Germany). The material was freeze-dried in two cycles using a freeze-dryer Epsilon 2-10D (Martin Christ GmbH, Osterode, Germany). For each cycle five trays were filled with about 500 g each of pre-cut mushrooms. In total 5.27 kg were dried, giving 570 g of dried mushroom, corresponding to a mass loss of about 89%.

Dried mushrooms were cryogenically milled using a Palla VM-KT vibrating mill (KDH, Humboldt-Wedag GmbH, Cologne, Germany). All grinding elements in this system were made of high-purity titanium to avoid contamination of the test material. After milling, this material was sieved over a 250 µm stainless steel sieve resulting in 522 g available for final mixing and homogenisation. Mixing was performed in a Dynamix CM-200 (WAB, Basel, Switzerland). Karl Fischer titration and laser diffraction analyses indicate that the material had a water content of 4% (m/m) with a top particle size below 200 µm, respectively.

Finally, portions of 2.5 g were filled using an automatic filling machine (Allfill, Sandy, UK) into acid-washed 20 ml amber glass vials. The vials were closed with acid washed inserts and aluminium caps.

Each vial was uniquely identified with a number and the name of the PT exercise.

Homogeneity and stability studies

The measurements for homogeneity and stability studies were performed by ALS Scandinavia AB (Sweden) using inductively coupled plasma sector field mass spectrometry (ICP-SFMS) after sample digestion with a mixture of HNO_3/HF. Homogeneity was evaluated according to ISO 13528 (ISO 2005). The material proved to be adequately homogeneous for the total mass fraction of As, Cd, Pb and Hg.

The stability study was conducted following an isochronous experimental design (Lamberty et al. 1998; Linsinger et al. 2001). The material proved to be adequately stable for the 8 weeks that elapsed between the dispatch of the samples and the deadline for submission of results and for all the four investigated total mass fractions (As, Cd, Pb and Hg).

The contributions to the uncertainty of the assigned value (u_{ref}), due to homogeneity (u_{bb}) and to stability (u_{st}), were calculated using the statistical software SoftCRM (SoftCRM). On the basis of previous experience (IMEP-107), it was assumed that total As and iAs are similarly homogeneously distributed and stable in the test item investigated. Therefore, the same contributions were used for total As and for iAs.

Instructions to participants

Participants were asked to perform two or three independent measurements, correct their measurements for recovery and for the moisture content, and report their calculated mean (expressed as mg kg^{-1} in dry mass) and its associated expanded measurement uncertainty (U_{lab}). The experimental protocol for the moisture content determination, described in the accompanying letter, was optimised to yield the same result as the one obtained by Karl-Fisher titration which is specific for water in contrast to oven methods.

Participants received an individual code to access the online reporting interface, to report their measurement results and to complete the related questionnaire. The questionnaire was used to gather additional information related to laboratories and measurements.

Participants were informed that the procedure used for the analysis should resemble as closely as possible their respective routine procedures for these measurands (defined by specific matrix, analyte and concentration level).

Assigned values and their uncertainties

Assigned values (X_{ref})

Five laboratories with demonstrated measurement capabilities (later referred as expert laboratories) analysed the test item in order to determine the assigned values (Table 1): Federal Institute for Materials Research and Testing, BAM, Germany; Laboratory of Public Health of Alicante, LSPA, Spain; Karl-Franzens-Universität Graz, KFUG, Austria; University of Barcelona, UB, Spain; and Instituto de Agroquímica y Tecnología de los Alimentos, Consejo Superior de Investigaciones Científicas, CSIC, Spain. Not every laboratory analysed all measurands.

Experts were asked to use the method of their choice; no further requirements were imposed regarding methodology. Experts were also asked to report their measurement uncertainty with a clear and detailed description on how the measurement uncertainty was estimated. A detailed description of the methods reported by the expert laboratories is presented in Table 1.

The mean of the means provided by the expert laboratories was used to derive the assigned values (X_{ref}) for these PTs according to ISO Guide 35 (ISO 2006).

Associated standard uncertainties (u_{ref})

The standard uncertainties associated to the assigned values (u_{ref}) were calculated according to ISO/IEC Guide 98:2008 (GUM) (ISO 2008) by combining the uncertainty of the characterisation (u_{char}) with a contribution for homogeneity (u_{bb}) and for stability (u_{st}) as follows:

$$u_{ref} = \sqrt{u_{char}^2 + u_{bb}^2 + u_{st}^2} \tag{1}$$

where u_{char} was calculated by combining the standard uncertainties reported by the expert laboratories (u_i):

$$u_{char} = \frac{1.25}{p} \sqrt{\sum\nolimits_1^p u_i^2} \tag{2}$$

where p is the number of expert laboratories used to assign the reference value.

Table 2 presents the average measurements reported by the expert laboratories (X_n), their expanded measurement uncertainties (U_n), assigned values, standard uncertainty contributions (from characterisation, homogeneity and stability) and combined uncertainties (u_{ref}) and the standard deviation for the PTs assessment.

Standard deviation for proficiency assessment (σ_p)

The standard deviations for the proficiency assessment (σ_{p}) for total Pb and iAs were calculated to be 20% and 19%, respectively, using the Horwitz equation modified by Thompson (2000). For the rest of the measurands, σ_{p} was set by the advisory board of this PT to 15% for total As and Hg and to 10% for total Cd, on the basis of previous performance on similar measurands (EURL-HM).

Evaluation of the results reported by laboratories taking part in IMEP-116 and IMEP-39

In IMEP-116, 37 out of the 38 NRLs (from 25 countries) having registered reported results. In IMEP-39 results were received from 62 (from 36 countries) of the 71 registered laboratories. Laboratories reporting 'less than X' were not scored. However, reported 'less than X' values were compared with the corresponding '$X_{\mathrm{ref}} - U_{\mathrm{ref}}$'. If the reported limit value X is lower than the corresponding $X_{\mathrm{ref}} - U_{\mathrm{ref}}$, this statement is considered incorrect, since the laboratory should have been able to detect the respective element.

Scoring and evaluation criteria

Individual laboratory performance is expressed in terms of z- and ζ-scores in accordance with ISO 13528 (ISO 2005):

$$z = \frac{x_{lab} - X_{ref}}{\sigma_p} \tag{3}$$

$$\zeta = \frac{x_{lab} - X_{ref}}{\sqrt{u_{ref}^2 + u_{lab}^2}} \tag{4}$$

where: x_{lab} is the measurement result reported by a participant; X_{ref} is the reference value (assigned value); u_{ref} is the standard uncertainty of the reference value; u_{lab} is the standard uncertainty reported by a participant; and σ_{p} is the standard deviation for proficiency assessment.

The interpretation of the z- and ζ-score is done as follows (according to ISO/IEC 17043 (ISO 2010):

Satisfactory performance = |score| ≤ 2
Questionable performance = 2 < |score| < 3
Unsatisfactory performance = |score| ≥ 3

The z-score compares the participant's deviation from the reference value with the standard deviation for

Table 1. Analytical methods used by the expert laboratories.

Certifier	Sample treatment/digestion/analytical method	Technique
BAM	Total As, Cd and Pb: 0.25 g of sample. Microwave-assisted digestion. 6 ml of HNO_3 (sub-boiling) in an Ultra Clave III. Power 1000 W, ramp 20 min. Hold 30 min. Digestion temperature 250°C at 100 bar. ICP equipped with a collision cell. Argon + helium as collision gas. Multi-point calibration from 0 to 10 μg l^{-1} (five points) for total As and Pb, 0–25 μg l^{-1} for Cd	ICP-MS
BAM	Total Hg: 0.25 g of sample. Microwave-assisted digestion. 6 ml of HNO_3 (sub-boiling) in an Ultra Clave III. Power 1000 W, ramp 20 min. Hold 30 min. Digestion temperature: 250°C at 100 bar. CV-AFS, amalgamation mode (gold trap). Argon as gas. Multi-point calibration from 0 to 125 μg l^{-1} (five points)	CV-AFS
BAM	Total Hg: 0.12 g of sample. Solid sampling cold-vapour AAS, combustion + amalgamation (gold trap). Advanced elementar Hg analyser (AMA-254) at the wavelength of 253.7 nm. Oxygen as gas mode. Multi-point calibration from 0.5 to 36 ng (nine points) and from 40 to 500 ng (nine points)	AMA-254
LSPA	Total As, Cd, Pb: the digestion of samples was carried out using a microwave digestion system, Ethos one (Milestone Inc., Shelton, CT, USA), equipped with the Q-20 Quartz Rotor Ultratrace Analysis (20 ml quartz tubes, 250ºC and 40 bars operating parameters). A unique sample digestion procedure was applied to all samples and analytes. 0.25 g of sample were weighted in quartz digestion vessels and 5 ml of HNO_3:H_2O 1:1 were added in a fume hood. The mixture was left to react over 1 h approximately until finishing the gas generation process. Analysis was performed on an ELAN DRC II ICP-MS (PerkinElmer) equipped with a perfluoroalcoxy standard nebuliser and a peltier cooled baffled glass cyclonic spray chamber (both Elemental Scientific, Omaha, NE, USA). Multi-element standard solutions were used for external calibration. Six standards in 2% (w/w) HNO_3 matrix for As, Cd and Pb were prepared at levels ranging from 0.1 to 50 μg l^{-1}. The calibration curve was drawn from six points, including the calibration blank and a weighted linear regression approach with internal standardisation was applied	ICP-MS
LSPA	Total Hg: 40 mg of sample were weighted directly in quartz samples boats and placed in the Hg analyser. To prevent explosions inside the catalyser, 500 μl of ultra-pure water were added in the quartz boats together with the samples. At least two quality control samples (CRM) were analysed in each sequence	Elemental Hg analyser
KFUG	Total As: a portion of the powdered samples (about 250 mg weighed with a precision of 0.1 mg) was weighed directly into 12 ml quartz tubes and concentrated nitric acid (2 ml) and H_2O (2 ml) were added. The tubes were transferred to a Teflon® rack of the Ultraclave microwave system (MLS GmbH, Leutkirch, Germany) and covered with Teflon caps. After closing the system, an argon pressure of 4–106 Pa was applied and the mixture was heated to 250°C for 30 min before being allowed to cool to RT. After mineralisation, the samples were transferred to 15 ml polypropylene tubes (Greiner, Bio-one, Frickenhausen, Germany) and diluted with water to 9 ml (based on mass). Finally, 1 ml of a solution containing 50% methanol (to enhance the As response) and 100 μg l^{-1} each of Ge and In as internal standards were added to all digested samples giving a final concentration of 5% methanol and 10 μg l^{-1} of Ge and In. All standards for total As determinations were prepared with 20% (v/v) of concentrated nitric acid and also 5% methanol for matrix matching with the digested samples. The As concentrations in the digests were determined by ICP-MS using helium as the collision cell gas	ICP-MS
KFUG	iAs: about 0.5 g of powder were weighed with a precision of 0.1 mg into 50 ml polypropylene tubes and a solution (10 ml) of 20 mmol l^{-1} trifluoracetic acid containing 50 μl of a 30% H_2O_2 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 RT 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	HPLC-ICP-MS

(*continued*)

Table 1. Continued.

Certifier	Sample treatment/digestion/analytical method	Technique
CSIC	iAs: 0.5–1 g of sample. Concentrated HCl is added and water. Reducing agent (2 ml of HBr and 1 ml of hydrazine sulphate) is added. 10 ml of $CHCl_3$. Agitate and separate the phases. Repeat the extraction three times. iAs is back-extracted with 10 ml of HCl. 2.5 ml of ashing aid suspension (20% w/v $Mg(NO_3).6H_2O$ and 2% w/v MgO) and 10 ml HNO_3 are added. Evaporated to dryness in a sand bath and place at a muffle at 150°C. Increase the temperature to 425 ± 25°C for 12 h. The white ash is dissolved in 6 mol l^{-1} HCl and reduced with pre-reducing solution (5% w/v KI and 5% w/v ascorbic acid). After 30 min, filter through Whatman No. 1 and dilute with 6 mol l^{-1} HCl. Samples are analysed by flow injection-hydride generation AAS	FI-HG-AAS
UB	iAs: a microwave digestion system (Ethos Touch Control, Milestone, Gomensoro, Barcelona, Spain), with a microwave power of 1000 W and temperature control, was used for the extraction procedure. An Agilent 7500ce ICPMS was coupled to an Agilent 1200 LC quaternary pump to determine iAs content. The analytical column Hamilton PRP-X100 (250 × 4.1 mm, 10 µm; Hamilton, Reno, NV, USA) was protected by guard column filled with the corresponding stationary phase. The outlet of the LC column was connected via PEEK capillary tubing to the nebuliser (BURGENER Ari Mist HP type) of the ICP-MS system, which was the As-selective detector. 0.25 g aliquots of the test material and three CRMs, for internal quality control, were weighed in PTFE vessels and then extracted by adding 10 ml of 0.2% (w/v) HNO_3 and 1% (w/v) H_2O_2 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 RT and centrifuged at 3500 rpm for 12 min. The supernatant was filtered through PET filters (pore size 0.45 µm) and analysed by HPLC-ICP-MS	HPLC-ICP-MS

Note: Certified reference materials (CRMs).

Table 2. Average measurements reported by the expert laboratories (X_n), their expanded measurement uncertainties (U_n), assigned values, standard uncertainty contributions (from characterisation, homogeneity and stability) and combined uncertainties (u_{ref}) and the standard deviation for the PTs assessment (mg kg^{-1}).

	Total As	Total Cd	Total Hg	Total Pb	iAs
$X_n \pm U_n$ ($k = 2$)	0.638 ± 0.026	4.42 ± 0.19	0.0782 ± 0.0032	0.274 ± 0.019	0.330 ± 0.014
	0.61 ± 0.06	3.99 ± 0.44	0.0781 ± 0.007	0.260 ± 0.016	0.286 ± 0.037
	0.69 ± 0.05		0.072 ± 0.007		0.348 ± 0.026
X_{ref}	0.646	4.21	0.076	0.267	0.321
u_{char}	0.017	0.15	0.002	0.008	0.010
u_{bb}	0.007	0.04	0.002	0.009	0.004
u_{st}	0.015	0.06	0.002	0.010	0.007
u_{ref}	0.024	0.17	0.004	0.016	0.013
U_{ref} ($k = 2$)	0.048	0.33	0.007	0.031	0.026
σ_p	0.10	0.42	0.011	0.05	0.06
σ_p (%)	15%	10%	15%	20%	19%

Note: Experts do not necessarily correspond to the order in which they were presented.

proficiency assessment (σ_p) used as a common quality criterion, defined in the previous section.

The ζ-score states if the laboratory result agrees with the assigned value within the respective uncertainty. The denominator is the combined uncertainty of the assigned value (u_{ref}) and the measurement uncertainty as stated by the laboratory (u_{lab}). The ζ-score includes all parts of a measurement result, namely the expected value (assigned value), its uncertainty and the unit of the result as well as the uncertainty of the reported values. An unsatisfactory ζ-score can be caused either by an incorrect measurement result or by an inappropriate estimation of its uncertainty, or both.

The standard measurement uncertainty of the laboratory was obtained by dividing the reported expanded uncertainty by the reported coverage factor, k. When no uncertainty was reported, it was set to zero ($u_{lab} = 0$). When k was not specified, the reported expanded uncertainty was considered as the half-width of a rectangular distribution; u_{lab} was then calculated by dividing this half-width by $\sqrt{3}$, as recommended by Eurachem and CITAC (Eurachem/CITAC 2012).

Uncertainty estimation is not trivial; therefore an additional assessment was provided to each laboratory reporting uncertainty, indicating how reasonable is their uncertainty estimate. The standard uncertainty from the laboratory (u_{lab}) is most likely to fall in a range between a minimum uncertainty (u_{min}) and a maximum allowed (u_{max}, case a). u_{min} is set to the standard uncertainty of the reference value (u_{ref}). It is unlikely that a laboratory carrying out the analysis on a routine basis would measure the measurand with a smaller uncertainty than the expert laboratories chosen to establish the assigned value. u_{max} is set to the standard deviation (σ_p) accepted for the PT assessment.

If u_{lab} is smaller than u_{min} (case b) the laboratory may have underestimated its uncertainty. However, such a statement has to be taken with care as each laboratory reported only measurement uncertainty, whereas the uncertainty of the reference value also includes contributions of homogeneity and stability. If those are large, measurement uncertainties smaller than u_{min} (u_{ref}) are possible and plausible.

If u_{lab} is larger than u_{max} (case c) the laboratory may have overestimated the uncertainty. An evaluation of this statement can be made by looking at the difference of the reported value and the assigned value: if the difference is smaller than U_{ref}, then overestimation is likely. If the difference is larger but x_{lab} agrees with X_{ref} within their respective expanded measurement uncertainties, then the measurement uncertainty is properly assessed resulting in a satisfactory z-score, though the corresponding z-score may be questionable or unsatisfactory. It should be pointed out that u_{max} is a normative criterion when set by legislation.

Laboratory results and scorings

Results as reported by the participants for total Cd, Pb, As, Hg and iAs mass fractions are summarised in Figures 1–5. They include the individual mean values and reported associated expanded uncertainties.

Figure 6 presents a general overview of z- and ζ-scores. In IMEP-116, 81% (iAs) to 97% (total Cd) of the NRLs performed satisfactorily ($z \leq 2$). The PT seems to have been more challenging for the laboratories taking part in IMEP-39 where 64% (iAs) to 72% (total Hg) of the reported results were satisfactory. As shown, the percentage of laboratories obtaining satisfactory z-scores is higher for all measurands in IMEP-116 than in IMEP-39, the largest differences between the two populations occurring for total Pb, total As and iAs.

Regarding ζ-scores, in IMEP-116 69% (total As) to 84% (Total Cd) performed satisfactorily. In IMEP-39, a

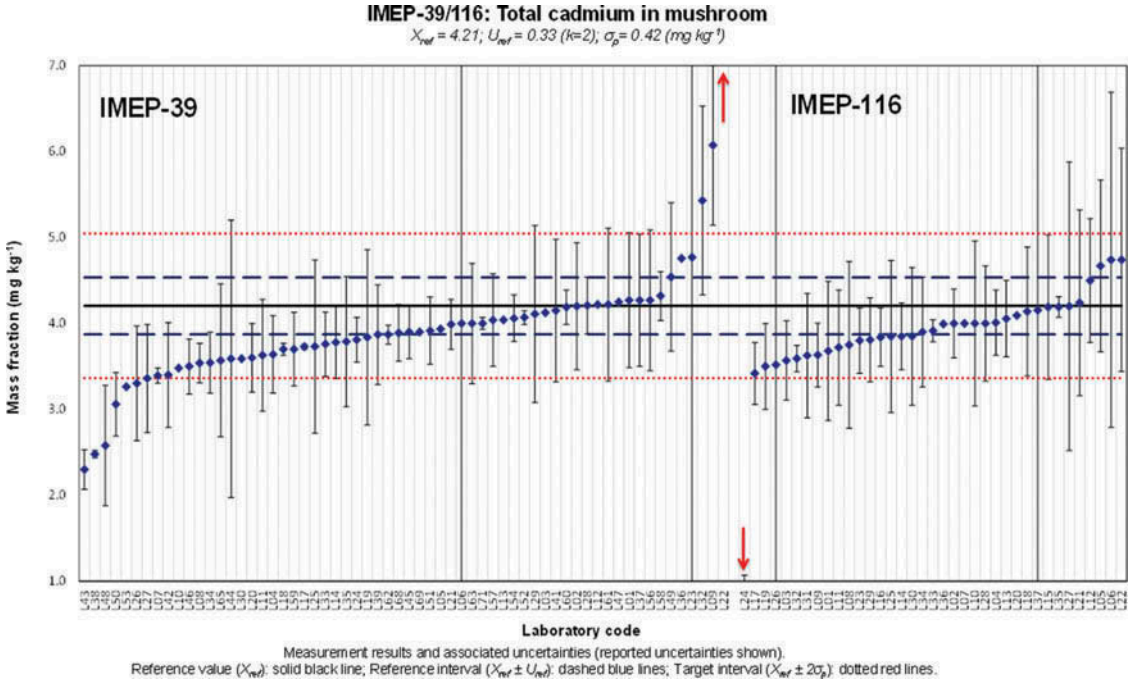

Figure 1. (colour online) X_{lab} and U_{lab} as reported by the participants in IMEP-39 and IMEP-116 for the total mass fraction of Cd.

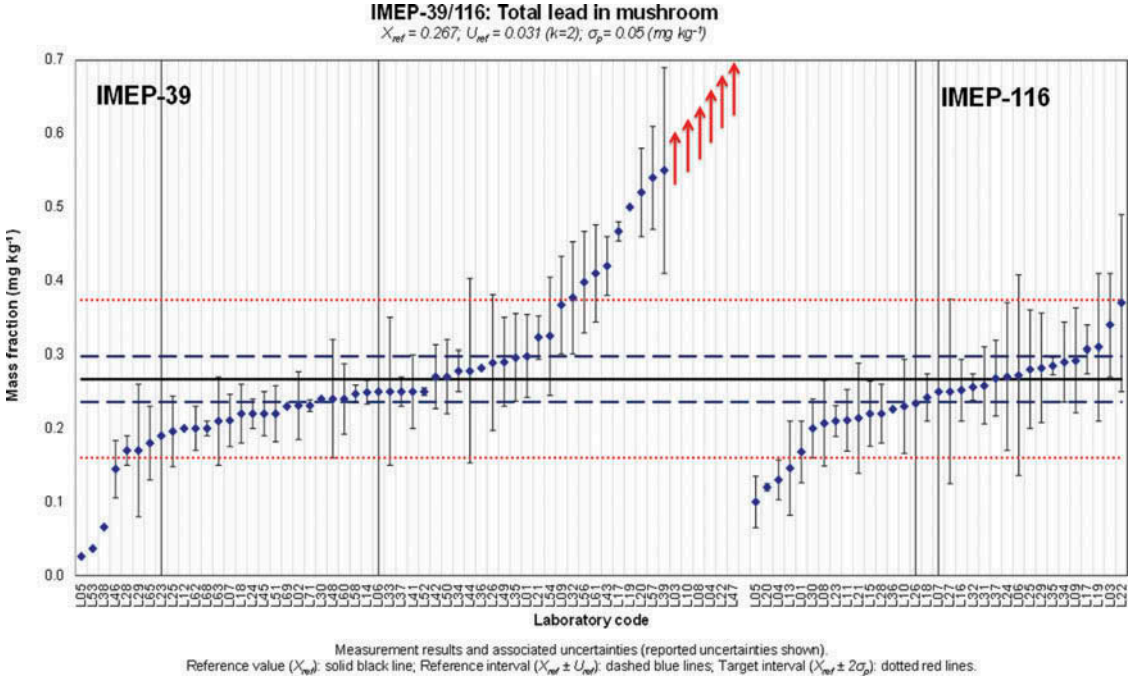

Figure 2. (colour online) X_{lab} and U_{lab} as reported by the participants in IMEP-39 and IMEP-116 for the total mass fraction of Pb.

lower percentage of the population performed satisfactorily (ranging from 44% to 66%, for total As and Cd mass fractions, respectively) with percentages of 46%, 52% and 55% for total Pb, Hg and iAs respectively. Thus laboratories should enhance their effort in the estimation of their measurement uncertainty.

As indicated in Scorings and evaluation criteria 'a', 'b' and 'c' scorings are just orientative assessments meant to

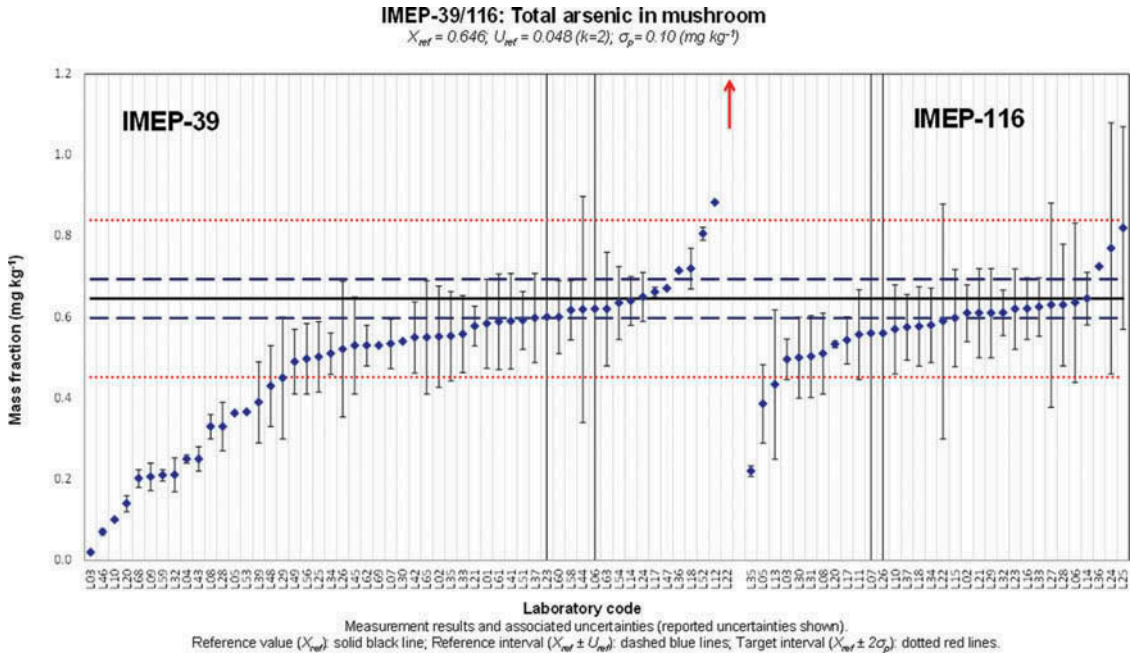

Figure 3. (colour online) X_{lab} and U_{lab} as reported by the participants in IMEP-39 and IMEP-116 for the total mass fraction of As.

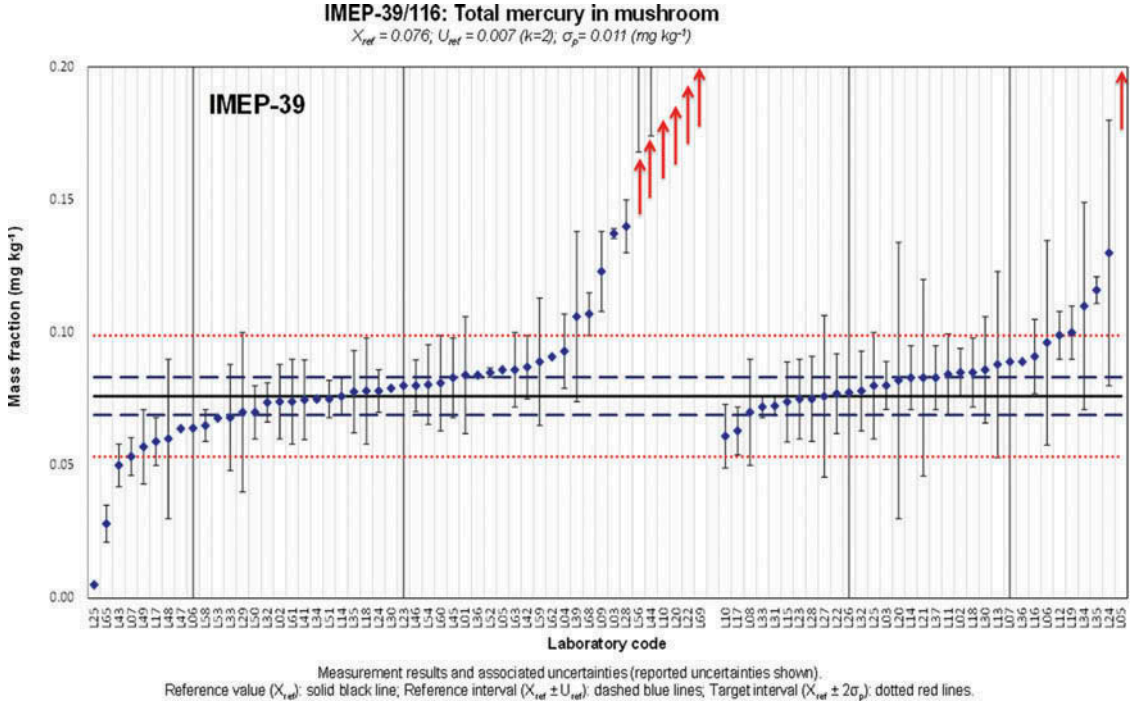

Figure 4. (colour online) X_{lab} and U_{lab} as reported by the participants in IMEP-39 and IMEP-116 for the total mass fraction of Hg.

help laboratories to evaluate the plausibility of their standard measurements.

The assessment of reported uncertainties presented in Table 3 is based on the three uncertainty categories defined in the chapter on Scorings and evaluation criteria: 'a' (realistic), 'b' (underestimated) and 'c' (overestimated/large). The first observation is that the percentage of laboratories reporting realistic uncertainties for all measurands is higher in IMEP-116 than in IMEP-39. The second observation is that while in

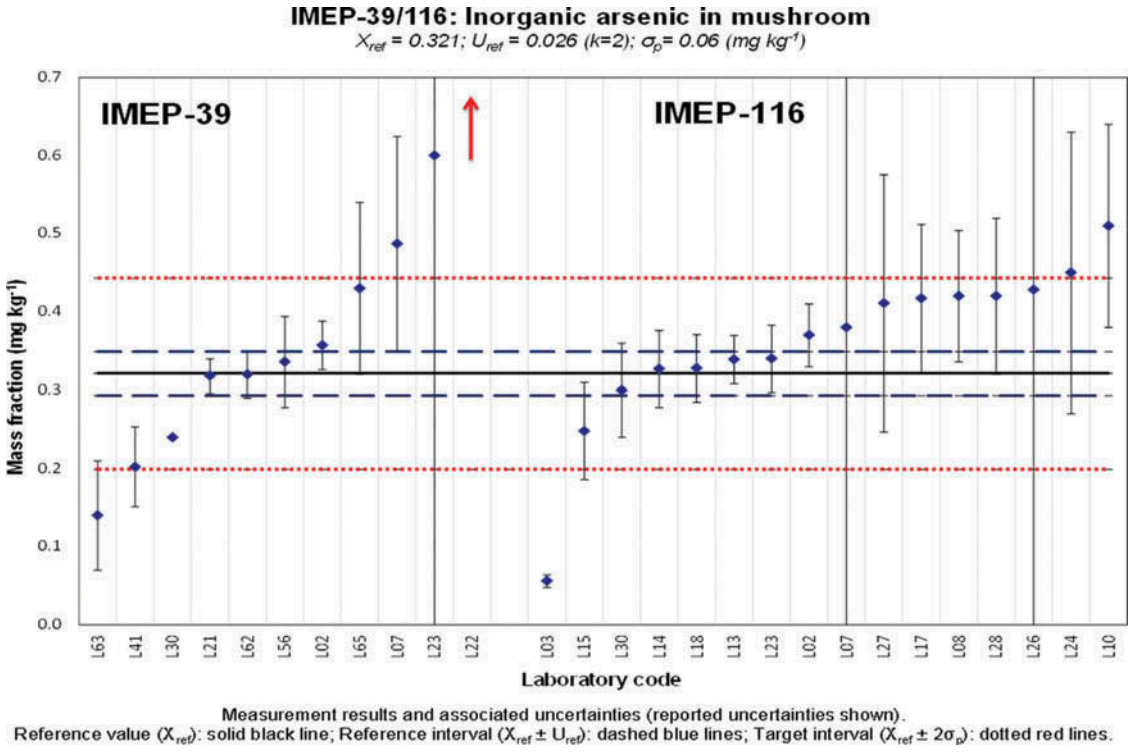

Figure 5. (colour online) X_{lab} and U_{lab} as reported by the participants in IMEP-39 and IMEP-116 for the total mass fraction of iAs.

IMEP-116 there is a clear tendency to overestimate the uncertainty, the opposite tendency took place in IMEP-39 where laboratories tended to underestimate the uncertainties associated with the reported results. Frequently underestimation of uncertainty occurs when repeatability is used as uncertainty. It also needs to be kept in mind that some laboratories did not report any uncertainties; in those cases IMEP considers the reported uncertainty to be zero and they are then counted as 'b'. This is done because Regulation (EC) No. 333/2007 (European Commission 2007) indicates that in official control analysis results are to be reported as $X \pm U$, where U is the expanded associated uncertainty. A proper estimation of the standard uncertainties is of paramount importance, for instance in cases of litigation. Along the years the EURL-HM organised several lectures providing NRLs with information about the different approaches that allow a sound estimation of the measurement uncertainties. Additionally, every PT organised by the EURL-HM for the network of NRLs was an opportunity to review the quality of their uncertainty estimation.

It is clear that the values used for σ_p have an impact on the percentage of uncertainties being assessed as overestimated for a given PT. The lower the σ_p the higher the chance that a laboratory would report an uncertainty assessed as overestimated. This could explain why most of the overestimated uncertainties were reported by the NRLs for total Cd and Hg.

In IMEP-116 the proportion of overestimated uncertainties for iAs (31%) could be explained by the fact that some NRLs have used an analytical method recently implemented, for which the laboratory is not fully confident, thus resulting in larger standard uncertainties. Such a tendency was not observed in IMEP-39 because, as discussed above, the majority of that population reported standard measurement uncertainties derived only from precision data.

Hg and As speciation

In the preparatory phase of the PTs, it was decided to perform some preliminary studies to evaluate the content of the most toxic species of Hg and As (methylmercury and iAs, respectively) in the test item.

The screening for methylmercury was performed by the Laboratory of Public Health of Alicante, using the analytical method validated by the EURL-HM in a collaborative trial (IMEP-115). The report of the collaborative trial (Cordeiro et al. 2013) and the standard operational procedure (SOP) (Calderón et al. 2013) can be downloaded from the EURL-HM webpage (EURL-HM).

For methylmercury, an approximate concentration of 0.0042 mg kg^{-1} was found, which corresponds to about

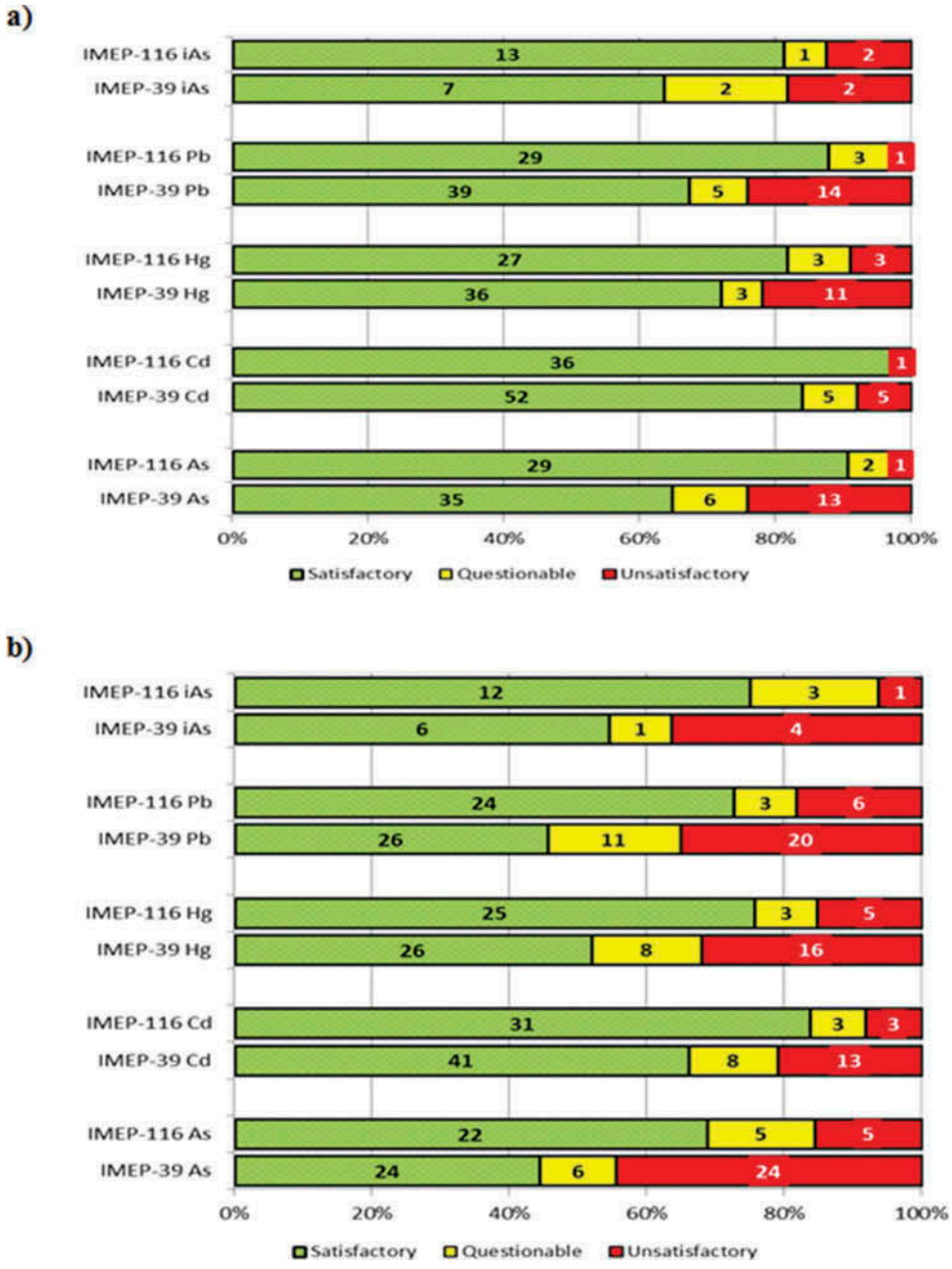

Figure 6. (colour online) Distribution of satisfactory, questionable and unsatisfactory (a) *z*- and (b) ζ-scores for IMEP-39 and IMEP-116.

Table 3. Uncertainty assessment. Proportion of participants in each study who received the 'a', 'b' or 'c' ratings (%).

	Case a		Case b		Case c	
Measurand	IMEP-116	IMEP-39	IMEP-116	IMEP-39	IMEP-116	IMEP-39
Total As	69	57	9	37	22	6
Total Cd	54	34	16	47	30	19
Total Hg	58	44	12	36	30	20
Total Pb	67	52	18	40	15	8
iAs	63	55	6	27	31	18

5% of the total content of Hg in the test item. This value can only be considered as approximate because the LOQ of the method used for the screening is 0.010 mg kg^{-1}. The concentration found is in agreement with the information published in the literature (Kalač & Svoboda 2000), mentioning that methylmercury is normally present at a low percentage, rarely more than 16%, of the total Hg mass fraction.

The screening of iAs performed by the UB indicates that around 50% of the total As mass fraction is present in the form of iAs. This was confirmed during the analysis

conducted to establish the assigned value for that measurand (Table 2). Two of the expert laboratories having determined iAs using HPLC-ICP-MS submitted chromatograms showing the distribution of As species in the test item (Figure 7). Both chromatograms show the same profile; iAs was identified by the two expert laboratories as the main As species in the mushroom (*Lentinula edodes*) analysed. Dimethylarsinic acid (DMA) was also clearly detected. Traces of monomethylarsonic acid were also present. The literature indicates that the main arsenocompound detected in some mushroom species was arsenobetaine (Kalač & Svoboda 2000), although it depends on the type of mushroom, for instance DMA is the main As species in *Laccaria laccata* and *Volvariealla volvacea* (Šlejkovc et al. 1997). In the test item used in the discussed PTs, arsenobetaine was not reported by any of the expert laboratories, although it has to be kept in mind that the chromatographic conditions used by the expert laboratories are those that best fit the determination of iAs (based on the use of an anion-exchange column), since that was the measurand in the discussed PTs. One expert laboratory also analysed the test item using a cation-exchange column (results not shown) and traces of arsenobetaine and some other cationic As species were detected.

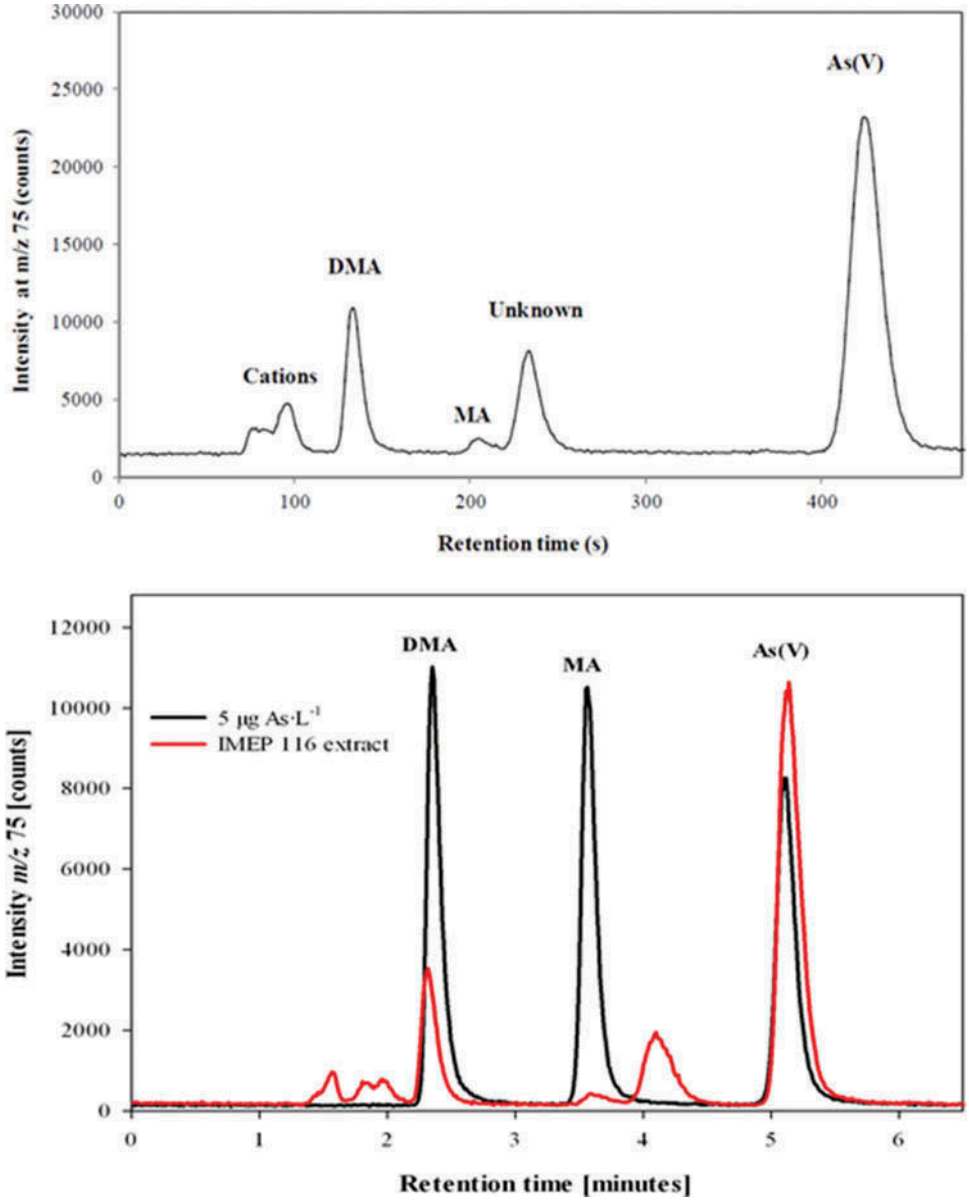

Figure 7. (colour online) Chromatograms showing the distribution of As species in the test item, as obtained by two expert laboratories using anion exchange-ICP-MS.

Analysis of the information reported by the laboratories in the questionnaire

When reporting their results participants were asked to answer a number of questions related to the analytical method used and to the quality assurance of their results. In order to allow the identification of all major potential sources of variability among the reported results, we investigated (for each measurand) the relation between each reported value and the set of responses provided in the questionnaire. The statistical data treatment was performed using The Unscrambler X 10.1 (CAMO Software AS, Oslo, Norway). Answers were first transformed into numerical variables, before applying partial least square regression modelling (PLS-R). Multivariate models succeed to 'explain' a reasonable percentage of the total covariance relating the reported results and the set of answers. Furthermore, the model errors were generally lower than the observed variability for each corresponding set of reported values (expressed as the respective standard deviation). Therefore, the multivariate models allowed reliable interpretations. Although no significant differences were observed among the participants, in general the better performing laboratories were characterised by: having used microwave digestion with nitric acid and hydrogen peroxide for sample digestion; some quality assurance issues (e.g. having a quality system in place, being accredited, use of certified reference materials for validation and/or calibration purposes and taking part regularly in PTs); and having experience with this type of analysis/matrices.

Two clear tendencies were observed in IMEP-39 (not present in IMEP-116), as follows.

Tendency to underestimate the total As mass fraction

At first glance this underestimation was directly related to the technique used, as illustrated in Figure 8. In general, participants using atomic absorption spectrometry (AAS)-based techniques reported lower values than the participants who used ICP-based techniques (ICP-MS and ICP-AES). The lower values reported by participants using AAS-based techniques resulted in a significantly lower percentage of satisfactory *z*-scores (35%) when compared with those obtained by laboratories using ICP-based techniques (87%). However, this clustering of results on the basis of the technique used could be due to a non-quantitative digestion of the matrix without being related to the technique used. Some organic species of As are difficult to digest and require digestion temperatures of around 280°C when microwave digestion is used (most of the participants in IMEP-39 used microwave digestion). Most of the laboratories that clearly failed to quantify the total As mass fraction used temperatures in the range 190–200°C with further hydride generation-AAS (HG-AAS).

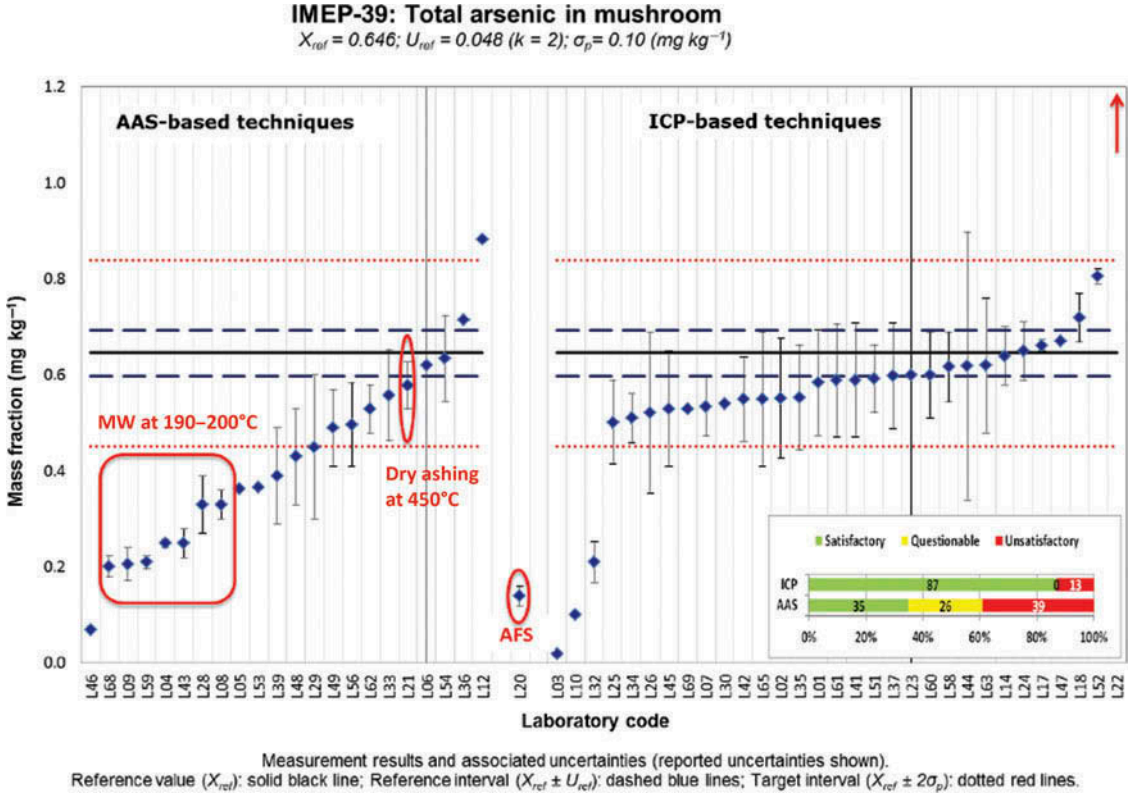

Figure 8. (colour online) Distribution of results reported for the total mass fraction of As on the basis of the technique used.

The high temperatures reached in the plasma would eliminate that problem when ICP-based techniques are used. The same would apply to methods that involve a final determination of total As using electrothermal atomic absorption spectrometry (ET-AAS), since atomisation temperatures in the graphite furnace are also very high. The problem of non-quantitative digestion would mostly affect the results obtained with hydride generation because only iAs species and, to a lesser extent, methylated As species can generate the hydride. This would also explain the underestimation of the total As mass fraction in the result reported by L20, which used atomic fluorescence spectrometry (AFS); the technique also requires generation of the As hydride before the final determination by AFS.

The observed underestimations are then not due to any effect directly related to AAS but to the use of low digestion temperatures. AAS-based techniques can be used if high temperatures are used for sample digestion (for instance dry ashing at 450°C), as shown by L21.

Laboratories using HG-AAS must also keep in mind that after digestion of the matrix with a mixture HNO_3 and H_2O_2 (mixture used by most of the participants in IMEP-39), if the digestion is quantitative, most As will be present in the form of As(V) and needs to be reduced to As(III) which is the As species generating the hydride with a higher yield. This means that a reduction step must be included and optimised prior to hydride generation to ensure quantitative reduction of As (V) to As(III).

For iAs determination, five out of the seven laboratories that obtained satisfactory *z*-scores in IMEP-39, used AAS-based techniques. If proper method validation is carried out AAS-based methods can be used and they are cheap and easy-to-use methods which can provide correct results. Regarding the selective determination of iAs using HPLC-ICP-MS, it has been reported in the literature that a significant decrease in the relative sensitivity of arsenite as opposed to arsenate has been observed at the low flow rates used for that type of hyphenation (Grotti et al. 2013). Hence a significant bias can be introduced if the oxidation state of iAs in the analysed sample is different from that in the standard solution used for calibration purposes. Laboratories using HPLC-ICP-MS should keep this information in mind when validating their methods for determination of iAs.

The influence of the technique used was not so significant for the total Cd, Pb and Hg mass fractions. However, it should be noted that the four lowest values reported for total Cd (L38, L43, L48 and L50) used AAS or ET-AAS. A similar observation was made for the total Pb mass fraction for which the three laboratories obtaining an unsatisfactory *z*-score due to a serious underestimation of this measurand (L05, L38 and L53) used AAS and ET-AAS. The majority of these participants used microwave assisted digestion with a mixture HNO_3 and H_2O_2 with temperatures between 190 and 200°C.

Tendency to overestimate the total Pb and Hg mass fractions

A relatively high number of laboratories reported unsatisfactory results in terms of *z*-scores for total Pb and Hg due to overestimation regardless the technique used. Four of the laboratories which obtained an unsatisfactory *z*-score for total Pb due to overestimation also did for total Hg (L10, L20, L22 and L56). Overestimation of the total Pb mass fraction could be due to contamination problems. Laboratories must pay attention to the purity of the reagents used via blank control, must use clean laboratory material and must carry out analyses in clean environments. It was not possible to find a suitable explanation for the overestimation of total Hg. Contamination in this case is not as likely to occur as in total Pb analysis. Nevertheless, regular blank controls must be regularly included in the analytical sequence.

Conclusions

The performance of the network of NRLs for all the investigated measurands can be considered satisfactory. The overall rates of satisfactory performance obtained by the NRLs (expressed as *z*-scores) ranged from 10% to 25% higher than the same rates in IMEP-39. When taking into consideration ζ-scores, the percentages of satisfactory performances are slightly lower than those for *z*-scores. This is particularly visible for the population of non NRLs. Only about half of the participants in IMEP-39 obtained satisfactory ζ-scores for total As, Pb and Hg and for iAs. This is closely related to the fact that a relatively high percentage of laboratories reported measurement uncertainties which were likely underestimated (case b).

Underestimation of the total As mass fraction can occur if not high enough temperatures (higher than 280°C) are used during the digestion of the sample. Laboratories using HG-AAS-based techniques for the final determination of As should be particularly careful. The high temperatures reached in the plasma when using ICP-based techniques would eliminate this bias.

Particularly interesting is the case of iAs. Sixteen NRLs reported values for this measurand (81% of which obtained a satisfactory *z*-score) which is a considerably higher number than in IMEP-107, the first PT organised by the EURL-HM in which iAs was covered. In IMEP-39, five out of the seven laboratories which obtained a satisfactory *z*-score for iAs, have used AAS-based techniques, showing that sound determinations of iAs can be made without the use of expensive sophisticated instrumentation.

Acknowledgements

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

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

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ABSTRACT

A collaborative trial, was conducted in accordance with international protocols 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) the subsequent extraction of the inorganic arsenic present in the acid medium using chloroform followed by back-extraction to acid medium. The final detection and quantification is done by flow injection hydride generation atomic absorption spectrometry (FI-HG-AAS).

Thirteen laboratories, from nine EU countries, registered for participation. All were experienced in the analysis of inorganic arsenic in various food commodities using FI-HG-AAS. The seven test items used in this exercise were all reference materials (either certified reference materials or test items of former IMEP proficiency tests) covering a broad range of matrices and concentrations: mussels tissue (EC-JRC-IRMM, ERM-CE278k), cabbage (IAEA, IAEA-359), seaweed (Hijiki) (NMIJ, CRM 7405a), fish protein (NRC, DORM-4), rice (EC-JRC-IRMM, IMEP-107), wheat (EC-JRC-IRMM, IMEP-112), mushrooms (EC-JRC-IRMM, IMEP-116) and finally rice (EC-JRC-IRMM, ERM-BC211), which was used as pre-test item for training purposes. The mass fraction of iAs was not known for all the test items used, for this reason five laboratories with recognised experience in the analysis of iAs were asked to analyse the test items using a method of their choice, different from the one being validated, in order to compare with the FI-HG-AAS method.

The relative standard deviation for repeatability of the validated method (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 %. The precision and trueness of the method made it fit-for-the-purpose of determining iAs mass fractions ranging from 0.074 to 7.55 mg kg^{-1}.

Keywords: Inorganic arsenic, collaborative trial, cereals, vegetables, mushrooms, mussels, fish, algae.

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 [1, 2, 3] or non-carcinogenic effects [4, 5]. Based on their chemical properties, the arsenic species are categorized as lipid-soluble or water-soluble arsenicals, the latter including both inorganic and organic compounds [6]. Inorganic arsenic species (As(III) and As(IV)) seem to be the most toxic and carcinogenic forms [7].

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) [8, 9, 10].

Until recently, most studies focused on the determination of total As in the diet [11]. 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 [4]. The 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 [12, 13]. 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 [14].

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) [15, 16]. 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 [17, 18]. 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 [19, 20]. 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) [21]. This method was validated in a collaborative trial in the frame of the IMEP-32 project [22] and has furthermore been used in studies on inorganic arsenic content in seafood and rice [17, 23]. Currently, CEN TC 275/WG 10 is validating a method for the selective

determination of iAs in food based on HPLC-ICP-MS. Other two standard methods have been published, GB/T 5009.11-2003 (in China) [24] and EN 15517:2008 [25], for the determination of abio arsenic in food and of iAs and in seaweed, respectively. Both methods are based on the selective determination of arsine 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 [26] (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, 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 [27].

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 non-expensive 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 [28] and applied to the determination of iAs in marine samples [29] 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 [30] can be downloaded from the webpage of the Institute for Reference Materials and Measurements of the Joint Research Centre (JRC-IRMM) [31]. 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 arseneous acid (MA). However, this species is typically only found as a minor arsenic species [32].

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) 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.

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

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, S2, S4 and S6 were relabelled to avoid their 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, S7 and S8, the supplied units were opened, pooled into a 5 L acid-washed plastic drum and placed in a 3D-mixer for 30 minutes (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 minutes, 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 NMIJ-7045a (S7) 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. 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.

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) HNO_3 and 1 % (v/v) H_2O_2 and left to stand overnight. Microwave irradiation was applied with the following temperature profile: 3 min ramp to 55 ^oC, 10 min at 55^oC, 2 min ramp to 75 ^oC, 10 min at 74 ^oC, 2 min ramp to 95^oC, 30 min at 95 ^oC. The extracts were centrifuged (10 min, 8000 rpm, 4 ^oC) 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} trifluoracetic acid containing 1 % (v/v) of a 30 % H_2O_2 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 % hydrogenperoxide (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 ^oC, 5 min at 50 ^oC, ramp in 2 min to 75 ^oC, 5 min at 75 ^oC, ramp in 4 min to 95 ^oC, 30 min at 95 ^oC). 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) HNO_3 and 1 % (w/v) H_2O_2 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.

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 [33].

In all cases (except for S5 and S8) the expert laboratories reported values with overlapping expanded measurement uncertainties, Figures 1a, 1b, 1c, 1e and 1.f. The uncertainty contribution due to characterization (u_{char}) was calculated according to ISO Guide 35 [33].

$$u_{char} = \frac{1}{p}\sqrt{\sum_1^p u_i^2} \qquad \text{Eq. 1}$$

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.

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

$$u_{char} = \frac{s}{\sqrt{p}} \qquad \text{Eq. 2}$$

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.

$$u_{CT} = \sqrt{u_{char}^2 + u_{bb}^2 + u_{st}^2} \qquad \text{Eq. 3}$$

For S7, u_{CT} was provided in the NMIJ certificate. For the former IMEP test items (S1, S2, S6) u_{bb} and u_{st} were extracted from the corresponding IMEP reports to participants. As for the remaining samples (S4, S5, S8) 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.

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 $Rec \pm 2u_{Rec}$ *(~ 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)

2.5 Organisation of the collaborative trial

A call for participants was published on the JRC-IRMM web site [31] 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 and S2), 8 g (S4), 5 g (S3, S5, S6, S7 and S8) 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 [30].

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 [34]. 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:

i) 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.

ii) 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;

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

Only the results reported by L03 for S4 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.

Table 4– List of identified outliers for the different matrices.

Sample	**Laboratory (number of outlying results)**	**Outlier type ****
S1 – Rice (IMEP-107)	L13 (6)	Cochran
S4 – Mussels (ERM-CE278k)	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

- 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 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) [30]. 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 as well as the high dispersion of data for S8 and S6 (in this sample about half of the total As mass fraction corresponds to organic compounds [35]) 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 and S8, while L13 had problems with S5 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, S5 and S8 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 for S4 and S8 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 [36]; and
- the overall analytical recovery R, is calculated as:

$$R = 100 \frac{X_{obs}}{X_{CT}} \quad \text{Eq. 4}$$

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

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

where $\cdot u_{obs}$ is the estimated standard deviation under reproducibility conditions (S_R).
$\cdot u_{CT}$ 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), 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.

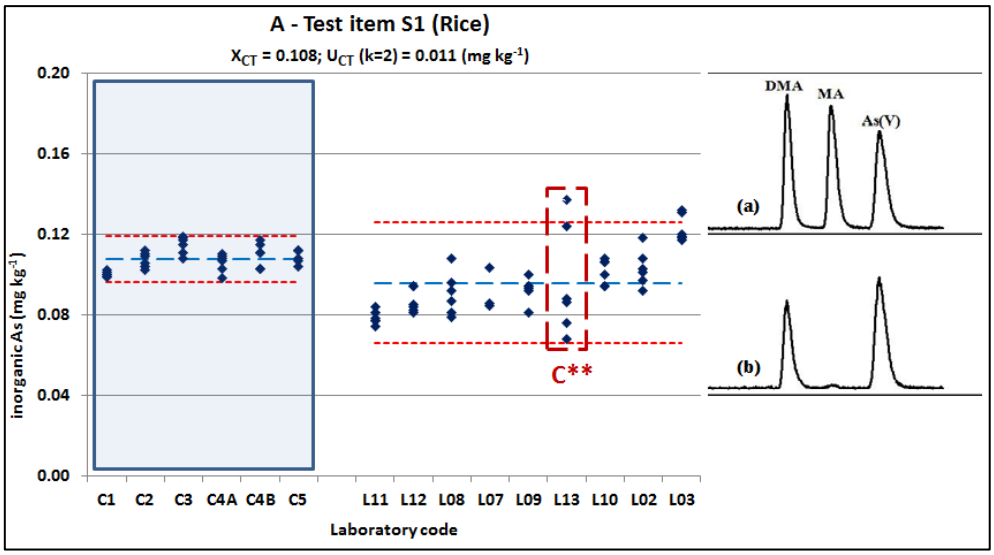
3.2 Degree of difficulty in the determination of iAs mass fraction in different types of matrices

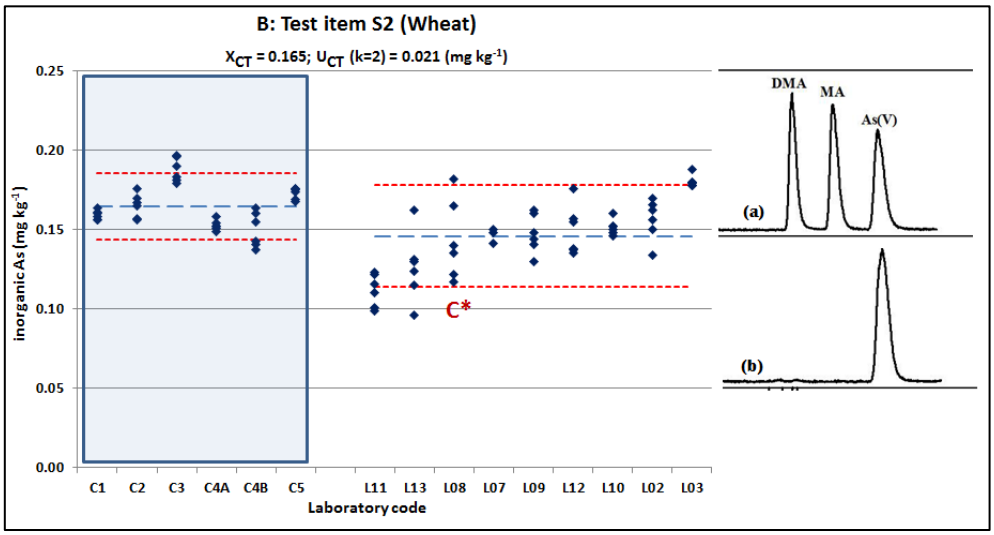
An evaluation of the results and of the comments reported by the participants in IMEP-41 on the method under validation, and by the expert laboratories using the method of their choice, made it possible to extract some conclusions about the inherent difficulty of iAs determination in different types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

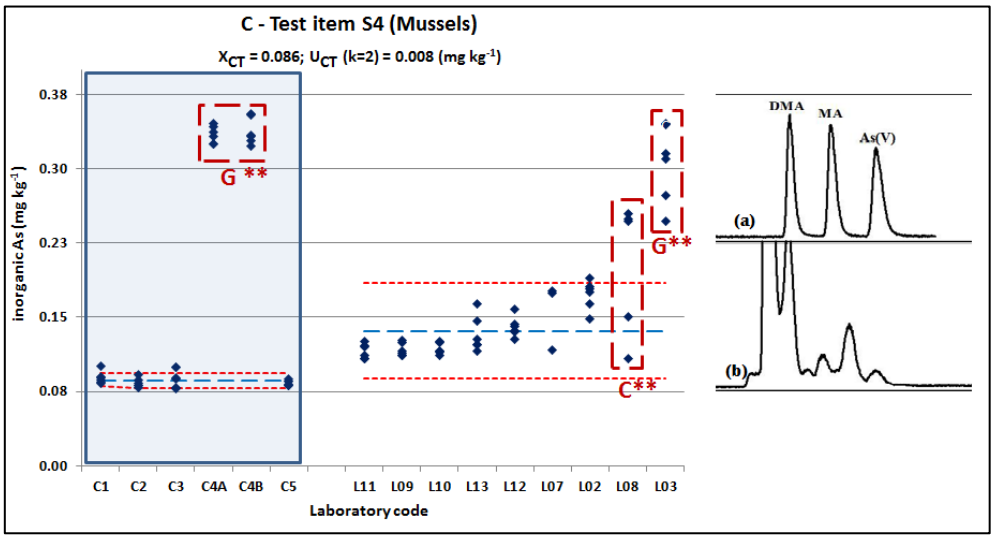
3.2.1 Matrices of marine origin

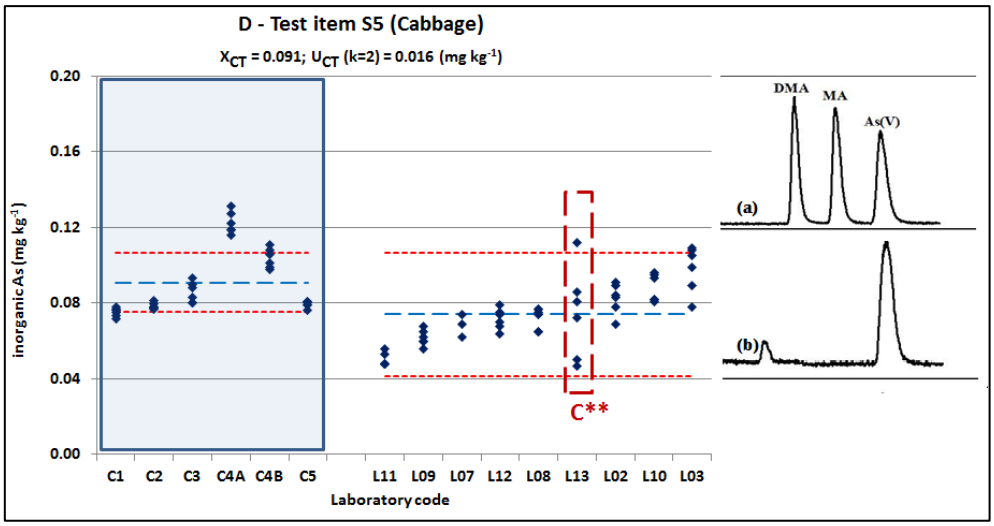
The selective determination of iAs seems to be particularly challenging in food of marine origin: mussels (S4), seaweed (S7) and fish (S8). In those samples iAs represents only a small fraction of the total As mass fraction (Figure 1 C, F and G). Samples of marine origin contain often a very large number 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 and S8, where the iAs mass fractions (0.086 ± 0.008 mg kg^{-1} and 0.27 ± 0.06 mg kg^{-1}, respectively), represent 1 and 4 % of the total As mass fraction (6.7 ± 0.4 mg kg^{-1} in S4 and 6.80 ± 0.64 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 ± 0.5 mg kg^{-1}) represents 28 % of the total As mass fraction (35.8 ± 0.9 mg kg^{-1}).

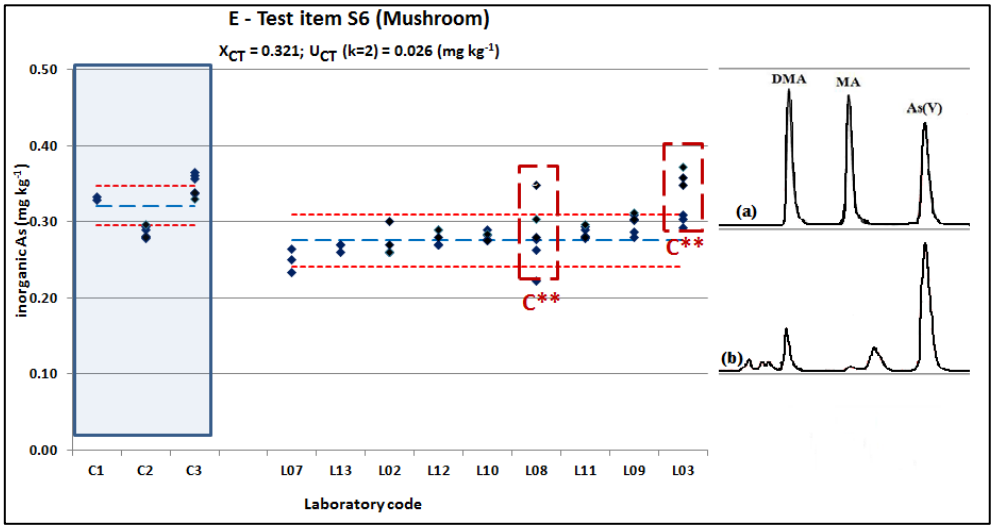
As discussed before, several laboratories (L03, L08 and L13) had problems with the analysis of S4 and/or S8, and reported results which were either biased or characterised by a large dispersion. The same difficulties were observed in the population of expert laboratories although only the results reported 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 that the results obtained by this laboratory for S4, using the two different techniques, for which the results are in good agreement.

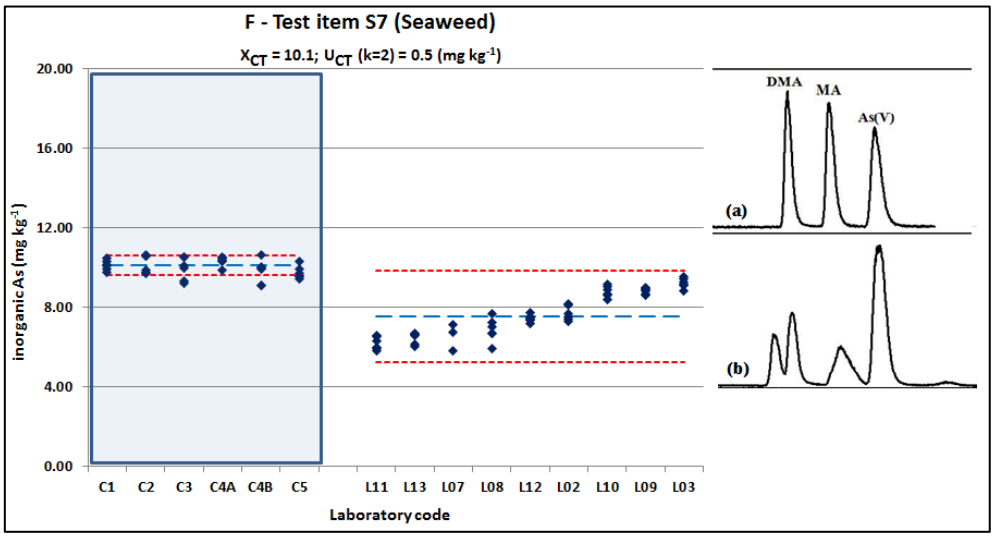

A - Test item S1 (Rice)
X_{CT} = 0.108; U_{CT} (k=2) = 0.011 (mg kg^{-1})
inorganic As (mg kg^{-1})
0.20
0.16
0.12
0.08
0.04
0.00
C1 C2 C3 C4A C4B C5 L11 L12 L08 L07 L09 L13 L10 L02 L03
Laboratory code
C**
DMA
MA
As(V)
(a)
(b)


B: Test item S2 (Wheat)
X_{CT} = 0.165; U_{CT} (k=2) = 0.021 (mg kg^{-1})
inorganic As (mg kg^{-1})
0.25
0.20
0.15
0.10
0.05
0.00
C1 C2 C3 C4A C4B C5 L11 L13 L08 L07 L09 L12 L10 L02 L03
Laboratory code
C*
DMA
MA
As(V)
(a)
(b)


C - Test item S4 (Mussels)
X_{CT} = 0.086; U_{CT} (k=2) = 0.008 (mg kg^{-1})
inorganic As (mg kg^{-1})
0.38
0.30
0.23
0.15
0.08
0.00
C1 C2 C3 C4A C4B C5 L11 L09 L10 L13 L12 L07 L02 L08 L03
Laboratory code
G **
G**
C**
DMA
MA
As(V)
(a)
(b)


D - Test item S5 (Cabbage)
X_{CT} = 0.091; U_{CT} (k=2) = 0.016 (mg kg^{-1})
inorganic As (mg kg^{-1})
0.20
0.16
0.12
0.08
0.04
0.00
C1 C2 C3 C4A C4B C5 L11 L09 L07 L12 L08 L13 L02 L10 L03
Laboratory code
C**
DMA
MA
As(V)
(a)
(b)


E - Test item S6 (Mushroom)
X_{CT} = 0.321; U_{CT} (k=2) = 0.026 (mg kg^{-1})
inorganic As (mg kg^{-1})
0.50
0.40
0.30
0.20
0.10
0.00
C1 C2 C3 L07 L13 L02 L12 L10 L08 L11 L09 L03
Laboratory code
C**
C**
DMA
MA
As(V)
(a)
(b)


F - Test item S7 (Seaweed)
X_{CT} = 10.1; U_{CT} (k=2) = 0.5 (mg kg^{-1})
inorganic As (mg kg^{-1})
20.00
16.00
12.00
8.00
4.00
0.00
C1 C2 C3 C4A C4B C5 L11 L13 L07 L08 L12 L02 L10 L09 L03
Laboratory code
DMA
MA
As(V)
(a)
(b)

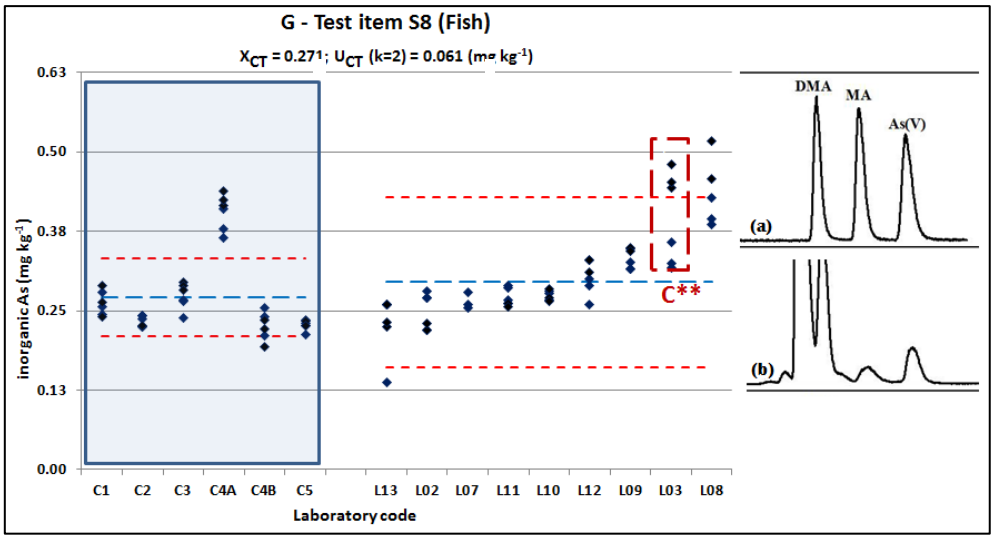


Figure 1 Measurement results of the expert laboratories (blue frame) and the CT participants. The blue dashed line corresponds to the mean of the respective results (X_{CT} in the case of the experts) and the red dashed lines the boundaries of the mean ($X_{CT} \pm U_{CT}$, for the expert laboratories and $X_{obs} \pm 2S_R$ for the participants of the CT). *C**, G** - Cochran and Grubbs outliers, C* - Cochran strangler, (a) - HPLC-ICP-MS chromatogram of standards, (b) -HPLC-ICP-MS chromatogram of the test item (Provided by expert C2).*

The results reported for S4 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 [32]. In the samples analyses by Muñoz et al. [29], MA did not exceed in any sample 0.4 % of the total As mass fraction. The content of MA in S4 (data provided by expert C2) is 0.183 ± 0.003 mg kg^{-1} (expressed as As), corresponding to 3 % of total As. The MA mass fraction in this test item is then 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.

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 by the other experts 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, 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 substraction of a reagent contribution to the blank without taking into consideration the dilution factor.
- Arsenosugars are the major arsenic compounds in marine algae [27]. Hijiki contains about 50 % arsenosugars which can be changed or completely destroyed by heating or acid treatment [37]. S7 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 ^{o}C. Under those conditions and according to the CRM producers [37], 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 hours, 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) [27]. 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 MMA. The pattern was slightly more complex in mushrooms (S6) where not only iAs, DMA and MMA 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 (Figure 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 obtain 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. The proposed method can be used to monitor iAs in food and help 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.

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Chapter 5

Occurrence of arsenic species in foodstuffs

Food provides nutrients, but also non-nutritional components and contaminants. To evaluate the risks and benefits associated to the intake of a given food, consumption level and the frequency should be taking into account. Food is generally consumed in processed form and is typically subjected to further culinary treatment before ingestion which may alter the concentration and chemical forms of an element. Humans are exposed to arsenic in the environment primarily through the ingestion of food and water [5]. Processing may cause a considerable increase or decrease in the real arsenic intake from food. Besides, for a better knowledge of the risks and benefits associated to food consumption, the estimation of arsenic bioavailability is needed. As a first step in bioavailability assessment is the study of bioaccessibility. The term bioavailability includes bioaccessibility and consequently bioaccessiblity studies could be an alternative approach to measure potential bioaccesibility. Therefore, accurate risk assessment studies should consider the effect of cooking as well as bioavailability (or bioaccessibility) of arsenic and arsenic species in foodstuffs.

Arsenic levels in food can vary by several orders of magnitude, with the arsenic present in many different molecular forms which vary substantially in toxicity. Some food commodities are able to accumulate high levels of arsenic which may represent a serious risk to consumer health. Arsenic is bioaccumulated in the marine food chain and tAs concentrations in the mg kg^{-1} range are usually found in marine organisms. Typical arsenic levels in algae and seaweeds are usually in the range of 1-100 mg As kg^{-1} depending of the algae species, meanwhile arsenic concentrations in the range of 1-30 mg As kg^{-1} have been reported in fish and shellfish. On the other hand, in samples of terrestrial origin the tAs concentration is typically < 0.3 mg As kg^{-1}. However, in certain cases concentrations up to 1 mg kg^{-1} have been reported [5]. Arsenic has a quite complex chemistry, especially in the marine environment, where more than 50 different naturally occurring As compounds have been identified, comprising both organic and inorganic forms. Among them and simplifying, the non-toxic AB was the major compound in fish and shellfish and the potentially toxic arsenosugars [100] were predominant in seaweeds. The speciation analysis in terrestrial foods is less complex than in marine foods, some arsenic species have been reported, but iAs usually predominates before other arsenic species, i.e. methylated species. Thus, toxic inorganic arsenic is usually predominant in rice, infant cereals and cereal products which are the most significant contributors to dietary exposure to inorganic arsenic.

Regarding EU maximum levels of iAs in foodstuffs, a maximum value of 10 μg L^{-1} is established for water intended for human consumption [139] without distinguishing forms of arsenic, while for natural mineral waters [140] a ML of 10 μg L^{-1} is laid down for total arsenic. Very recently, the European Union established maximum levels of iAs in rice and rice-based

products [107]. However, to date there are no maximum levels established for arsenic in foodstuffs other than rice at EU level, although some MLs are laid down in national legislation in some Member States. Although arsenic toxicology depends mostly on the chemical form, most countries food's regulation does not define a maximum allowed concentration in terms of specific As species, but only in terms of total As. Regulators have been reluctant to set maximum levels (ML) for arsenic species in food because of the molecular diversity of the arsenic species present.

With those considerations in mind and due to the toxicity of arsenic depends of their chemical species, we aimed to provide reliable arsenic speciation data in a several foodstuffs. This speciation studies may be useful for further studies on risk assessment and also in ongoing discussions in the European Commission and the CODEX Alimentarius for establishing and implementing future maximum levels of inorganic arsenic in food commodities. All these speciation studies are presented in **Chapter 5.**

As a summary, publications included in **Chapter 5** are presented below:

- ✓ **Article VI**
  Measurement of arsenic compounds in littoral zone algae from the Western Mediterranean Sea. Occurrence of arsenobetaine.
  Llorente-Mirandes, T., Ruiz-Chancho, M.J., Barbero, M., Rubio, R., López-Sánchez, J. F.
  Chemosphere, 2010, 81, 867–875.

- ✓ **Article VII**
  Determination of Water-Soluble Arsenic Compounds in Commercial Edible Seaweed by LC-ICPMS.
  Llorente-Mirandes, T., Ruiz-Chancho, M.J., Barbero, M., Rubio, R., López-Sánchez, J. F
  Journal of Agricultural and Food Chemistry, 2011, 59, 12963–12968.

- ✓ **Article VIII**
  Establishment of a method for determination of arsenic species in seafood by LC-ICP-MS.
  Zmozinski, A.V., **Llorente-Mirandes , T**., López-Sánchez, J.F., da Silva, M.M.
  Food Chemistry, 2015, 173, 1073–1082.

- ✓ **Article IV**
  Occurrence of inorganic arsenic in edible Shiitake (Lentinula edodes) products.
  Llorente-Mirandes, T., Barbero, M., Rubio, R., López-Sánchez, J.F.
  Food Chemistry, 2014, 158, 207–215.

- ✓ **Article V**

  Assessment of inorganic arsenic exposure in commercial edible mushrooms and mushroom supplements by HPLC-ICPMS.

  Llorente-Mirandes, T., Barbero, M., Rubio, R., López-Sánchez, J.F.

  Awaiting publication

- ✓ **Article IX**

  Assessment of arsenic bioaccessibility in raw and cooked edible mushrooms by a PBET method.

  Llorente-Mirandes, T., Llorens-Muñoz, M., Funes-Collado, V., Sahuquillo, A., López-Sánchez, J. F.

  Food Chemistry, 2016, 194, 849–856

5.1 Measurement of arsenic compounds in littoral zone algae from the Western Mediterranean Sea. Occurrence of arsenobetaine

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Measurement of arsenic compounds in littoral zone algae from the Western Mediterranean Sea. Occurrence of arsenobetaine

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Measurement of arsenic compounds in littoral zone algae from the Western Mediterranean Sea. Occurrence of arsenobetaine

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ABSTRACT

The determination of arsenic compounds in algae collected on the Catalan coast (Western Mediterranean) is reported. Ten algae species and the seagrass *Posidonia oceanica* were analyzed. Total arsenic in the samples was determined by microwave digestion and inductively coupled plasma mass spectrometry (ICPMS). Arsenic speciation in water extracts of samples was analyzed by liquid chromatography with both anionic and cationic exchange with ICPMS detection (LC–ICPMS). The total arsenic content of the algae samples ranged from 2.96 to 39.0 mg As kg^{-1}. The following compounds were detected: arsenite (As(III)), arsenate (As(V)), methylarsonate (MA), dimethylarsinate (DMA), sulfonate sugar (SO_3-sug), sulfate sugar (SO_4-sug), phosphate sugar (PO_4-sug), arsenobetaine (AB), arsenocholine (AC), trimethylarsine oxide (TMAO) and glycerol sugar (Gly-sug). The main arsenic species found were arsenosugars. Significant percentages of arsenobetaine (0.54 mg As kg^{-1}, 28% of the extractable arsenic and 0.39 mg As kg^{-1}, 18% of the extractable arsenic) were found in *Ulva rigida* and *Enteromorpha compressa*. These results are discussed in relation to the presence of epiphytes.

1. Introduction

Arsenic is present in the environment as a consequence of both anthropogenic and natural processes. It occurs in seawater mainly as inorganic arsenic, in the trivalent [As(III)] and pentavalent [As(V)] states, in the low μg L^{-1} range. Algae can accumulate and biotransform inorganic arsenic to arsenosugars (derivatives of dimethylarsinoylribosides and trimethylarsinoribosides), and among them the derivatives of dimethylarsinoylribosides commonly named glycerol sugar, phosphate sugar, sulfonate sugar and sulfate sugar are the most frequently occurring arsenic species in algae (Shibata et al., 1987; Francesconi and Edmonds, 1997). As well as these compounds, other organo arsenicals like MA, DMA, AB and AC and inorganic arsenic (As(III) and As(V)) can also be found in marine algae, but generally in lower amounts than arsenosugars. TMAO and tetramethylarsonium ion (TETRA) have also been reported in a very few studies (Hirata and Toshimitsu, 2007; Thomson et al., 2007a). Since the information obtained from total arsenic determination in a sample is not enough to assess the toxicological risk in environmental studies, analytical speciation of arsenic is necessary. This is especially important since algae are widely consumed in some countries due to their high mineral content and recognized therapeutic properties. The toxicity of arsenic in environmental and biological systems is strongly dependent on the chemical species (Irvin and Irgolic, 1995). The inorganic forms of arsenic are highly toxic, but arsenosugars are considered non-toxic although there are no reliable data (Niegel and Matysik, 2010). Detailed information concerning analytical methods for arsenic speciation can be found in several reviews (Szpunar, 2000; Francesconi and Kuehnelt, 2004; Niegel and Matysik, 2010). The main technique used in arsenic speciation analysis is separation of the species by LC, followed by detection with ICPMS (Francesconi and Edmonds, 1998; Madsen et al., 2000; Kohlmeyer et al., 2003; Slejkovec et al., 2006; Hirata and Toshimitsu, 2007; Ruiz-Chancho et al., 2010). Several research groups that have studied arsenic speciation in marine organisms agreed that AB is the main arsenic species found in animal tissues, but that it is unusual in algae. However, recent studies detected AB in some algae, at low concentrations (Nischwitz and Pergantis, 2005; Raab et al., 2005). Slejkovec et al. (2006) and Grotti et al. (2008) reported 12% and 17% of the extracted arsenic, respectively. Another researcher, Thomson et al. (2007a), reported as much

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as 47% of extracted arsenic. The metabolism of arsenic species in marine organisms has been intensively investigated. The most interesting aspect concerns the pathway for the biosynthesis of arsenobetaine, which is one of the crucial questions in arsenic biogeochemistry in marine systems (Francesconi et al., 1998). Several pathways for transforming arsenosugars to AB in algae have been put forward (Francesconi et al., 1999). Such a transformation is viable because, as has been demonstrated, arsenosugars become rapidly and quantitatively degraded by microbial activity into dimethyloxarsylethanol, which is a precursor of AB (Edmonds et al., 1982) or AC (Francesconi et al., 1992). Several authors (Nischwitz and Pergantis, 2005; Slejkovec et al., 2006; Thomson et al., 2007a) argued that the presence of AB in algae could be related to epiphytes not removed in sample pre-treatment.

The goal of our study was to assess, for the first time, arsenic speciation in a natural population of common littoral zone algae from the Western Mediterranean coast of Catalonia, focusing in particular on the arsenosugar species in these algae. The following algae were selected: *Ulva rigida*, *Codium effusum*, *Codium vermilara*, *Halopteris filicina*, *Halopteris scoparia*, *Enteromorpha compressa*, *Jania rubens*, *Cystoseira mediterranea*, *Cladophora prolifera* and *Alsidium corallinum* to assess the distribution of As compounds. Arsenic speciation in the flowering plant *Posidonia oceanica* was also carried out.

2. Materials and methods

2.1. Reagents and standards

All solutions were prepared with doubly deionised water obtained from Millipore water purification systems (Elix&Rios) (18.2 MΩ cm^{-1} resistivity and TOC, Total Organic Carbon <30 μg L^{-1}). Nitric acid (69%) (Panreac, Hiperpur), 98% formic acid (Panreac, p.a.), ammonium dihydrogen phosphate (Panreac, p.a.) and 25% aqueous ammonia solution (Panreac, p.a.), pyridine (Scharlau, p.a.) and 31% hydrogen peroxide (Merck, Selectipur) were used. Stock standard solutions (1000 mg L^{-1}) were prepared as follows: arsenite, from As_2O_3 (NIST, USA, Oxidimetric Primary Standard 83d, 99.99%) dissolved in 4 g L^{-1} NaOH (Merck, Suprapure); arsenate, from $Na_2HAsO_4{\cdot}7H_2O$ (Carlo Erba) dissolved in water; MA, prepared from $(CH_3)AsO(ONa)_2{\cdot}6H_2O$ (Carlo Erba) dissolved in water; DMA, prepared from $(CH_3)_2AsNaO_2{\cdot}3H_2O$ (Fluka) dissolved in water. AC from $(CH_3)_3As^+(CH_2)\ CH_2OHBr^-$ was supplied by the "Service Central d'Analyse" (CNRS Vernaison, France); a certified reference material of arsenobetaine (AB) from $(CH_3)_3As^+CH_2COO^-$ was supplied by BCR, CRM 626, standard solution; and TMAO was prepared from $(CH_3)_3AsO$ (Argus Chemicals srl) dissolved in water.

All the stock solutions were kept at 4 °C, and further diluted solutions for the analysis were prepared daily. Arsenate, arsenite, DMA, MA, AC, TMAO and AB were standardised against As_2O_3 (NIST Oxidimetric Primary Standard 83d) for our internal quality control. Arsenic standard solution from NIST High-Purity Standards with a certified concentration of 1000 ± 2 mg As L^{-1} was used as calibrant in the determination of total arsenic content with ICPMS.

NIES CRM 09 Sargasso (*Sargassum fulvellum*) seaweed, supplied by the National Institute for Environmental Studies (Japan), had a certified total arsenic content of 115 ± 9.2 mg As kg^{-1}.

An aliquot freeze-dried extract of *Fucus serratus*, kindly donated by Prof. K.A. Francesconi (Karl-Franzens University, Graz, Austria) (Madsen et al., 2000), containing the four common arsenosugars, i.e. phosphate (PO_4-sug), sulfate (SO_4-sug), sulfonate (SO_3-sug), and glycerol (Gly-sug), was used to assign the arsenosugar peaks in the chromatograms.

2.2. Instruments

A microwave digestion system, Milestone Ethos Touch Control, with a microwave power of 1000 W and temperature control, was used for digestion. An Agilent 7500ce ICPMS with a micro-flow nebuliser (Agilent, Germany) was used to measure total arsenic content. For arsenic speciation, LC–ICPMS was used with an Agilent 1200 LC quaternary pump, equipped with an autosampler. The analytical columns Hamilton PRP-X100 (250 × 4.1 mm, 10 μm, Hamilton, USA) and Zorbax-SCX300 (250 × 4.6 mm, 5 μm, Agilent) were protected by guard columns filled with the corresponding stationary phases. Table 1 summarises the chromatographic systems and conditions used in the study. The table reported the arsenic species able to be separated with both chromatographic systems. However in the present study As(III) co-elutes with some cationic arsenic species present in the extracts and further discussion on peak assignment of As(III) and the estimation of its concentration is reported. The outlet of the LC column was connected *via* PEEK capillary tubing to the nebuliser (BURGENER Ari Mist HP type) of the ICPMS system (Agilent 7500ce), which was the arsenic-selective detector. The ion intensity at *m*/*z* 75 (^{75}As) was monitored using time-resolved analysis software. Additionally, the ion intensities at *m*/*z* 77 ($^{40}Ar^{37}Cl$ and ^{77}Se) were monitored to detect possible argon chloride ($^{40}Ar^{35}Cl$) interference at *m*/*z* 75.

2.3. Sample collection

Ten algae species were collected in November 2008 in the zone of Lloret de Mar, on the Catalan coast (Spain) in the Western Mediterranean Sea (Fig. 1). The samples were selected on the basis of availability and of their different morphological and physiological characteristics. We sampled a variety of species and homogeneous samples representative of the entire population of algae that live in the area studied. Macroalgae samples of different sizes were carefully collected from the rocks with a flat-bladed knife and scissors, identified and separated according to their species, as shown in Table 2. For each species collected, a sample containing the thallus was set aside for identification. The samples were transported to the lab in plastic bags containing sea water in a refrigerator to prevent decomposition and changes of arsenic species and to maintain the conditions of their natural habitat. The samples were stored in the lab at 4 °C in plastic containers with sea water till sample preparation (up to 24 h). The marine plant *P. oceanica*, collected by scuba divers from Blanes beach, Catalan coast (Spain), in a previous

Table 1
Chromatographic conditions used for the separation of arsenic species.

Chromatography	Anionic exchange	Cationic exchange
LC	Quaternary pump, Agilent 1200 equipped with an autosampler	
Column	Hamilton PRP-X100 (250 mm × 4.1 mm, 10 μm)	Zorbax 300-SCX. (250 mm × 4.6 mm, 5 μm)
Pre column	Hamilton PRP-X100. (20 × 2.0 mm i.d., 10 μm)	Zorbax 300-SCX. (12.5 mm × 4.6 i.d., 5 μm)
Mobile phase	20 mM $NH_4H_2PO_4$ pH = 5.8 (adjusted with aqueous ammonia)	20 mM pyridine pH = 2.6 (adjusted with formic acid)
Flow rate (mL min^{-1})	1.5	1.5
Injection volume (μL)	20	20
Column temperature	Room temperature	Room temperature
Pressure (bar)	106	127
Arsenic species	As(III), As(V), MA, DMA, PO_4-sug, SO_3-sug and SO_4-sug	AB, AC, TMAO and Gly-sug

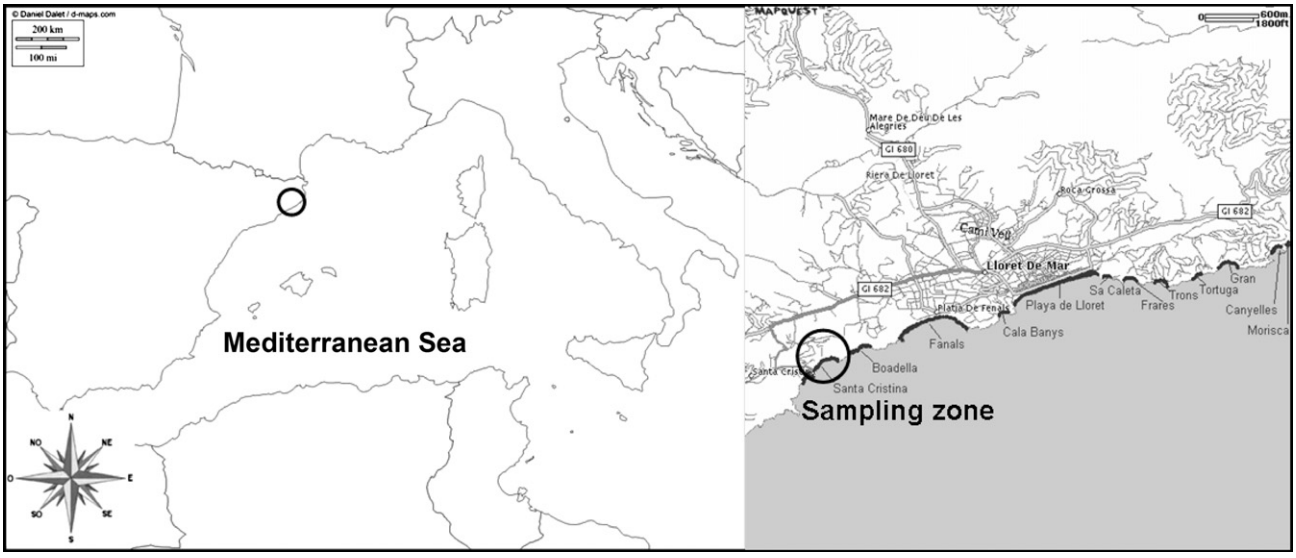

Fig. 1. Sampling zone in Catalan coast.

sampling by the Centre for Advanced Studies of Blanes (CEAB-CSIC), was also analyzed in the present study.

2.4. Sample pre-treatment

Seaweeds were washed with doubly deionised water to remove impurities (stones, sediments, salts, shells and small invertebrates) and the majority of mesofauna contamination. All the different algae species were observed under a stereomicroscope and then identified and classified according to taxonomic group. Epiphytes were eliminated manually under a stereomicroscope, as recommended (Rubio et al., 2010). Molluscs and other epiphytes (organisms that live on the surface of algae) that covered algae were finally eliminated by scraping samples with razor blades, scalpels and stainless steel tweezers cleaned with ethanol. Even so, some algae were fully covered by large numbers of epiphytes (red algae) on their surface, which were difficult to remove. Therefore, *C. vermilara* and *H. scoparia* were analyzed with and without epiphytes. Hence, we prepared two sub-samples for these algae, one cleaned by the procedure described above and the other one not cleaned (see Table 2). Then, the algae were filtered and washed thoroughly with deionised water to remove salts and subsequently dried in an oven at 40 °C for 24 h. The samples were further ground to a fine powder in a tungsten carbide disc mill for all subsequent analyses.

2.5. Procedures

2.5.1. Moisture determination

The moisture of the samples was determined in triplicate by oven drying 0.5 g aliquots at 100 ± 5 °C to constant weight. Moisture ranged from 3% to 10% and further results are referred to dry mass.

2.5.2. Total arsenic analysis

The total arsenic content in the algae samples and in the water extracts were determined by ICPMS after microwave digestion (Ruiz-Chancho et al., 2010), as follows: 0.2 g aliquots of the samples and the CRM (*S. fulvellum*) were weighed in the digestion vessels, and 8 mL of concentrated nitric acid and 2 mL of hydrogen peroxide were added. Mixtures were digested according to the following programme: 10 min from room temperature to 90 °C, maintained for 5 min at 90 °C, 10 min from 90 °C to 120 °C, 10 min from 120 °C to 190 °C and 10 min maintained at 190 °C. After cooling to room temperature, the digested samples were filtered through ash-free filter papers (Whatman 40) and diluted in water up to 20 mL. Before measurement of total As a dilution of 1/20 of the extract was made. He gas was used in the collision cell to remove interferences in ICPMS measurements. ^{103}Rh was used as internal standard. Triplicate analyses were performed for each sample. Samples were quantified by an external calibration curve (0–100 ng As mL^{-1}). For quality control purposes the standards of the calibration curve were run before and after each sample series. Digestion blanks are also prepared in each sample digestion series. In-between calibration checks were performed after every eighth samples.

2.5.3. Arsenic speciation analysis

The dried pulverised and homogenized samples (0.1 g for all the samples and the CRM) were weighed in 15-mL (polypropylene) tubes. Ten millilitres of water were added to each tube. The tubes were placed in an end-over-end shaker operating at 30 rpm for 16 h at room temperature. The resulting mixtures were centrifuged at 2800 rpm (10 min) and the supernatants filtered through PET filters (Chromafil® PET, Macherey–Nagel, pore size 0.45 μm). The extracts were diluted with water (1/10 or 1/20, depending on the sample) and total arsenic was determined by ICPMS (as described previously) and arsenic speciation was carried out in the extracts by LC–ICPMS by using the method previously applied to marine algae (Ruiz-Chancho et al., 2010). After extraction two chromatographic modes were used for separation of the arsenic species. Arsenite, arsenate, DMA, MA, PO_4-sug, SO_4-sug and SO_3-sug were analyzed by anion exchange chromatography, based on the method described by Gailer et al. (1999). AB, AC, TMAO and Gly-sug were analyzed by cation-exchange chromatography, based on the method described by Madsen et al. (2000). For both chromatographic systems, the performance characteristics are described in Table 1. Arsenic species in extracts were identified by comparison of retention times with standards. External calibration curves were used to quantify MA, DMA, arsenite, arsenate, AB, TMAO and AC with the corresponding standards. Extraction blanks were also analyzed by LC–ICPMS in each work session. The element response of the ICPMS detection system is independent of the species (Francesconi and Sperling, 2005). Thus, phosphate sugar was quantified with the calibration curve of MA standard, sulfate sugar and

Table 2
Taxonomical data of the algae studied. Total arsenic contents in the present study and from algae of different species and geographical zones but belonging to the same genus reported in the literature.

Alga species	Type	Phylum	Family	Total As (mg kg^{-1})	Reference
Cladophora sp.	Green	Chlorophyta	Cladophoraceae	4.2–9.3	Tukai et al. (2002) and Schaeffer et al. (2006)
Cladophora prolifera	Green	Chlorophyta	Cladophoraceae	13.9	Present study
Enteromorpha sp.	Green	Chlorophyta	Ulvaceae	1.4–49.5	Vasquez and Guerra (1996), Guven et al. (1998), Almela et al. (2002, 2006), Hansen et al. (2003), Serfor-Armah et al. (2006), Slejkovec et al. (2006)
Enteromorpha compressa	Green	Chlorophyta	Ulvaceae	6.2	Present study
Ulva sp.	Green	Chlorophyta	Ulvaceae	0.8–8.7	Vasquez and Guerra (1996), Guven et al. (1998), Serfor-Armah et al. (1999, 2006), Almela et al. (2002), Caliceti et al. (2002), Al-Masri et al. (2003), Hansen et al. (2003), Slejkovec et al. (2006), Moreda-Pineiro et al. (2007), Perez et al. (2007), Thomson et al. (2007a)
Ulva rigida	Green	Chlorophyta	Ulvaceae	5.3	Present study
Codium sp.	Green	Chlorophyta	Codiaceae	0.6–20.8	Francesconi and Edmonds (1998), Tukai et al. (2002), Hansen et al. (2003)
Codium effusum	Green	Chlorophyta	Codiaceae	20.4	Present study
Codium vermilara	Green	Chlorophyta	Codiaceae	27.0	Present study
Codium vermilara epiphytes	Green	Chlorophyta	Codiaceae	36.2	Present study
Halopteris hordacea	Brown	Heterokontophyta (Cl. Phaeophyceae)	Stypocaulaceae	16.2	Vasquez and Guerra (1996)
Halopteris filicina	Brown	Heterokontophyta (Cl. Phaeophyceae)	Stypocaulaceae	9.4	Present study
Halopteris scoparia	Brown	Heterokontophyta (Cl. Phaeophyceae)	Stypocaulaceae	9.1	Present study
Halopteris scoparia epiphytes	Brown	Heterokontophyta (Cl. Phaeophyceae)	Stypocaulaceae	11.5	Present study
Cystoseira sp.	Brown	Heterokontophyta (Cl. Phaeophyceae)	Cystoseiraceae	4.20–242	Guven et al. (1998), Caliceti et al. (2002), Al-Masri et al. (2003), Slejkovec et al. (2006)
Cystoseira mediterranea	Brown	Heterokontophyta (Cl. Phaeophyceae)	Cystoseiraceae	39.0	Present study
Alsidium corallinum	Red	Rhodophyta	Rhodomelaceae	11.0	Present study
Jania rubens	Red	Rhodophyta	Corallinaceae	0.97–10.6	Al-Masri et al. (2003) and Serfor-Armah et al. (2006)
Jania rubens	Red	Rhodophyta	Corallinaceae	2.0	Present study
Posidonia sp.	Angiosperms	Spermatophyta	Posidoniaceae	0.35–44.0	Grauby et al. (1991), Gosselin et al. (2006), Thomson et al. (2007b), Ruiz-Chancho et al. (2010)
Posidonia oceanica (seagrass)	Angiosperms	Spermatophyta	Posidoniaceae	2.96	Present study

sulfonate sugar were quantified with the calibration curve of As(V) standard, and glycerol sugar was quantified with the calibration curve of AC standard. The assignment of the AB peak was performed by matching the retention times of the standard. Moreover spiking of AB at two different concentrations levels were performed and the results were agree on the corresponding increases of the original AB concentration in the extract.

3. Results and discussion

3.1. Total arsenic determination

Total arsenic concentrations in the present study range from 2.0 to 39.0 mg As kg^{-1} and are given in Table 2. Moreover, results reported in the literature from algae of different species and geographical zones but belonging to the same genus are also shown for comparison purposes. Although in some cases reports from the same species could not be found, the comparison with algae from the same genus is relevant. Seaweed absorbs arsenic directly from sea water and then bio transforms it. Some factors influencing the distribution of As in algae and growth rates in macroalgae are the light intensity, turbidity, temperature, depth, salinity and nutrient uptake, among others. These factors may contribute to the variability of the results obtained in this study, as seen in Table 2. No results on total arsenic contents have been found in the literature for *A. corallinum*.

Some researchers (Morita and Shibata, 1990; Almela et al., 2002, 2006) affirm that there is a relationship between As content and group of algae, following the sequence (brown > red > green). The number of algae analyzed in the present study is too low to see a clear correlation between total arsenic content and algal type. According to our results, within the algae that were submerged at the same depth (always submerged), algae belonging to Chlorophyta (green algae) contain higher As content than other phyla, such as Rhodophyta (red algae) and Heterokontophyta (Phaeophyceae) (brown algae). *E. compressa* and *U. rigida* (green algae) were collected from rocky areas not always covered by water. Normally, such algae grow in areas disturbed by anthropogenic pollution, in zones where the wave action is moderate, on rocks and polluted by organic matter. The fact that these algae are less in contact with water than those that are completely submerged during their growth could explain the lower absorption of arsenic from sea water. The low arsenic content found in *J. rubens* (red algae) may be due to the obstruction of arsenic absorption, attributable to calcium carbonate, since this species is a calcified and articulated seaweed due to the accumulation of carbonate in the cell wall. For *C.*

Table 3
Concentrations expressed as mg As kg^{-1} on dry mass (mean ± SD, $n = 3$) of total arsenic and arsenic species in algae and seagrass samples. Detection and quantification limits for the As species in algae (mg As kg^{-1}).

Alga species	Total As	Arsenic species												Column Recovery (%)	Extraction efficiency (%)
		As(III)	DMA	MA	As(V)	AB	AC	PO_4-sug	SO_3-sug	SO_4-sug	Gly-sug	TMAO	Unknown anion	Unknown cation	
Cladophora prolifera	13.9 ± 0.8	<LOD	0.07 ± 0.01	<LOD	0.83 ± 0.03	0.24 ± 0.01	<LOD	<LOD	<LOD	<LOD	3.84 ± 0.05	<LOD	0.56 ± 0.06 (t_R = 236 s)	0.03 ± 0.01 (t_R = 388 s)	74.7	53.4
Enteromorpha compressa	6.2 ± 0.2	<LOD	0.03 ± 0.01	<LOD	0.14 ± 0.01	0.39 ± 0.01	<LOD	<LOD	<LOD	<LOD	1.56 ± 0.07	<LOD	0.04 ± 0.01 (t_R = 177 s)	0.03 ± 0.01 (t_R = 394 s)	75.3	46.7
Ulva rigida	5.3 ± 0.8	<LOD	0.07 ± 0.01	<LOD	0.11 ± 0.01	0.54 ± 0.02	<LOD	0.06 ± 0.01	<LOD	<LOD	0.84 ± 0.01	0.17 ± 0.08	0.08 ± 0.01 (t_R = 178 s)	0.04 ± 0.04 (t_R = 376 s)	78.7	45.6
Codium effusum	20.4 ± 0.7	<LOD	<LOD	<LOD	0.60 ± 0.03	0.05 ± 0.01	0.14 ± 0.03	0.33 ± 0.06	<LOD	1.30 ± 0.13	5.12 ± 0.16	<LOD	<LOD	<LOD	53.4	69.4
Codium vermilara	27.0 ± 2.3	1.36 ± 0.13	0.44 ± 0.01	0.22 ± 0.01	4.32 ± 0.23	0.10 ± 0.01	<LOD	<LOD	<LOD	<LOD	0.69 ± 0.03	0.07 ± 0.01	<LOD	0.51 ± 0.01 (t_R = 266 s)	58.7	47.4
Codium vermilara with epiphytes	36.2 ± 5.8	0.64 ± 0.34	1.73 ± 0.04	0.73 ± 0.04	10.4 ± 0.1	0.24 ± 0.06	<LOD	<LOD	<LOD	<LOD	1.40 ± 0.06	0.50 ± 0.06	<LOD	0.34 ± 0.03 (t_R = 264 s)	78.4	56.8
Halopteris filicina	9.4 ± 2.3	0.20 ± 0.09	<LOD	<LOD	1.50 ± 0.07	0.27 ± 0.01	<LOD	<LOD	1.45 ± 0.04	<LOD	0.99 ± 0.05	<LOD	<LOD	0.03 ± 0.01 (t_R = 373 s)	83.2	55.6
Halopteris scoparia	9.1 ± 3.4	<LOD	0.09 ± 0.03	<LOD	0.87 ± 0.07	0.16 ± 0.01	<LOD	<LOD	2.48 ± 0.32	<LOD	1.28 ± 0.07	<LOD	<LOD	<LOD	77.5	68.9
Halopteris scoparia with epiphytes	11.5 ± 0.5	0.02 ± 0.03	0.17 ± 0.01	<LOQ	1.15 ± 0.04	0.13 ± 0.02	<LOD	<LOD	1.77 ± 0.07	<LOD	0.94 ± 0.04	<LOD	<LOD	<LOD	72.0	50.9
Cystoseira mediterranea	39.0 ± 1.2	0.49 ± 0.19	0.26 ± 0.03	<LOD	1.17 ± 0.06	<LOD	<LOD	0.39 ± 0.04	19.6 ± 0.2	<LOD	2.21 ± 0.21	<LOD	1.17 ± 0.10 (t_R = 960 s)	<LOD	73.2	88.6
Alsidium corallinum	11.0 ± 0.1	<LOD	0.15 ± 0.01	<LOD	1.25 ± 0.02	0.22 ± 0.01	<LOD	0.13 ± 0.05	4.51 ± 0.25	<LOD	0.84 ± 0.06	<LOD	<LOD	<LOD	75.2	85.8
Jania rubens	2.0 ± 0.1	0.04 ± 0.01	<LOD	<LOD	0.12 ± 0.01	0.06 ± 0.01	<LOD	<LOD	<LOQ	<LOD	0.50 ± 0.02	<LOQ	<LOD	<LOD	86.9	52.3
Posidonia oceanica	2.96 ± 0.09	0.90 ± 0.07	<LOD	<LOD	<LOD	0.03 ± 0.01	<LOD	<LOD	<LOD	<LOD	0.06 ± 0.01	<LOD	<LOD	<LOD	77.4	43.2
Fucus serratus extract[a]		<LOD	0.01 ± 0.01	<LOD	<LOD	<LOD	<LOD	0.09 ± 0.01	0.64 ± 0.02	0.40 ± 0.01	0.10 ± 0.01	<LOD	<LOD	<LOD		
Sargassum fulvellum (CRM)	110.3 ± 0.7	<LOD	2.1 ± 0.1	<LOD	69.9 ± 1	<LOD	<LOD	2.2 ± 0.1	1.8 ± 0.1	9.0 ± 0.7	1.2 ± 0.2	<LOD	<LOD	<LOD	85.0	92.1
Detection limits (LOD)	0.033	0.005	0.007	0.009	0.017	0.002	0.005	0.015	0.061	0.089	0.008	0.009				
Quantification limits (LOQ)	0.109	0.016	0.025	0.031	0.058	0.007	0.018	0.050	0.205	0.297	0.028	0.030				

[a] Values for *F. serratus* extract are given as absolute amount for extract μg.

mediterranea we only analyzed its *caulidium*, which is perennial and, therefore, absorbs more arsenic from water than other algae. This might explain its higher arsenic concentration than other brown algae's.

The present study also examined the influence of epiphytes on the arsenic contents. As discussed above (Section 2.4). Two different algae species were analyzed: *C. vermilara* and *H. scoparia* were selected and two sub-samples (one containing the original epiphytes and another one in which the epiphytes were removed) were prepared. The results obtained for total arsenic were as follows: 36.2 mg kg^{-1} and 27.0 mg kg^{-1} in *C. vermilara* in sub-samples containing epiphytes and with epiphytes removed, respectively and 11.5 mg kg^{-1} and 9.1 mg kg^{-1} in *H. scoparia* in sub-samples containing epiphytes and with epiphytes removed, respectively. Thus, our results show that the presence of epiphytes in these algae increases total arsenic content.

3.2. Arsenic species extraction and occurrence

The arsenic compounds determined in our study are very polar and soluble in water. This is especially true for arsenosugars, some of which have strong polar character. Thus, a suitable solvent for extraction of arsenic species is probably water, provided that it can penetrate the sample's matrix (Francesconi and Kuehnelt, 2004). Thus, we chose water for arsenic species extraction. Wide ranges of extraction efficiencies are reported, even using the same extractant, depending on the particular algae type (Francesconi and Kuehnelt, 2004; Rubio et al., 2010). In the present study, extraction efficiencies (calculated as the ratio of total arsenic in the aqueous extract to total arsenic in the algae) are comparable with those reported and are shown in Table 3. The values ranged from 45.6% (*U. rigida)* to 88.6% (*C. mediterranea*). The lowest percentage among the samples studied corresponds to the seagrass *P. oceanica* (43.2%). The fraction of arsenic not extracted with water could be associated with lipids and might account for up to 50% of total arsenic in algae (Francesconi, 2003).

Table 3 shows the results on arsenic speciation and also the detection and quantification limits for arsenic species in algae. The results clearly show that arsenosugars are the most abundant arsenic compounds quantified, percentages ranging from 47.4% to 89.5% in most algae, except in *C. vermilara* and *C. vermilara* with epiphytes. However, from the results, no relationship can be established between the algae type (green, red or brown) and arsenosugar contents. Fig. 2a and b shows, as an example, two chromatograms obtained with both anion and cation-exchange chromatography of *C. mediterranea* extracts, in which the high proportion of SO_3-sug (19.6 mg As kg^1, corresponding to 78% of the arsenic extracted) can be seen. Table 3 reports some unknown peaks for both anionic and cationic columns, together with their retention time. Arsenic concentrations of these unknown compounds were estimated by using the calibration curves of the nearest eluting standard compounds, but we could not identify them. In Fig. 3a and b and for two algae species extracts, the retention time of the eluted peak near to 400 s could be attributed to TETRA, although from the literature consulted this compound has been analyzed under different cationic columns and elution conditions (different pH of the mobile phases, and different temperatures) (Kuehnelt et al., 2000; Madsen et al., 2000; Thomson et al., 2007a), so different retention times for this compound are reported.

In all the analyzed algae samples, Gly-sug was found. In some algae, Gly-sug is the predominant species, whereas in others it is minor (below 10% extracted arsenic). The range of percentages is wide, from 8.7% to 71.2% of the extracted As. SO_3-sug was detected in some algae within a range from 18% to 78% of the extracted As. Phosphate sugar was found in a few algae such as *U. rigida*, *C. effusum*, *C. mediterranea* and *A. corallinum*, but always in small proportions (below 5%). Sulfate sugar was only detected in *C. effusum*: 17.2% of the extracted As.

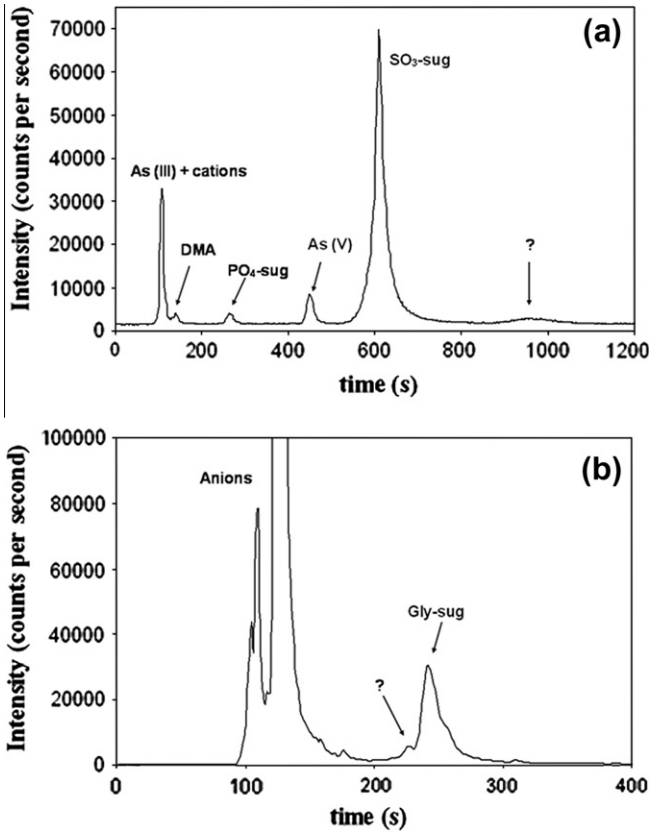

Fig. 2. Chromatograms of *Cystoseira mediterranea* extract from anion exchange (a) and cation exchange (b) by LC–ICPMS.

Regarding other arsenic compounds, DMA was identified in some algae but always in small proportions and below 11% of the extracted As. MA was only detected in small percentages in *C. vermilara*.

Arsenocholine is not a common compound in marine algae and is usually found at trace levels. Arsenocholine was only found in *C. effusum* at trace levels (0.14 mg As kg^{-1}), which confirms other reported values found in the literature (Hirata and Toshimitsu, 2007; Thomson et al., 2007a,b). TMAO has also been detected in small proportions in some algae such as *U. rigida*, *C. vermilara* and *C. vermilara* with epiphytes. To our surprise, AB was found in some algae in significant percentages, which is discussed in depth in the next section (see Section 3.3).

Some algae showed high concentrations of inorganic arsenic. In *C. vermilara* were found: 4.3 mg As kg^{-1} of As(V), corresponding to 57% of the extracted As. In the same alga with an epiphytic community, the highest content of As(V) was found: 10.4 mg As kg^{-1}, accounting for 64.2% of total As in extract. Moreover, *C. vermilara* also contains As(III): 1.4 mg As kg^{-1} (18% of extracted arsenic). In other algae, As(III) was also found, but below 5% of the extracted As. Even though the peak could correspond to some cationic specie which eluted in the front peak. The peak was identified as As(III) by comparison with the retention time of the standard in the anionic column. Its quantification in six algae and in *P. oceanica* (see Table 3) has been estimated by difference between the concentration corresponding to the integrated peak (As(III) + cations) eluted in the anionic column and the sum of cations eluted in the cationic column.

In *P. oceanica*, although the total arsenic content was very low, the main species found was As(III) (91.4%), whilst AB (2.6%) and Gly-sug were also detected. Only one arsenic speciation study on

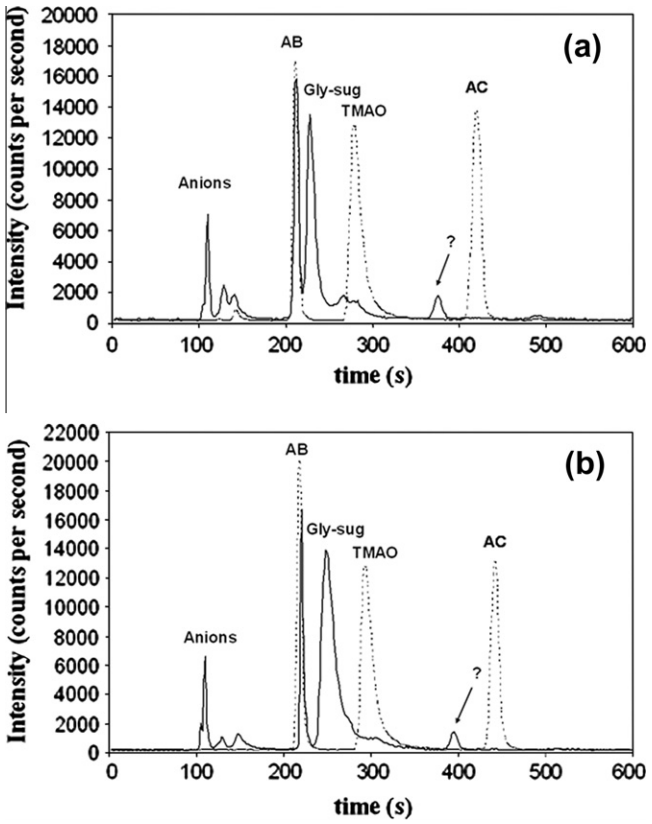

Fig. 3. Chromatograms of *Ulva rigida* (a) and *Enteromorpha compressa* (b) overlapped with the corresponding chromatograms of the standards both from cation exchange by LC–ICPMS. Continuous line: extracts. Dotted line: standards.

P. oceanica has been reported in the literature (Ruiz-Chancho et al., 2010), in which As(V) and Gly-sug were found as majority species. However, in both studies AB was detected. *P. oceanica* is well-known for the high quantity of epiphytes that cover its surface in some periods of the year (Dauby and Poulicek, 1995).

3.3. Occurrence of arsenobetaine: relationship with epiphytes

As stated above, the main arsenic compounds in algae are arsenosugars. Arsenobetaine, which is mainly present in marine animals and in many cases accounts for over 90% of the total arsenic, is not usual in algae. However, recent studies have detected AB in some algae at low concentrations (Nischwitz and Pergantis, 2005; Raab et al., 2005). Slejkovec et al. (2006) reported that AB accounted for 12% of the extracted As and another author reported 17% of the extracted arsenic in *Phyllophora antarctica* (Grotti et al., 2008). Thomson et al. (2007a) reported as much as 47% of the extracted arsenic (0.20 mg As kg^{-1}). Several authors (Nischwitz and Pergantis, 2005; Slejkovec et al., 2006; Thomson et al., 2007a) reported that the presence of arsenobetaine in algae might be related to epiphytes that were not removed in the sample pretreatment. In the present study, AB was detected in several samples and in two samples in particular: *U. rigida* and *E. compressa*. As it has been stated in Section 3.1 we observed that for two algae *C. vermilara and H. scoparia* the sub-samples containing epiphytes showed higher arsenic concentration than the sub-samples in which epiphytes were removed. The results of speciation proved that such higher contents of arsenic were unrelated to an increase in AB (see Table 3). Thus, *C. vermilara*, with and without epiphytes, showed 0.10 and 0.24 mg As kg^{-1} as AB, respectively. The higher values of total As in the uncleaned subsample is attributable to other arsenic compounds such as As(V) or Gly-sug. In *H. scoparia* we found similar AB concentration (0.16 mg As kg^{-1}) in the clean subsample and in the subsample with epiphytes (0.13 mg As kg^{-1}). Thus, according to these results in the algae species studied, the AB content cannot be attributed to the presence of epiphytes. In the present study, *U. rigida* and *E. compressa*, in which the highest percentages of AB were found, were collected in an area near the water surface and frequented by human. These algae are opportunistic species and grow in areas where there are disturbances, moderately beaten by the waves and on the rocks. The disturbance anthropogenic (contamination) increases the nutrient enrichment. It is generally accepted that bacterial populations decline in size from estuaries and inshore areas of greater organic and inorganic enrichment toward the more oligotrophic open sea (Ducklow, 2000). Fig. 3a and b shows two chromatograms obtained with cation-exchange chromatography of *U. rigida* and *E. compressa*, overlapped with the corresponding chromatograms of the standards; in which the presence of relatively high amounts of AB is clear. In the process of algae sample cleaning, we certainly removed all epiphytic material visible on a stereomicroscope, but not microorganisms (bacteria) and microparticles that are impossible to see on a stereomicroscope. Thus, we postulate that, in samples with high AB, the presence of microorganisms could transform arsenosugars into AB. There is support for such a hypothesis in studies arguing that microbial activity is responsible for arsenosugar transformation (Edmonds et al., 1982; Francesconi et al., 1992). Foster et al. (2008) suggested that in *Ecklonia radiata* the AB detected came from arsenosugar degradation by bacterial activity. In the present study we detected the presence of TMAO (unusual As compound in algae) in *U. rigida* and *E. compressa* (see Fig. 3), which could be due to some microbial degradation of AB. In all the samples in the present study AB was found except in *C. mediterranea* and in this particular case we could only collect the *caulidium*, the only part of this alga remaining in autumn. In the cleaning step it was decorticated and only the medulla was selected for further arsenic speciation, eliminating the possible bacterial population and other associated particles that could be at the origin of possible AB presence. This might explain that the SO_3-sug (78% of the arsenic extracted) found could not be degraded to AB. In summary, we think that AB origin in the algae studied is due to the presence of microorganisms and we rule out epiphytic origin. However, we cannot be sure whether microorganisms (bacteria) living on the algae surface accumulate AB or if they transform arsenosugars into AB. This is a question of current debate. Further interdisciplinary studies are needed for more consistent conclusions to be reached.

3.4. Quality assessment in the determination of arsenic species

3.4.1. Column recovery

In speciation studies mass balance between total element content and the total element extracted provides an estimation of the extraction yield. For quality assessment, column recovery must also be established, to guarantee the correctness of the chromatographic separation. With this aim, we calculated the ratio of the sum of the species eluted from the chromatographic columns with the total arsenic in the extract injected into the column. This parameter, assessed in replicates with good reproducibility, allowed us to evaluate correctly the quantification of the arsenic species. The values obtained for column recoveries, shown in Table 3, ranged between 53.4% and 86.9%. Low column recoveries could indicate the presence of other species that cannot be evaluated with the chromatographic separations used in the present study, which could be a subject for further research in this area.

3.4.2. Certified reference material (CRM)

CRMs are used throughout the study to assess the accuracy and the reliability of analytical results obtained. For the CRM NIES no. 9

S. fulvellum certified for total As content (115 ± 9 mg kg^{-1}), the result obtained (110.3 ± 0.7 mg As kg^{-1}) shows no significant differences with the certified value and demonstrates the accuracy of the analytical method. Regarding arsenic speciation in this CRM, most of the arsenic was found in the form of inorganic arsenic (69% of the extracted As), but we also detected and quantified the four arsenosugars commonly found in algae (sulfonate sugar, sulfate sugar, phosphate sugar and glycerol sugar) and DMA. The results are reported in Table 3. Our results corroborate the only study found in the literature in which arsenosugars were analyzed in this CRM (Ruiz-Chancho et al., 2010). Other authors (Garcia Salgado et al., 2006) analyzed this CRM and found 61% of inorganic arsenic.

3.4.3. Analysis of F. serratus extract

We used an extract from the brown seaweed *F. serratus* (Madsen et al., 2000) to identify arsenosugars present in our algae. For quality control, we quantified As species in *F. serratus* extracts: the results (Table 3) confirm those reported by Madsen et al. (2000) and other values listed in the literature on the same extract (Kohlmeyer et al., 2003; Slejkovec et al., 2006; Ruiz-Chancho et al., 2010). We detected, as well as the four arsenosugars and DMA, traces of MA and As(V).

4. Conclusions

For the first time, several algae species from the Catalan coast, on the Western Mediterranean Sea, were investigated for their arsenic species distribution. In the samples studied, the following species were found: As(III), As(V), MA, DMA, AB, TMAO, AC and arsenosugars, but the predominant arsenic fraction in water extracts of samples consisted of arsenosugars, except in *C. vermilara* samples in which inorganic arsenic predominated. Significant percentages of AB were detected in *U. rigida* and *E. compressa* and, according to our results, the AB content cannot be attributed to the presence of epiphytes and is probably due to the presence of microorganisms that transform arsenosugars into AB. The total arsenic content can be influenced by the presence of epiphytes in the samples. The results reported in the present study contribute to existing data on the distribution and metabolism of arsenic compounds in marine algae and the seagrass *P. oceanica*.

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5.2 Determination of Water-Soluble Arsenic Compounds in Commercial Edible Seaweed by LC-ICPMS

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Determination of Water-Soluble Arsenic Compounds in Commercial Edible Seaweed by LC-ICPMS

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Supporting Information

ABSTRACT: This paper reports arsenic speciation in edible seaweed (from the Galician coast, northwestern Spain) produced for human consumption. *Chondrus crispus*, *Porphyra purpurea*, *Ulva rigida*, *Laminaria ochroleuca*, *Laminaria saccharina*, and *Undaria pinnatifida* were analyzed. The study focused on arsenosugars, the most frequently occurring arsenic species in algae. As(III) and As(V) were also determined in aqueous extracts. Total arsenic in the samples was determined by microwave digestion and inductively coupled plasma mass spectrometry (ICPMS). For arsenic speciation, a water extraction especially suitable for arsenosugars was used, and the arsenic species were analyzed by liquid chromatography with both anionic and cationic exchange and ICPMS detection (LC-ICPMS). The total arsenic content of the alga samples ranged from 5.8 to 56.8 mg As kg^{-1}. The mass budgets obtained in the extracts (column recovery × extraction efficiency) ranged from 38 to 92% except for *U. pinnatifida* (4%). The following compounds were detected in the extracts: arsenite (As(III)), arsenate (As(V)), methylarsonate (MA), dimethylarsinate (DMA), sulfonate sugar (SO_3-sug), phosphate sugar (PO_4-sug), arsenobetaine (AB), and glycerol sugar (Gly-sug). The highest concentrations corresponded to the arsenosugars.

KEYWORDS: arsenic speciation, LC-ICPMS, edible seaweed, inorganic arsenic, arsenosugars

INTRODUCTION

Marine algae have high contents of iodine, minerals, and vitamins and form part of the human diet, especially in Asian countries, due to their recognized therapeutic properties.[1] Several countries cultivate seaweed for industrial purposes because they are used as a source of agar, carrageenans, and alginates; China, Korea, Japan, and the Philippines are the leaders in such production. Each algal species requires different farming methods as several factors, such as the morphology and regeneration capacity of the thallus, as well as the complex interactions between irradiance, temperature, nutrients, and water movements, are responsible for the success of large-scale seaweed production.[2] In the European Union (EU), seaweeds are considered novel foods, defined as food that does not have a significant history of consumption within the EU before May 15, 1997,[3] although it is a subject of controversy in some EU countries.[4] Nowadays different types of edible seaweed are increasingly consumed in many European countries. In Spain, seaweed is not a widespread constituent of the diet, although the number of consumers has increased considerably in recent years. The edible seaweed sold in Spain is mainly cultivated in the northern area of the country, although it is also imported from Asian countries. Many types of seaweed are consumed raw or after only light cooking, for example, *Porphyra* spp. (red algae, commercialized as "nori"), which is frequently consumed and rich in proteins and vitamins B and C. However, *Chondrus* spp. (red algae, commercialized as "Irish moss") is an industrial source of carrageenan, which is commonly used as a thickener and stabilizer in milk products such as ice cream and processed foods including luncheon meats and is also commonly eaten raw in salads and cooked in soups. *Ulva rigida* (green algae, commonly called "sea lettuce") is eaten raw in salads, cooked in soups, or served as a side dish to accompany fish or seafood; it is high in protein, soluble dietary fiber, and a variety of vitamins and minerals, especially iron. Brown algae, *Laminaria* spp. (generic commercial name, "kombu", with more than 12 species), and *Undaria* spp. (generic name "wakame") are consumed the most worldwide.

Seaweed can absorb arsenic (mainly inorganic) from seawater and can accumulate and biotransform this arsenic into less toxic organo-arsenicals. The arsenic species usually identified in seaweed are arsenosugars (derivatives of dimethylarsinoylribosides and trimethylarsonioribosides).[5,6] The structures of the four arsenosugars most reportedly found in algae are presented in Figure 1. As well as these compounds, other organo-arsenicals such as methylarsonate (MA), dimethylarsinate (DMA), trimethylarsine oxide (TMAO), the tetramethylarsonium ion (TETRA), arsenobetaine (AB), arsenocholine (AC), and inorganic arsenic (As(III) and As(V)) can also be found in some seaweed. Although arsenosugars are the most abundant arsenic compounds found in algae, some researchers have detected high contents of inorganic arsenic in some seaweed,[7] mainly in *Hizikia fusiforme*.[8−11] Speciation of arsenic in food analysis is

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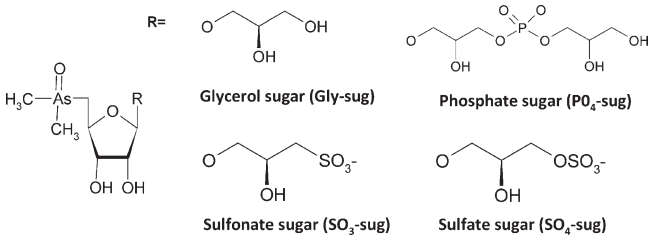

Figure 1. Structures of the four arsenosugars commonly found in algae.

necessary to evaluate the toxicological risk, which is strongly related to the specific chemical molecule.[12] The International Agency for Research on Cancer (IARC) considers inorganic arsenic compounds (arsenite and arsenate) highly toxic and classifies them as group I (human carcinogens).[13] The same considerations are reported by the Joint FAO/WHO Expert Committee on Food and Additives[14] and by the European Food Safety Authority.[15,16] Organic forms of arsenic, such as arsenobetaine and arsenosugars, are considered to be nontoxic, although there are no reliable data on arsenosugars.[17] Recent publications suggest that arsenosugars should be reported as potentially toxic arsenic compounds due to the fact that they are metabolized by humans and so far there are no conclusive results about their toxicity.[11,18] Specific regulations for toxic elements in edible seaweed have been established in the United States with 3 mg kg^{-1} (dw) as the maximum permitted inorganic arsenic.[19] Other countries such as Australia and New Zealand have established different limits for inorganic As in seaweed: 1 mg As kg^{-1} (dw).[20] In the EU the Commission Regulations do not establish maximum levels for arsenic in food.[21] France was the first European country to regulate the human consumption of seaweed as a nontraditional food substance, and the French limit for inorganic As in edible seaweed is 3 mg As kg^{-1} (dw). Currently, 12 macroalgae (6 brown algae, 4 red algae, 2 green algae) and 2 microalgae are licensed in France as vegetables and condiments.[19,22] There is no specific legislation regarding seaweed in Spain, and the only Spanish legislation concerns seaweed for animal consumption; it establishes a maximum level of 2 mg As kg^{-1} (dw) for inorganic As and warns of the possible risk of *H. fusiforme*.[23]

The aim of the present study is to determine the total arsenic content, inorganic arsenic as well as organoarsenicals (some of them potentially toxic), in commercially available edible seaweed and to evaluate the safety and assess the risk associated with its consumption. This may contribute to increase the availability of reliable results, which will be necessary for establishing and implementing future EU directives on inorganic and organic arsenic compounds in edible seaweed and for further studies on risk assessment. This study also focuses on the speciation of arsenic in different types of edible seaweed. To carry out the study, six seaweed (*Chondrus crispus* (Irish moss), *Porphyra purpurea* (nori), *Ulva rigida* (sea lettuce) *Laminaria ochroleuca* (kombu), *Laminaria saccharina* (kombu), and *Undaria pinnatifida* (wakame)) were selected and purchased in retail stores in Barcelona (Spain). Water was used as the extracting reagent, and a coupled technique, LC-ICPMS, was applied to quantify the arsenic species detected using both anionic and cationic chromatographic systems. We used an extract from the seaweed *Fucus serratus*, in which four dimethylarsinoylribosides were previously identified and quantified,[24] to identify the arsenosugars present in our seaweed samples. Total arsenic in the samples was also determined using microwave digestion and ICPMS. The certified reference material (CRM) NIES 9 *Sargassum fulvellum* was used throughout the study to assess the accuracy and the reliability of the analytical results.

MATERIALS AND METHODS

Reagents and Standards. All solutions were prepared with doubly deionized water obtained from Millipore water purification systems (Elix & Rios) (18.2 MΩ cm^{-1} resistivity and total organic carbon < 30 $\mu g\ L^{-1}$). Nitric acid (69%) (Panreac, Hiperpur), 98% formic acid (Panreac, p.a.), ammonium dihydrogen phosphate (Panreac, p.a.), 25% aqueous ammonia solution (Panreac, p.a.), pyridine (Scharlau, p.a.), and 31% hydrogen peroxide (Merck, Selectipur) were used. Stock standard solutions (1000 mg L^{-1}) were prepared as follows: arsenite, from As_2O_3 (NIST, USA, Oxidimetric Primary Standard 83d, 99.99%) dissolved in 4 g L^{-1} NaOH (Merck, Suprapure); arsenate, from $Na_2HAsO_4 \cdot 7H_2O$ (Carlo Erba) dissolved in water; MA, prepared from $(CH_3)AsO(ONa)_2 \cdot 6H_2O$ (Carlo Erba) dissolved in water; and DMA, prepared from $(CH_3)_2AsNaO_2 \cdot 3H_2O$ (Fluka) dissolved in water. AC from $(CH_3)_3As^+(CH_2)CH_2OHBr^-$ was supplied by the "Service Central d'Analyse" (CNRS Vernaison, France); arsenobetaine (AB) from $(CH_3)_3\ As^+CH_2COO^-$ was supplied by BCR, as CRM 626 standard solution; and TMAO was prepared from $(CH_3)_3AsO$ (Argus Chemicals srl) dissolved in water.

All of the stock solutions were kept at 4 °C, and further diluted solutions for the analysis were prepared daily. Arsenate, arsenite, DMA, MA, AC, TMAO, and AB were standardized against As_2O_3 (NIST Oxidimetric Primary Standard 83d) as our internal control. Arsenic standard solution from NIST High-Purity Standards with a certified concentration of 1000 ± 2 mg As L^{-1} was used as the calibrant in the determination of total arsenic content using ICPMS.

CRM NIES 9 Sargasso (*S. fulvellum*) seaweed, supplied by the National Institute for Environmental Studies (Japan), had a certified total arsenic content of 115 ± 9.2 mg As kg^{-1}.

An aliquot of freeze-dried extract of *F. serratus*, containing the four common arsenosugars, that is, phosphate (PO_4-sug), sulfate (SO_4-sug), sulfonate (SO_3-sug), and glycerol (Gly-sug),[24] was used to identify the arsenosugar peaks in the chromatograms.

Instruments. A microwave digestion system, Milestone Ethos Touch Control, with a microwave power of 1000 W and temperature control, was used for digestion. An Agilent 7500ce ICPMS with a microflow nebulizer (Agilent, Germany) was used to measure total arsenic content. For arsenic speciation, LC-ICPMS was used with an Agilent 1200 LC quaternary pump, equipped with an autosampler. The analytical columns, a Hamilton PRP-X100 (250 × 4.1 mm, 10 μm, Hamilton, USA) and Zorbax-SCX300 (250 × 4.6 mm, 5 μm, Agilent), were protected by guard columns filled with the corresponding stationary phases. Chromatographic conditions are reported.[25] The outlet of the LC column was connected via PEEK capillary tubing to the nebulizer (BURGENER Ari Mist HP type) of the ICPMS system (Agilent 7500ce), which was the arsenic-selective detector. The ion intensity at m/z 75 (^{75}As) was monitored using time-resolved analysis software. Additionally, the ion intensities at m/z 77 ($^{40}Ar^{37}Cl$ and ^{77}Se) were monitored to detect possible argon chloride ($^{40}Ar^{35}Cl$) interference at m/z 75.

Samples. Dry algae (from the Galician coast) were purchased in a food market in Barcelona, Spain. Six seaweed samples were analyzed in this study: *C. crispus*, *P. purpurea*, *U. rigida*, *L. ochroleuca*, *L. saccharina*, and *U. pinnatifida*. Details of algal taxonomy can be found in the Supporting Information (Table SI-1). The samples were dried in an oven at 40 °C for 24 h and then ground to a fine powder in a tungsten carbide disk mill.

Table 1. Concentrations (Mean ± SD, $n = 3$) of Total Arsenic and Arsenic Species in Alga Samples as Well as Detection (LOD) and Quantification Limits (LOQ) for the As Species in Algae[a]

trade name	algae species	total As	arsenic species (mg As kg^{-1}, dw)													
			As(III)	DMA	MA	As(V)	inorganic As[a]	AB	AC	PO_4-sug	SO_3-sug	SO_4-sug	Gly-sug	TMAO	column recovery (%)	extraction efficiency (%)
Irish moss	*Chondrus crispus*	18.2 ± 0.5	0.5 ± 0.05	<LOD	<LOD	<LOD	0.51	<LOD	<LOD	9.2 ± 0.3	<LOD	<LOD	8.4 ± 0.2	<LOD	92.1	99.5
nori	*Porphyra purpurea*	40.7 ± 2.8	<LOD	0.53 ± 0.07	<LOD	<LOD	<LOD	<LOD	<LOD	27.6 ± 3.0	<LOD	<LOD	2.02 ± 0.04	<LOD	75.2	98.6
sea lettuce	*Ulva rigida*	5.8 ± 0.4	<LOD	0.05 ± 0.04	<LOD	0.30 ± 0.01	0.30	0.10 ± 0.01	<LOD	0.29 ± 0.02	<LOD	<LOD	1.45 ± 0.02	<LOD	72.0	52.6
kombu	*Laminaria ochroleuca*	56.8 ± 2.4	<LOD	0.26 ± 0.08	<LOD	<LOD	<LOD	0.20 ± 0.02	<LOD	6.2 ± 0.1	39.4 ± 1.6	<LOD	2.71 ± 0.04	<LOD	98.2	87.4
kombu	*Laminaria saccharina*	52.4 ± 2.1	<LOD	0.67 ± 0.02	0.21 ± 0.03	<LOD	<LOD	0.09 ± 0.02	<LOD	6.9 ± 0.2	30.7 ± 1.2	<LOD	2.9 ± 0.1	<LOD	71.7	110.6
wakame	*Undaria pinnatifida*	41.0 ± 2.6	<LOD	0.13 ± 0.03	<LOD	0.29 ± 0.03	0.29	<LOD	<LOD	0.30 ± 0.02	<LOD	<LOD	0.87 ± 0.03	<LOD	13.3	29.1
sargasso	*Sargassum fulvellum* (CRM)	110.3 ± 0.7 (certified value: 115 ± 9)	<LOD	2.1 ± 0.1	<LOD	69.9 ± 1.0	69.9	<LOD	<LOD	2.2 ± 0.1	1.80 ± 0.10	9.0 ± 0.7	1.2 ± 0.2	<LOD	85.0	92.1
fucus	*Fucus serratus* extract[b]		<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	0.09 ± 0.01	0.64 ± 0.02	0.40 ± 0.01	0.10 ± 0.01	<LOD		
LOD (mg As kg^{-1})		0.007	0.005	0.007	0.009	0.017		0.002	0.005	0.015	0.061	0.089	0.008	0.009		
LOQ (mg As kg^{-1})		0.024	0.016	0.025	0.031	0.058		0.007	0.018	0.050	0.205	0.297	0.028	0.030		

[a] Inorganic arsenic = [As(III) + As(V)]. [b] Values for *F. serratus* extract are given as absolute amount for extract in μg.

Procedures. *Moisture Determination.* The moisture of the samples was determined in triplicate by oven-drying 0.5 g aliquots at 100 ± 5 °C to constant weight. Moisture ranged from 7 to 10%, and all further results refer to dry mass.

Total Arsenic Analysis. The total arsenic content of the algal samples and CRM *S. fulvellum* was determined in triplicate by ICPMS measurement after digestion[25,26] and in the aqueous extracts obtained for speciation purposes. For this, 0.2 g aliquots of the samples or the CRM (*S. fulvellum*) were weighed in the digestion vessels, and 8 mL of concentrated nitric acid and 2 mL of hydrogen peroxide were added. The mixtures were digested according to the following program: 10 min from room temperature to 90 °C, maintained for 5 min at 90 °C, 10 min from 90 to 120 °C, 10 min from 120 to 190 °C, and 10 min maintained at 190 °C. After cooling to room temperature, the digested samples were filtered through ash-free filter papers (Whatman 40) and diluted in water to 20 mL. For the final measurements further dilution was carried out when necessary. He gas was used in the collision cell to remove interferences in the ICPMS measurements. ^{103}Rh was used as the internal standard. The samples were quantified by means of an external calibration curve (0−100 ng As mL^{-1}). For quality control purposes, the standards of the calibration curve were run before and after each sample series. Digestion blanks were also prepared in each sample digestion series. Calibration checks were performed after every eight samples.

Arsenic Speciation Analysis. The dried pulverized and homogenized samples and the CRM (0.1 g) were weighed in 15 mL (polypropylene) tubes. Ten milliliters of water was added to each tube. The tubes were placed in an end-over-end shaker operating at 30 rpm for 16 h at room temperature. The resulting mixtures were centrifuged at 2800 rpm (10 min) and the supernatants filtered through PET filters (Chromafil PET, Macherey-Nagel, pore size = 0.45 μm). The extracts were diluted with water (1/10 or 1/20, depending on the total arsenic content of the sample). Total extracted arsenic was determined by ICPMS (as described above) and arsenic speciation was carried out on the extracts by LC-ICPMS using a method previously applied to marine algae.[25,26] Arsenic species in the chromatograms were identified by comparison of the retention times with those of the standards. External calibration curves were used to quantify MA, DMA, arsenite, arsenate, AB, TMAO, and AC against the corresponding standards. Extraction blanks were also analyzed by LC-ICPMS in each session. The element response of the ICPMS detection system is independent of the species,[27] and arsenosugars with no standards available were quantified using the calibration curves of the nearest eluting standard compound. Thus, PO_4-sug was quantified with the calibration curve of the MA standard, SO_4-sug and SO_3-sug were quantified with the calibration curve of the As(V) standard, and Gly-sug was quantified with the calibration curve of the AC standard.

RESULTS AND DISCUSSION

Quality Assessment in the Determination of Arsenic Species. *Column Recovery.* In speciation studies, the mass balance between total element content and the total element extracted provides an estimation of the extraction yield. For quality assessment, column recovery must also be established to guarantee the correctness of the chromatographic separation. To this end, we calculated the ratio of the sum of the species eluted from the chromatographic column to the total arsenic in the extract injected into the column. This parameter, assessed in replicates with good reproducibility, allowed us to evaluate our quantification of the arsenic species. The values obtained for column recoveries, shown in Table 1, ranged between 72 and 98% with one exception. That was *U. pinnatifida* (13.3%) and

could indicate the presence of other species in this sample that cannot be evaluated with the chromatographic separations used in the present study; this could be the subject of further research in this area.

Certified Reference Material. CRM NIES 9 *S. fulvellum* was used throughout the study to assess the accuracy and the reliability of the analytical results. For total As, the result obtained (110.3 ± 0.7 mg As kg^{-1}) shows good agreement with the certified value content (115 ± 9 mg kg^{-1}) and demonstrates the accuracy of the analytical method. With regard to arsenic speciation in this CRM, most of the arsenic was found in the form of inorganic arsenic (69% of extracted As), but we also detected and quantified the four arsenosugars commonly found in algae (sulfonate sugar, sulfate sugar, phosphate sugar, and glycerol sugar) and DMA. The results are shown in Table 1 and are in agreement with those obtained in a previous study in which we analyzed the arsenosugars in this CRM.[26]

Analysis of F. serratus Extract. The extract from the brown seaweed *F. serratus* was analyzed to identify the arsenosugars present in the samples. Gly-sug, PO_4-sug, SO_4-sug, and SO_3-sug were identified. DMA and traces of MA and As(V) were also detected. The results, shown in Table 1, are in agreement with those reported in ref 24 and with other values for the same extract in the literature.[26,28]

Total Arsenic Content. Total arsenic concentrations in the algae ranged from 5.8 to 56.8 mg As kg^{-1} and are given in Table 1. We compare our results for total arsenic with those in the literature for algae belonging to the same genera (as shown in the Supporting Information, Table SI-1). The results obtained are in the ranges reported in the literature for the same genus of alga. Seaweed absorbs arsenic directly from seawater and then biotransforms it. Complex abiotic and biotic factors influence the distribution of algae and can affect growth rates of macroalgae; these include light intensity, turbidity, temperature, hydrodynamics, depth, salinity, and nutrient availability.[29−31] Furthermore, seasonal variations in arsenic content have been reported for some edible algae.[32] Green and red seaweed have low concentrations of polyphenols[19] compared to brown seaweed species, which have high concentrations of the phenol group phlorotannin.[4] The higher arsenic content in brown seaweeds could be due to the higher content of phlorotannins. These compounds could act as chelating agents and might influence the retention of the metal in the seaweed matrix.[33] All of these factors might contribute to variability in the results obtained in this study and those reported in the literature. In general, brown algae accumulate higher arsenic levels than green or red algae,[8,34,35] and some researchers[8,35,36] propose a relationship between As content and algae type, namely, a gradation of total As in relation to algal type: brown > red > green. In the present study, the number of algae analyzed is too low to establish a clear correlation between total arsenic content and algal type.

Arsenic Speciation. *Organoarsenic Compounds.* The arsenic compounds determined in our study are very polar and soluble in water; this is especially so for arsenosugars. Thus, water is probably a suitable solvent for the extraction of arsenic species, provided it can penetrate the sample matrix.[37] Thus, we chose water for arsenic species extraction. Wide ranges of extraction efficiencies are reported, even using the same extractant, depending on the particular algae type.[38] In the present study, extraction efficiencies (calculated as the ratio of total arsenic in the aqueous extract to total arsenic in the algae) are reported in Table 1. The values are generally high, but as an exception the lowest figure for

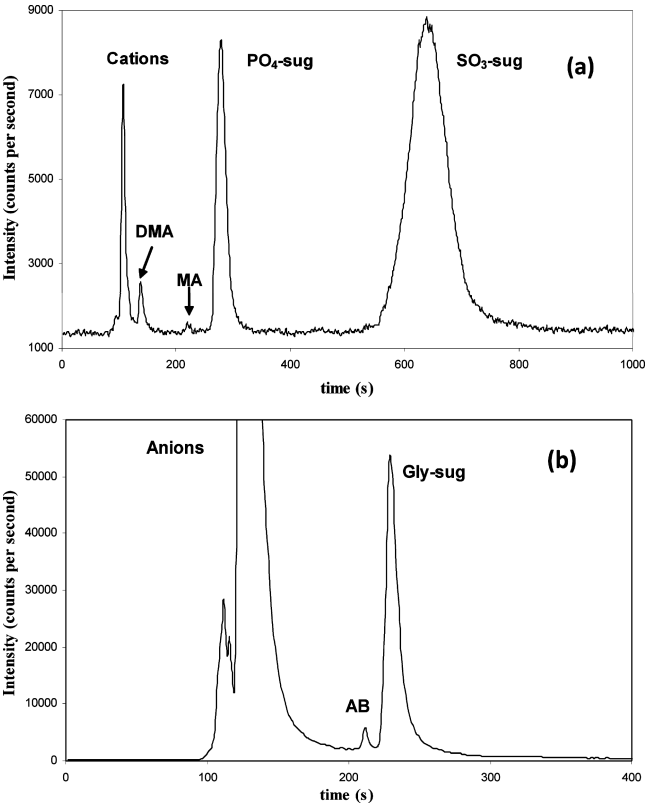

Figure 2. Chromatograms of *Laminaria saccharina* extract from anion exchange (a) and cation exchange (b) by LC-ICPMS.

the samples studied corresponds to *U. pinnatifida* (29%). The variations in the extraction efficiencies may be attributable to the species of alga analyzed as well as the extraction agent. Other researchers also report large differences in extractable arsenic (5 and 49% in *U. pinnatifida*[39]). The fraction of arsenic not extracted with water could be associated with lipids and might account for up to 50% of the total arsenic in algae.[40] The detection and quantification limits obtained for the arsenic compounds (calculated according to the standard deviation of the values obtained for each chromatographic peak of the baseline for different extracts) are also reported in Table 1. Arsenic is mainly present in marine algae in the form of derived carbohydrates. In general, PO_4-sug and SO_4-sug (see Figure 1) are the most common arsenic compounds in green and red algae, whereas SO_3-sug and SO_4-sug are the most abundant in brown algae.[41] Table 1 summarizes our results for arsenic speciation and clearly shows that arsenosugars are the most abundant arsenic compounds quantified in most algae. In *C. crispus*, *P. purpurea*, *L. ochroleuca*, and *L. saccharina*, arsenosugars accounted for >90% of the arsenic extracted: 97.0, 98.3, 99.0, and 98.5%, respectively. However, our results reveal no relationship between the algal type (red, green, and brown) and the type of arsenosugars. The main species found in *C. crispus* were PO_4-sug and Gly-sug, which accounted for >90% of the arsenic extracted, and the percentage of inorganic arsenic was low (<1 mg As kg^{-1}). In *P. purpurea*, PO_4-sug was the most common As species (corresponding to 91.6% of extracted As), whereas Gly-sug and DMA were also detected in low percentages (<2%). Different As species were found in *U. rigida*, with Gly-sug being the most abundant (66.2% of extracted As), whereas AB was detected in small proportions

(<5% of extracted As). Although AB is a very common arsenic compound in marine animals, in algae it is not a common compound. However, recent studies have detected AB, which is the major form in fish and most seafood, in some algae at low concentrations.[25,28,42,43] Another researcher[44] reported 47% of the extracted arsenic was AB. In *L. ochroleuca*, the most abundant species was SO_3-sug (which accounted for 80.9% of extracted As) but PO_4-sug and Gly-sug were also found. In *L. saccharina*, the most abundant species were SO_3-sug (74.5% of extracted As) and PO_4-sug (16.8% of extracted As). As an example, chromatograms of the *L. saccharina* extract obtained using anion exchange and cation exchange are shown in Figure 2. The anion exchange chromatogram shows the presence of DMA and MA and also the major contribution of SO_3-sug and PO_4-sug, whereas the chromatogram obtained from cation exchange reveals the presence of Gly-sug and AB. The most abundant species in *U. pinnatifida* was Gly-sug (54.4% of extracted As), and the other arsenic compounds found were PO_4-sug, As(V), and DMA.

The availability of data on arsenosugar contents in seaweed is of interest because, even though no information is available regarding their toxicity, such compounds are similar to toxic inorganic arsenic in terms of metabolite formation and tissue accumulation and might be potentially toxic.[11,18]

Inorganic Arsenic. The safety of edible seaweed is currently evaluated after determining the content of inorganic arsenic, which includes the most toxic and carcinogenic species of arsenic (arsenite and arsenate). Hence, we calculated inorganic arsenic in edible seaweed to check for possible toxicity from alga intake.

Inorganic arsenic was not detected in *P. purpurea*, *L. ochroleuca,* or *L. saccharina*. However, in other edible seaweed we found low concentrations of inorganic arsenic: 0.51 mg As kg^{-1} (3% of extracted As) in *C. crispus*, 0.30 mg As kg^{-1} (14% of extracted As) in *U. rigida* and 0.29 mg As kg^{-1} (18% of extracted As) in *U. pinnatifida*. These results for inorganic arsenic are in agreement with other values found in the literature for the same seaweed genera. In kombu (*Laminaria* sp.), wakame (*U. pinnatifida*), sea lettuce (*Ulva* sp.), Irish moss (*Chondrus* sp.) and nori (*Porphyra* sp.) inorganic arsenic contents range from 0.12 to 1.44 mg kg^{-1} dw[8,35] Checking the inorganic arsenic as a measure of toxic arsenic might underestimate the risk, because a major proportion of the arsenic in seaweed might be present in a form with unknown toxicity (that is potentially toxic).[18] Furthermore, it cannot be ruled out that part of the unextracted arsenic in this study was in inorganic form, because water may be an ineffective extractant for tightly bound (e.g., protein-bound) As(III).[37] That having being said, if we compare our inorganic arsenic results with the values established by legislation, we find that none of the samples analyzed exceed the French limit (=3.0 mg As kg^{-1} dw). *U. pinnatifida*, *C. crispus*, *Ulva* spp., and *P. umbilicalis* (which belongs to the same genus as *P. purpurea*) are authorized for human consumption in France.[22]

According to ref 16, rice and rice products are a major source of risk because the inorganic arsenic content is usually much higher than that of DMA and MA (which are less toxic than inorganic arsenic but could also be present in rice). Therefore, arsenic speciation methods allowing the quantification of arsenite and arsenate are suitable to determine the risk associated with rice consumption. In a recent proficiency test it was shown that inorganic arsenic can be evaluated with different analytical methods, and it was concluded that the introduction of a maximum level for inorganic As in rice should not be postponed because of analytical concerns.[45] Nevertheless, the situation for seaweed is very different. Seaweed contains more arsenic than rice, the major arsenic compounds are arsenosugars, and inorganic arsenic is usually a small proportion of total arsenic. Speciation analysis in this matrix requires different and complex analytical methods, and a robust, simple, and affordable method is not yet envisaged. Accordingly, there is an urgent need for a validated analytical method for inorganic arsenic in food of marine origin and for the preparation of suitable reference materials certified for inorganic arsenic content.[11,18]

■ ASSOCIATED CONTENT

Supporting Information. Table SI-1, providing taxonomical data of the algae studied and comparative results of total arsenic contents in the present study and from other algae reported in the literature. This material is available free of charge via the Internet at http://pubs.acs.org.

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Supporting Information

Table SI-1

Taxonomical data of the algae studied. Comparative results of total arsenic contents in the present study and from algae of different species but belonging to the same genus in several geographical zones reported in the literature.

Algae species	Trade name	Type	Phylum	Family	Total As (mg kg^{-1})	Reference
Laminaria sp.	Kombu	Brown	Heterokontophyta (Cl. Phaeophyceae)	Laminariaceae	8.4-134	(1-7)
Laminaria saccharina	Kombu	Brown	Heterokontophyta (Cl. Phaeophyceae)	Laminariaceae	52.4 ± 2.1	present study
Laminaria ochroleuca	Kombu	Brown	Heterokontophyta (Cl. Phaeophyceae)	Laminariaceae	56.8 ± 2.4	present study
Undaria pinnatifida	Wakame	Brown	Heterokontophyta (Cl. Phaeophyceae)	Alariaceae	6.2-70.0	(1, 2, 4, 5, 8, 9, 10)
Undaria pinnatifida	Wakame	Brown	Heterokontophyta (Cl. Phaeophyceae)	Alariaceae	41.0 ± 2.6	present study
Porphyra sp.	Nori	Red	Rhodophyta	Bangiaceae	0.85-58.3	(1, 2, 4, 5, 8, 10, 11, 12)
Porphyra purpurea	Nori	Red	Rhodophyta	Bangiaceae	40.7 ± 2.8	present study
Chondrus sp.	Irish moss	Red	Rhodophyta	Gigartinaceae	5.7-16.7	(2, 11)
Chondrus crispus	Irish moss	Red	Rhodophyta	Gigartinaceae	18.2 ± 0.5	present study
Ulva sp.	Sea lettuce	Green	Chlorophyta	Ulvaceae	0.8-8.7	(1, 3, 4, 10, 11, 12, 13, 14, 15, 16)
Ulva rigida	Sea lettuce	Green	Chlorophyta	Ulvaceae	5.8 ± 0.4	present study

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5.3 Establishment of a method for determination of arsenic species in seafood by LC-ICP-MS

Establishment of a method for determination of arsenic species in seafood by LC-ICP-MS

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Establishment of a method for determination of arsenic species in seafood by LC-ICP-MS

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ABSTRACT

An analytical method for determination of arsenic species (inorganic arsenic (iAs), methylarsonic acid (MA), dimethylarsinic acid (DMA), arsenobetaine (AB), trimethylarsine oxide (TMAO) and arsenocholine (AC)) in Brazilian and Spanish seafood samples is reported. This study was focused on extraction and quantification of inorganic arsenic (iAs), the most toxic form. Arsenic speciation was carried out via LC with both anionic and cationic exchange with ICP-MS detection (LC-ICP-MS). The detection limits (LODs), quantification limits (LOQs), precision and accuracy for arsenic species were established. The proposed method was evaluated using eight reference materials (RMs). Arsenobetaine was the main species found in all samples. The total and iAs concentration in 22 seafood samples and RMs ranged between 0.27–35.2 and 0.02–0.71 mg As kg^{-1}, respectively. Recoveries ranging from 100% to 106% for iAs, based on spikes, were achieved. The proposed method provides reliable iAs data for future risk assessment analysis.

1. Introduction

The rapid expansion in trade of seafood products makes this an important market worldwide (De Silva & Bjondal, 2013). The increase in global consumption of seafood is associated with several benefits such as a reduction in risk of several diseases (Innis, 2007; Zmozinski et al., 2013). On the other hand, concerns about human health have arisen since several arsenic species have been detected in seafood (Leufroy, Noël, Dufailly, Beauchemin, & Guérin, 2011). The toxicity of As is dependent on its chemical species, with inorganic species (iAs) such as arsenite (As(III)) and arsenate (As(V)) being the most toxic (Geng et al., 2009). Other arsenic species such as monomethylarsonic acid (MA) and dimethylarsenic acid (DMA) are less toxic to humans, with asenobetaine (AB) being considered non-toxic (Feldmann & Krupp, 2011; Geng et al., 2009).

Seafood contains intrinsically more total arsenic than terrestrial foods, and more than 50 species of arsenic were identified in seafood (Francesconi, 2010). Inorganic As species in seafood are commonly present as low percentages of the total amount of As (Borak & Hosgood, 2007). However, high concentrations have been reported in some types of seafood, e.g. in bivalve mussels, where concentrations of up to 5 mg As kg^{-1} were found (Sloth & Julshamn, 2008). The different toxicities of the As species reinforce the importance of its chemical speciation, as the total amount of As does not provide enough information about the toxicity of the analysed sample.

The analysis of arsenic species usually involves many steps, including extraction, separation and detection. Several methods have been employed to perform As speciation analysis: high-performance liquid chromatography (HPLC) and detection by inductively coupled plasma-optical emission spectrometry (ICP-OES), inductively coupled plasma-mass spectrometry (ICP-MS), hydride generation-atomic absorption spectrometry (HG-AAS) and hydride generation-atomic fluorescence spectrometry (HG-AFS) (Francesconi & Kuehnelt, 2004).

Countries such as New Zealand and Australia have legislation for the maximum levels of inorganic arsenic (iAs) in seafood and established a maximum level of inorganic arsenic of 2 mg kg^{-1} for crustaceans and fish, and 1 mg kg^{-1} for molluscs and seaweed (Australia New Zealand Food Authority, 2013). The Republic of China establishes a maximum level of inorganic arsenic of 0.1 mg kg^{-1} for fish and 1.0 mg kg^{-1} for shells, shrimps and crabs (dry weight), respectively (MHC, 2005). On the other hand, the Brazilian government through the Ministry of Agriculture, Livestock and Food Supply (MAPA) establishes a reference value of 1 mg kg^{-1} for total As in fish (National Program for Residue, 2012). However, the European Union has not established a limit for total or inorganic As in fish and seafood in its legislation (Commission Regulation, 2006).

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Aware of this situation, the EFSA (European Food Safety Authority) published in 2009 and 2014, two reports about the dietary exposure to arsenic in the European population (European Food Safety Authority, 2009, 2014). Both reported the urgent need for further data on arsenic species, particularly iAs data, in particular in fish and seafood, and in food groups that provide a significant contribution to the dietary exposure to iAs (e.g. rice and wheat-based products) to reduce the uncertainty of the exposure assessments to iAs. Thus, the need to introduce specific legislation is becoming evident (European Food Safety Authority, 2009; Feldmann & Krupp, 2011). Furthermore, the need to create certified reference materials for seafood and to develop arsenic speciation methods for a large range of food samples and arsenic species was also emphasised (European Food Safety Authority, 2009). The increased focus on inorganic arsenic in food has led to several initiatives towards development of methods for selective determination of inorganic arsenic in seafood. For this purpose, the Institute for Reference Materials and Measurements (IRMM) organised two proficiency tests (PT) in 2010 for measuring iAs, and trace metals in seafood (IMEP-109 and IMEP-30). The determination of iAs in seafood test material presented serious analytical problems. The expert laboratories were not able to agree on a value for the iAs within a reasonable degree of uncertainty (Baer et al., 2011). It was concluded that more research in extraction and chromatographic procedures was required to quantify the iAs in seafood (Baer et al., 2011). The complexity of the seafood matrix requires accurate and robust procedures. However, the analytical procedures used to date do not comply with these requirements (Feldmann & Krupp, 2011).

Some authors reported inorganic arsenic values in several seafood CRM collected from previously published studies (Leufroy et al., 2011; Pétursdóttir, Gunnlaugsdóttir, Krupp, & Feldmann, 2014; Pétursdóttir, Gunnlaugsdóttir, Jörundsdóttir, Mestrot, et al., 2012; Pétursdóttir, Gunnlaugsdóttir, Jörundsdóttir, Raab, et al., 2012). The results of iAs varied widely according to the extraction and detection method. This emphasises the need for the development of reliable methods for the determination of iAs in seafood and a certified value of inorganic As in a seafood-based reference material.

The goal of this work was to establish a method for the determination of total As and As species in seafood samples comprising fish, crustaceans and bivalves. Due to the increasing focus on inorganic arsenic in food, the study was focused on the extraction, identification, separation and accurate quantification of inorganic arsenic (iAs), the most toxic form, which was selectively separated and determined using anion exchange LC-ICP-MS. Finally, due to the lack of CRMs for iAs in seafood samples, previously published values were compared with results obtained in the present study.

2. Materials and methods

2.1. Instruments

For total As, all measurements were carried out using an Agilent 7500ce ICP-MS (Agilent, Germany) with a BURGENER Ari Mist HP type nebuliser. For As speciation, LC-ICP-MS was used with an Agilent 1200 LC quaternary pump, equipped with an auto sampler. The analytical columns Hamilton PRP-X100 (250 × 4.1 mm, 10 μm, Hamilton, USA) and Zorbax-SCX300 (250 × 4.6 mm, 5 μm, Agilent, Germany) were protected by guard columns filled with the corresponding stationary phases. The outlet of the LC column was connected via PEEK capillary tubing to the nebuliser of the ICP-MS system. A microwave (Milestone Ethos Touch Control) was used for digesting and extracting the samples. The fish samples supplied by MAPA (Brazil) were lyophilised in a ModulyonD Freeze Dryer lyophiliser (Thermo Electron Corporation, USA) and milled in an A 11 Basic micro-mill (IKA – Werke, Germany).

2.2. Reagents and standards

Analytical grade reagents were used exclusively. Deionised water with a specific resistivity of 18 MΩ cm^{-1} from a Milli-Q water purification system (Millipore, Bedford, MA, USA) was used for the preparation of all solutions. Formic acid (98%) (Panreac, p.a., Barcelona, Spain), ammonium dihydrogen phosphate (Panreac, p.a., Barcelona, Spain), aqueous ammonia solution (25%) (Panreac, p.a., Barcelona, Spain), and pyridine (Scharlau, p.a., Barcelona, Spain) were used for the preparation of mobile phases. The following reagents were used for sample digestion and extraction: 31% H_2O_2 (Merck, Selectipur, Darmstadt, Germany) and 69% HNO_3 (Panreac, Hiperpur, Barcelona, Spain). External calibration standards for total As were prepared daily by dilution of a standard stock solution traceable to the National Institute of Standards and Technology (Gaithersburg, USA) with a certified concentration of 1001 ± 5 mg As L^{-1} (Inorganic Ventures Standards, Christiansburg, USA). A solution of ^{9}Be, ^{103}Rh and ^{205}Tl was used as the internal standard in ICP-MS measurements. An arsenate standard solution of 1000 ± 5 mg As L^{-1} (Merck, Darmstadt, Germany) was used for internal quality control in total arsenic and arsenic speciation measurements. Stock standard solutions (1000 mg As L^{-1}) for arsenic speciation were prepared as follows: As(III), from As_2O_3 (NIST, Gaithersburg, USA, Oxidimetric Primary Standard 83d, 99.99%) dissolved in 4 g L^{-1} NaOH (Merck, Suprapure, Darmstadt, Germany); As(V), from $Na_2HAsO_4{\cdot}7H_2O$ (Carlo Erba, Milano, Italy) dissolved in water; MA, prepared from $(CH_3)AsO(ONa)_2{\cdot}6H_2O$ (Carlo Erba, Milano, Italy) dissolved in water; DMA, prepared from $(CH_3)_2AsNaO_2{\cdot}3H_2O$ (Fluka, Buchs, Switzerland) dissolved in water. Arsenocholine (AC) from $(CH_3)_3As^{+}(CH_2)\ CH_2OHBr^{-}$ was supplied by the "Service Central d'Analyse" (CNRS Vernaison, Solaize, France) and trimethylarsine oxide (TMAO) was prepared from $(CH_3)_3AsO$ (Argus Chemicals, Vernio, Italy) dissolved in water. The certified reference material of arsenobetaine (AB) from $(CH_3)_3\ As^{+}CH_2COO^{-}$ was supplied by NMIJ (Tsukuba, Japan) as a standard solution, NMIJ CRM 7901-a. For our internal quality control, the As concentration in in-house prepared As speciation standards was determined by ICPMS. For this, As(V), As(III), DMA, MA, AC, TMAO and AB were standardised against two arsenic certified standard solutions (Merck, Darmstadt, Germany and Inorganic Ventures, Christiansburg, USA) as well as against As_2O_3 solution. All stock solutions were kept at 4 °C, and further diluted solutions for the analysis were prepared daily.

2.3. Reference materials and samples

The following certified reference materials (CRM) were used for method development: DOLT-4 (Dogfish), TORT-2 (Lobster Hepatopancreas) (both from the National Research Council, Canada); NIST SRM 2976 (Mussel Tissue) and NIST SRM 1566b (Oyster Tissue) (National Institute of Standards and Technology, Gaithersburg, MD, USA); BCR-627 (Tuna fish), ERM-BC211 (Rice) and ERM-CE278 (Mussel Tissue) (Institute for Reference Materials and Measurements of the European Commission's Joint Research Centre, Geel, Belgium). The reference material (RM) 9th PT on fish from the Community Reference Laboratory-Istituto Superiore di Sanità (CRL-ISS, Rome, Italy) was also analysed.

Four fresh fish muscle samples were provided by the Laboratory of Trace Metals and Contaminants (LANAGRO/RS) of the Ministry of Agriculture, Livestock and Supply (MAPA/Brazil). The total amount of these four samples were initially washed with Milli-Q water, cut and then lyophilised for a period of 5 h. They were then

ground in a vibratory mill and sieved through polyester mesh of 85 μm to improve the particle size distribution.

Ten fish samples and a clam sample were supplied by the Laboratory of the Public Health Agency of Barcelona (ASPB, Barcelona, Spain). Three crustacean samples and four bivalve samples were purchased from local supermarkets in Barcelona, Spain, during 2013. All these samples were analysed in a raw state (wet weight) without lyophilisation or other pretreatments. Only edible parts of each fish and seafood were used for the analysis. Samples were washed with Milli-Q water, cut, and homogenised using a blender (non-contaminating kitchen mixer; Multiquick 5 Hand Processor, Braun, Barcelona, Spain). After homogenisation, samples were stored in the refrigerator at 4–10 °C until analysis (before 2 days).

2.4. Procedures

2.4.1. Moisture determination

The moisture of fresh samples was determined in triplicate by drying 0.5 g aliquots in an oven at 102 ± 3 °C until constant weight. Moisture ranged from 45% to 94%, and all results are expressed as dry mass.

2.4.2. Total arsenic analysis

The total arsenic content in seafood and CRM samples was determined by ICP-MS following microwave digestion. Initially, 0.5 g and 2 g aliquots of lyophilised and fresh samples, respectively, were weighed in digestion vessels, after which 8 mL of concentrated nitric acid and 2 mL of hydrogen peroxide were added. The microwave digestion procedure was carried out according to the following programme: 10 min from room temperature to 90 °C, maintained for 5 min at 90 °C, 10 min from 90 °C to 120 °C, 10 min from 120 °C to 190 °C and 10 min maintained at 190 °C. After cooling to room temperature, the digested samples were diluted in water up to 25 mL. Helium gas was used in the collision cell to avoid interferences in the ICP-MS measurements. A solution of ^{9}Be, ^{103}Rh and ^{205}Tl was used as the internal standard. The samples were quantified by means of an external calibration curve from As(V) standards. Triplicate analyses were performed for each sample. For quality control purposes, the standards of the calibration curve were run before and after each sample series. The corresponding digestion blanks (one for each sample digestion series) were also measured. Quality control standard solutions at two concentrations were measured after constructing the calibration curve. To assess the accuracy of the ICP-MS method, seven CRMs (DOLT-4, TORT-2, SRM 2976, SRM 1566b, BCR-627, ERM-BC211 and ERM-CE278) and one RM (9th PT) were analysed.

2.4.3. Arsenic speciation analysis

The extraction of As species was based on our previous study (Llorente-Mirandes, Calderón, Centrich, Rubio, & López-Sánchez, 2014). For this, 0.2 g and 1.0 g aliquots of lyophilised and fresh samples, respectively, were weighed in digestion vessels and 10 mL of a solution containing 0.2% (w/v) of nitric acid and 1% (w/v) of hydrogen peroxide were added to perform a microwave assisted extraction (MAE) at temperature of 95 °C. Samples were cooled to room temperature and centrifuged at 3500 rpm for 25 min. The supernatant was filtered through PET filters (Chromafil, Macherey–Nagel, pore size 0.45 μm). Triplicate analyses were performed for each sample. This extraction method completely oxidises As(III) into As(V), without conversion of the other organoarsenic species into inorganic arsenic (iAs). The iAs was identified and quantified as As(V) in the extracts by comparing the chromatographic peak for the samples with the peak of As(V) standard solution. Total arsenic in the extracts was determined by ICP-MS (as described previously). Arsenic speciation was carried out in the extracts by LC-ICP-MS. Two chromatographic separation methods were used for separation of the arsenic species. As(III), As(V), DMA and MA were analysed by anion exchange chromatography. AB, AC and TMAO were analysed by cation-exchange chromatography. The performance characteristics of anion-exchange chromatographic system are previously described (Llorente-Mirandes et al., 2014). The main chromatographic conditions of cation-exchange chromatography were: mobile phase of 20 mM pyridine, pH = 2.6, flow rate at 1.5 mL min^{-1}, and injection volume of 50 μL. Arsenic species in extracts were identified by comparison of retention times with standards. External calibration curves were used to quantify MA, DMA, As(III), As(V), AB, TMAO and AC according to the corresponding standards. Extraction blanks were also analysed by LC-ICP-MS in each work session. The ion intensity at m/z 75 (^{75}As) was monitored using time-resolved analysis software. Additionally, the ion intensities at m/z 77 (^{40}Ar^{37}Cl) and m/z 35 (^{35}Cl) were monitored to detect possible argon chloride (^{40}Ar^{35}Cl) interference at m/z 75. In each speciation run, an As(V) certified standard solution (Merck, Darmstadt, Germany) and a certified reference material solution were measured every ten samples and at the end of the sequence to ensure stable instrument sensitivity.

3. Results and discussion

3.1. Quality control

3.1.1. Analysis of the total As concentration

To evaluate the accuracy of the method, several CRMs were analysed. Seafood CRMs (TORT-2, DOLT-4, SRM 2976, SRM 1566b, BCR-627, ERM-BC211 and ERM-CE278) and one material reference (9th) were analysed during the study (Table 1). For quality control of acid digestion, a CRM was analysed in every batch of samples measurements (total As concentration). The comparison between each obtained value of total As with its corresponding certified value (Table 1) showed no significant difference at a 95% confidence level when Student's t-test was applied. The repeatability (six times within a day, $n = 6$) was assessed for the results obtained by analysis of different replicates of CRMs (Table 1). The RSD (%) values were: 4.9% for TORT-2 and 1.2% for DOLT-4. The detection (LOD) and quantification limits (LOQ) were calculated as three times the standard deviation (3σ) and ten times the standard deviation signal (10σ) of ten digestion blanks, respectively (Llorente-Mirandes et al., 2014). The results obtained were as follows: 0.006 mg As kg^{-1} dry weight basis for method detection limit and 0.021 mg As kg^{-1} dry weight basis for method quantification limit.

3.1.2. Analysis of As species

Extraction efficiencies. The extraction efficiency was evaluated by calculating the ratio between total arsenic present in the extracts and the total arsenic present in the samples, given by the acid digestion. The extraction efficiencies are presented in Table 1 for the CRMs and Table 2 for the real samples. The efficiency obtained in this work varied between 73% and 104% with an average of 89%, which is consistent with the literature (Amayo et al., 2011; Pétursdóttir et al., 2014; Zheng & Hintelmann, 2004). Thus, the solution containing 0.2% (w/v) of HNO_3 and 1% (w/v) of H_2O_2 proved to be an effective solvent in the extraction of As species in seafood. A recent study compared nine extraction methods for determination of iAs in seafood, including the HNO_3/H_2O_2 (Pétursdóttir et al., 2014). The highest extraction efficiency for all samples was achieved by HNO_3/H_2O_2 method, which corroborate with this work. An average extraction efficiency of 93% was obtained for most samples, with the exception of DOLT-4, ERM CE278 and salmon-2, for which the average was 75%. According

Table 1
Total arsenic and arsenic species in reference materials; concentrations are expressed as mg As kg^{-1} dry mass (mean ± SD, $n = 3$ and $^{*}n = 6$).

Reference materials	Total As	Total extracted As	Arsenic species									Sum of As species	Extraction efficiency (%)	Column recovery (%)
			DMA	MA	UA-B[c]	iAs	AB	TMAO	AC	UC-A[d]	UC-B[e]			
TORT-2*	22.4 ± 1.1	21.9 ± 1.7	1.57 ± 0.05	0.20 ± 0.01	0.12 ± 0.02	0.71 ± 0.04	13.1 ± 0.45	0.19 ± 0.02	0.05 ± 0.004	0.94 ± 0.05	0.08 ± 0.02	17.0 ± 0.64	98	78
Certified value[a]	21.6 ± 1.8													
DOLT-4*	9.64 ± 0.11	7.39 ± 0.39	0.45 ± 0.07	0.10 ± 0.02	0.07 ± 0.01	0.02 ± 0.003	5.17 ± 0.51	0.32 ± 0.01	<LOD	0.10 ± 0.01	<LOD	6.24 ± 0.63	77	84
Certified value[a]	9.66 ± 0.62													
ERM-CE278	6.09 ± 0.21	4.46 ± 0.23	0.62 ± 0.04	0.10 ± 0.02	0.03 ± 0.007	0.07 ± 0.003	2.27 ± 0.17	<LOD	<LOD	0.09 ± 0.005	0.17 ± 0.012	3.36 ± 0.26	73	75
Certified value[a]	6.07 ± 0.13													
NIST 1566	7.67 ± 0.13	6.85 ± 0.19	0.84 ± 0.06	<LOD	0.45 ± 0.02	0.05 ± 0.001	2.63 ± 0.07	<LOD	<LOD	<LOD	<LOD	3.97 ± 0.15	89	58
Certified value[a]	7.65 ± 0.65													
NIST 2976	13.7 ± 0.25	13.3 ± 0.52	0.41 ± 0.05	0.12 ± 0.002	0.30 ± 0.04	0.11 ± 0.013	10.3 ± 0.20	<LOD	<LOD	0.14 ± 0.02	0.13 ± 0.012	11.5 ± 0.33	97	86
Certified value[a]	13.30 ± 1.8													
9th PT (CRL-ISS)	7.00 ± 0.32	6.89 ± 0.06	0.5 ± 0.06	0.05 ± 0.01	0.25 ± 0.04	0.24 ± 0.02	4.3 ± 0.19	0.23 ± 0.01	<LOD	0.16 ± 0.03	<LOD	5.73 ± 0.36	98	83
Assigned value[b]	6.65 ± 0.71													
BCR-627	4.84 ± 0.13	4.75 ± 0.08	0.13 ± 0.02	0.02 ± 0.004	0.03 ± 0.006	0.02 ± 0.002	3.8 ± 0.07	<LOD	0.05 ± 0.008	0.05 ± 0.003	0.06 ± 0.006	4.16 ± 0.11	98	88
Certified value[a]	4.80 ± 0.3		0.15 ± 0.02				3.9 ± 0.2							
ERM-BC211	0.263 ± 0.011	0.265 ± 0.010	0.128 ± 0.006	0.016 ± 0.004	<LOD	0.119 ± 0.005	<LOD	<LOD	<LOD	<LOD	<LOD	0.262 ± 0.01	101	99
Certified value[a]	0.260 ± 0.013		0.119 ± 0.013			0.124 ± 0.011								

[a] Certified value: mean ± uncertainty.
[b] Assigned value: mean ± uncertainty.
[c] Unknown anion arsenic species (UA-B) with a retention time of 251 s.
[d] Unknown cation arsenic species (UC-A) with a retention time of 279 s.
[e] Unknown cation arsenic species (UC-B) with a retention time of 360 s.

to Pétursdóttir, Gunnlaugsdóttir, Jörundsdóttir, Raab, et al. (2012) and Amayo et al. (2011) this difference in extraction efficiencies can be attributed to the different amount of lipids in the samples. Salmon has a high lipid content and possibly contained arsenolipids that could not be extracted by the present extractant. Zheng and Hintelmann (2004) attributed the remaining arsenic (lower efficiencies in the extraction procedures) to the arsenolipids, which is not soluble in the methanol/water solvent. For DOLT-4 extraction efficiency, the value of 77% found in this work is similar to (78%) reported by Pétursdóttir et al. (2014) that used the same extraction method. On the other hand, whitefish and swordfish, which have low lipid content, had high extraction efficiencies of 97% and 95%, respectively.

Column recovery. Column recovery is expressed as the ratio of total As (sum of all arsenic species) eluted from the chromatographic column to the total As in the extract injected into the chromatographic column. Measurement of column recovery is essential to provide a control of chromatographic separation and to evaluate the quantification of the As species. The column recovery values ranged from 58% to 99% for CRMs (Table 1) and 70% to 104% for all samples (Table 2). These values are in agreement with those reported by Zheng and Hintelmann (2004), which found values from 85% to 110% using HPLC-ICP-SFMS and methanol/water as extracting agent.

Recovery of inorganic arsenic. Standards of As(III) and As(V) were spiked in solid samples of red porgy, tuna-1, clam-1, mussel and CRM TORT-2 and then homogenised. Samples were taken for extraction 30 min after spiking. Quantitative oxidation of As(III) to As(V) was achieved since only As(V) was found as iAs in the spiked samples. Thus, anion LC-ICP-MS was used to quantify the As(V) as iAs in the samples. The recoveries found for red porgy, tuna-1, clam-1, mussel and TORT-2 were 102 ± 2, 100 ± 5, 100 ± 4, 101 ± 2 and 106 ± 2 (mean% ± standard deviation, $n = 3$), respectively. These recovery values were calculated according to the literature (Llorente-Mirandes et al., 2014) and show quantitative recovery of iAs. As an example, Figs. 1a and 2a show the chromatograms of clam-1 and red porgy extracts, respectively. The clam-1 was fortified with 0.200 mg As kg^{-1} of As(III) and As(V); the red porgy with 0.250 mg As kg^{-1} of As(III) and As(V). As can be seen, iAs was recovered successfully as As(V) from the two samples.

Accuracy. In order to verify the accuracy of the proposed speciation method, two CRMs were analysed and evaluated: BCR-627 (Tuna fish) and ERM-BC211 (Rice). The CRM BCR-627 has a certified value of 3.9 ± 0.22 mg As kg^{-1} for AB and 0.15 ± 0.02 mg As kg^{-1} for DMA. To assess the accuracy of the inorganic arsenic results, the ERM-BC211 rice material was analysed because there is no CRM for measurement of inorganic arsenic in seafood. The ERM-BC211 has a certified value of 0.124 ± 0.011 mg As kg^{-1} for iAs and 0.119 ± 0.013 mg As kg^{-1} for DMA. The values found for the ERM-BC211 and CRM BCR-627 are shown in Table 1 and did not differ significantly from certified values at a 95% confidence level.

Limits of detection and quantification. Limits of detection (LOD) and quantification (LOQ) were estimated for each As species. To calculate these parameters, the standard deviation of the base line and the chromatographic peak base of each analyte multiplied by 3 or 10 (LOD and LOQ respectively) were interpolated in the slope of the height calibration curve. The instrumental limits were converted to sample limits by multiplying by the extraction dilution factor. The LODs for As(III), DMA, MA, As(V), AB, TMAO and AC were 0.0010, 0.0014, 0.0017, 0.0024, 0.0010, 0.0028 and

Table 2
Arsenic speciation analysis of selected seafood samples; concentrations are expressed as mg As kg^{-1} dry mass (mean ± SD, $n = 3$).

Sample	Total extracted As	Arsenic species										Sum of As species	Extraction efficiency (%)	Column recovery (%)
		DMA	MA	UA-A[a]	UA-B[b]	iAs	AB	TMAO	AC	UC-A[c]	UC-B[d]			
Fish														
White fish	34.3 ± 0.89	<LOD	0.014 ± 0.001	<LOD	<LOD	<LOD	33.5 ± 2.95	0.04 ± 0.005	<LOD	<LOD	0.1 ± 0.004	33.6 ± 2.96	97	98
Red porgy	33.8 ± 1.84	<LOD	0.010 ± 0.001	<LOD	<LOD	<LOD	33.2 ± 2.71	0.04 ± 0.004	<LOD	0.94 ± 0.062	<LOD	34.1 ± 2.77	97	101
Hake-1	6.70 ± 0.16	<LOD	<LOD	<LOD	<LOD	<LOD	6.58 ± 0.19	0.03 ± 0.004	<LOD	0.04 ± 0.002	<LOD	6.65 ± 0.39	94	99
Hake-2	3.80 ± 0.03	0.13 ± 0.02	0.012 ± 0.001	<LOD	<LOD	<LOD	3.2 ± 0.20	<LOD	0.07 ± 0.026	<LOD	<LOD	3.41 ± 0.25	90	89
Forkbeard	27.6 ± 1.22	0.24 ± 0.02	<LOD	<LOD	<LOD	<LOD	20.3 ± 1.12	<LOD	<LOD	4.53 ± 0.29	<LOD	25.0 ± 1.43	86	89
Sardine	6.88 ± 0.27	0.16 ± 0.015	<LOD	<LOD	<LOD	<LOD	5.27 ± 0.13	0.07 ± 0.003	<LOD	<LOD	<LOD	6.0 ± 0.14	93	87
Salmon-1	1.45 ± 0.04	0.012 ± 0.0010	<LOD	<LOD	<LOD	<LOD	1.18 ± 0.04	0.024 ± 0.015	<LOD	<LOD	<LOD	1.21 ± 0.056	86	85
Salmon-2	1.38 ± 0.08	0.03 ± 0.006	<LOD	<LOD	<LOD	<LOD	0.86 ± 0.08	0.03 ± 0.007	<LOD	<LOD	<LOD	0.93 ± 0.009	76	70
Tuna-1	1.41 ± 0.09	0.05 ± 0.008	<LOD	<LOD	<LOD	<LOD	0.90 ± 0.037	0.08 ± 0.002	<LOD	0.05 ± 0.007	<LOD	1.08 ± 0.054	98	77
Tuna-2	1.71 ± 0.06	0.02 ± 0.006	<LOD	<LOD	<LOD	<LOD	1.43 ± 0.09	0.01 ± 0.009	<LOD	<LOD	<LOD	1.46 ± 0.10	94	86
Louvar	4.65 ± 0.07	0.04 ± 0.008	<LOD	<LOD	<LOD	<LOD	4.15 ± 0.31	<LOD	<LOD	0.09 ± 0.007	<LOD	4.3 ± 0.32	104	93
Swordfish-1	5.20 ± 0.08	0.16 ± 0.008	<LOD	<LOD	<LOD	<LOD	4.20 ± 0.16	0.008 ± 0.006	0.02 ± 0.005	0.35 ± 0.05	<LOD	4.73 ± 0.22	102	91
Swordfish-2	3.00 ± 0.11	0.05 ± 0.009	<LOD	<LOD	<LOD	<LOD	1.73 ± 0.02	0.01 ± 0.002	<LOD	0.89 ± 0.02	<LOD	2.68 ± 0.05	93	104
Swordfish-3	2.58 ± 0.05	0.05 ± 0.007	<LOD	<LOD	<LOD	<LOD	1.96 ± 0.05	<LOD	<LOD	0.16 ± 0.04	<LOD	2.17 ± 0.09	90	84
Crustaceans														
Prawn-1	2.0 ± 0.07	0.06 ± 0.008	0.08 ± 0.009	<LOD	<LOD	0.06 ± 0.08	1.44 ± 0.023	<LOD	<LOD	0.01 ± 0.004	<LOD	1.66 ± 0.12	87	83
Prawn-2	2.9 ± 0.05	<LOD	0.012 ± 0.002	<LOD	0.007 ± 0.001	0.037 ± 0.002	2.21 ± 0.039	<LOD	0.016 ± 0.003	0.054 ± 0.002	0.040 ± 0.001	2.37 ± 0.050	94	82
Shrimp	1.0 ± 0.09	<LOD	0.016 ± 0.001	<LOD	<LOD	0.033 ± 0.003	0.61 ± 0.017	<LOD	0.005 ± 0.001	0.020 ± 0.002	0.016 ± 0.002	0.70 ± 0.024	83	70
Bivalves														
Clam-1	16.8 ± 0.94	0.25 ± 0.006	<LOD	0.18 ± 0.02	2.07 ± 0.08	0.35 ± 0.009	11.7 ± 0.73	<LOD	0.29 ± 0.03	0.33 ± 0.06	<LOD	15.4 ± 0.91	99	92
Clam-2	10.5 ± 0.06	0.14 ± 0.02	<LOD	<LOD	1.86 ± 0.44	0.20 ± 0.005	7.93 ± 0.27	<LOD	0.02 ± 0.009	0.04 ± 0.006	0.03 ± 0.004	10.21 ± 0.13	86	97
Mussel	10.3 ± 0.08	0.07 ± 0.007	<LOD	0.04 ± 0.005	0.65 ± 0.10	0.08 ± 0.006	8.79 ± 0.07	<LOD	0.08 ± 0.006	0.26 ± 0.009	0.03 ± 0.009	10.0 ± 0.10	80	97
Cockle	7.5 ± 0.45	<LOD	0.13 ± 0.009	<LOD	0.16 ± 0.008	0.27 ± 0.008	4.01 ± 0.193	<LOD	<LOD	0.38 ± 0.011	0.50 ± 0.024	5.5 ± 0.24	90	73
Oyster	21.7 ± 0.28	0.10 ± 0.009	0.08 ± 0.006	<LOD	0.29 ± 0.021	0.10 ± 0.009	15.9 ± 0.75	0.06 ± 0.007	0.06 ± 0.005	0.46 ± 0.076	<LOD	17.1 ± 0.84	88	79

[a] Unknown anion arsenic species (UA-A) with a retention time of 148 s.
[b] Unknown anion arsenic species (UA-B) with a retention time of 251 s.
[c] Unknown cation arsenic species (UC-A) with a retention time of 279 s.
[d] Unknown cation arsenic species (UC-B) with a retention time of 360 s.

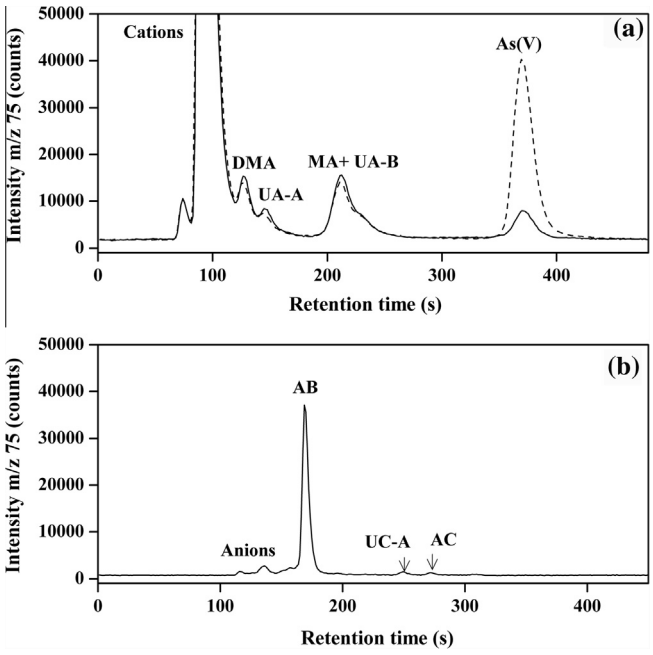

Fig. 1. Chromatograms of clam-1 extract from anion exchange (a) (continuous line: non-spiked sample and dotted line: sample spiked with iAs) and cation exchange (b) by LC-ICP-MS.

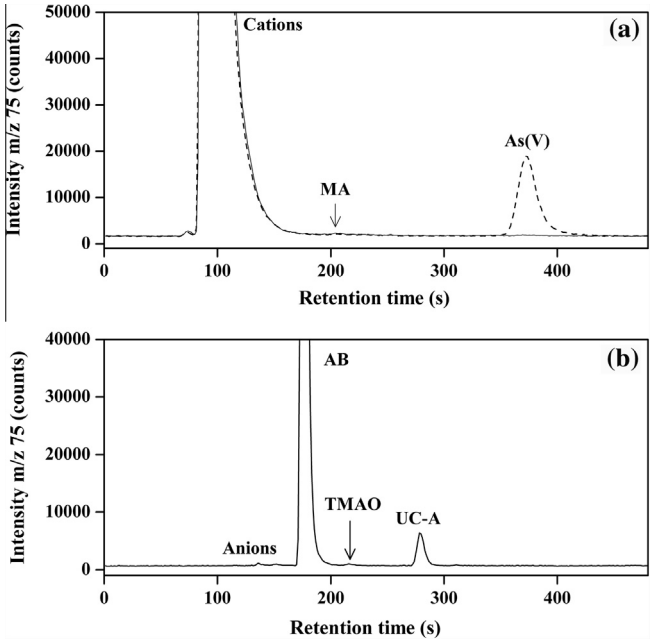

Fig. 2. Chromatograms of red porgy extract from anion exchange (a) (continuous line: non-spiked sample and dotted line: sample spiked with iAs) and cation exchange (b) by LC-ICP-MS.

0.0018 mg As kg^{-1} dry weight basis, respectively. The LOQs for As(III), DMA, MA, As(V), AB, TMAO and AC were 0.0033, 0.0047, 0.0056, 0.0080, 0.0033, 0.0093, 0.0060 mg As kg^{-1} dry weight basis, respectively.

3.2. Comparison of inorganic arsenic in seafood reference materials

The concentrations of iAs in TORT-2, DOLT-4, BCR-627 and SRM 1566b CRMs found in the literature since 2005 are given in Table 3. These concentrations vary widely according to the extraction and detection method. According to Table 3, the concentrations of iAs ranged from 0.09 to 1.233 mg kg^{-1} for TORT-2, 0.010 to 0.152 mg kg^{-1} for DOLT-4, 0.004 to 1.161 mg kg^{-1} for SRM 1566b and 0.015 to 0.192 mg kg^{-1} for BCR-627. No iAs concentrations were found in the literature for NIST SRM 2976, ERM-CE278 and 9th PT RMs, however the concentrations found in this work are given in Table 1.

The international measurement evaluation programme (IMEP) and the EU-RL-HM performed two proficiency tests in 2010 for the determination of trace metals, methylmercury and iAs, in seafood. In these proficiency tests, CRM DOLT-4 was used as the test material and the iAs values reported by expert laboratories using different extraction methods and techniques (Baer et al., 2011) ranged between 0.040 and 0.152 mg kg^{-1} (Table 3), highlighting strong discrepancies among the reported results. In other words, it was not possible to establish an assigned value for iAs, which was clearly more difficult to analyse in the seafood matrix than other matrices (Baer et al., 2011). Due to these problems, Pétursdóttir et al. have been published several works about determination of iAs concentration in CRMs using different extraction and detection methods (Pétursdóttir, Gunnlaugsdóttir, Jörundsdóttir, Mestrot, et al., 2012; Pétursdóttir, Gunnlaugsdóttir, Jörundsdóttir, Raab, et al., 2012; Pétursdóttir et al., 2014). In the most recent study, nine different extraction methods were used to extract DOLT-4 and TORT-2 (Pétursdóttir et al., 2014). The reported values ranged between 0.010–0.036 mg kg^{-1} and 0.315–0.823 mg kg^{-1} for DOLT-4 and TORT-2, respectively (Table 3). This fact illustrates that solvent plays a role in the extraction of iAs, and therefore, a difficulty in obtaining a consistent value of iAs in DOLT-4 and TORT-2. The concentrations of iAs found in the present study for DOLT-4 (0.020 ± 0.003 mg kg^{-1}) and TORT-2 (0.71 ± 0.04 mg kg^{-1}) are concordant with Pétursdóttir et al. (2014) work (0.017 ± 0.003 mg kg^{-1} and 0.714 ± 0.092 mg kg^{-1} for DOLT-4 and TORT-2, respectively), which used a similar extraction method (MAE, 2% HNO_3 in 3% H_2O_2). On the other hand, Leufroy et al. (2011) used two MAE methods (water and methanol/water) and found a mean concentration of 1.183 mg kg^{-1} iAs for TORT-2 that is higher than found in HNO_3/H_2O_2 extraction method.

For CRM BCR-627, the concentration found in this study was 0.02 ± 0.002 iAs. Leufroy et al. (2011) found 0.074 ± 0.014 mg kg^{-1} iAs with water and 0.192 ± 0.071 mg kg^{-1} iAs with methanol/water. Santos et al. (2013) using MAE (methanol/water) method found 0.325 mg kg^{-1} iAs. Sloth and Julshamn (2008) using MAE (ethanol/NaOH) method found 0.015 mg kg^{-1} iAs. The latter concentration was the most similar to that found in this work.

In relation to SRM 1566b, the concentration of iAs found was 0.05 ± 0.001 mg kg^{-1}, different from that reported by Santos (1.161 mg kg^{-1}) and Sloth (0.004 mg kg^{-1}) (Santos et al., 2013; Sloth & Julshamn, 2008).

In summary, the concentrations of iAs found in this work (Table 1) are within the range reported by several authors (Table 3), which show that proposed method give comparable results. However, the large variability of iAs concentration illustrates that it is difficult to obtain a consistent value for iAs in these CRMs. Therefore, the lack of a CRM for iAs in seafood limits the comparison and validation of values found by different authors. The development of seafood CRMs would help in the validation of speciation data and in the creation of legislation that could establish the maximum amount of iAs (Pétursdóttir, Gunnlaugsdóttir, Jörundsdóttir, Raab, et al., 2012).

3.3. Total arsenic in samples

Total As was determined in 22 seafood samples, four of which were Brazilian fish samples and the remainder Spanish seafood

Table 3
Inorganic arsenic (iAs) concentrations in TORT-2, DOLT-4, BCR 627 and SRM 1566b CRMs found in literature since 2005.

CRMs	Techniques	Extractions	iAs (mg kg^{-1})	References
TORT-2	HPLC-ICP-MS	MAE/(HCl/H_2O_2)	0.648	Pétursdóttir, Gunnlaugsdóttir, Jörundsdóttir, Mestrot, et al. (2012), Pétursdóttir, Gunnlaugsdóttir, Jörundsdóttir, Raab, et al. (2012)
		MAE/(HNO_3)	0.663	
		MAE/(NaOH/EtOH)	0.417	
	HPLC-HG-ICP-MS	MAE/(HCl/H_2O_2)	0.614	
		MAE/(HNO_3)	NM[a]	
		MAE/(NaOH/EtOH)	0.453	
	IEC/ICP-MS	MAE/(H_2O)	1.133	Leufroy et al. (2011)
		MAE/($MeOH/H_2O$)	1.233	
	HPLC–ICP-MS	MAE($MeOH/H_2O$)	0.320	Foster, Maher, Krikowa, and Apte (2007)
		MAE/(HNO_3)	0.780	
	HPLC–ICP-MS	MAE/(H_2O)	0.100	Hirata, Toshimitsu, and Aihara (2006)
	HPLC–ICP-MS	MAE/(EtOH/NaOH)	0.190	Sloth et al. (2005)
	HPLC–ICP-MS	SON/(acetone/MeOH/HCl)	0.09	Cao et al. (2009)
	HPLC–ICP-MS	MAE/(EtOH/NaOH)	0.340	Pétursdóttir, Gunnlaugsdóttir, Jörundsdóttir, Mestrot, et al. (2012), Pétursdóttir, Gunnlaugsdóttir, Jörundsdóttir, Raab, et al. (2012)
	HPLC–HG-ICP-MS		0.470	
	HPLC–HG-AFS		0.369	
	HPLC-ICP-MS	MAE/(EtOH/NaOH)	0.188	Larsen et al. (2005)
	HPLC-HG-AFS	Mineralization/(HCl/KI/ascorbic acid)	0.320	Baeyens et al. (2009)
	HPLC-HG-AFS	Shaking/(H_3PO_4)	0.450	Geng et al. (2009)
	CT-HG AAS	Alkaline digestion/(NaOH)	ND[b]	
	HPLC-HG-ICP-MS	MAE/(HCl/H_2O_2)	0.614	Pétursdóttir et al. (2014)
		MAE/(H_2O/MeOH)	0.676	
		SON and MAE/(TFA/H_2O_2)	0.315	
		Described in reference	0.331	
		MAE/(HNO_3)	0.823	
		MAE/(HNO_3/H_2O_2)	0.714	
		MAE/(H_2O)	0.611	
		SON/(H_2O)	0.470	
		MAE/(NaOH/EtOH)	0.453	
DOLT-4	HPLC-ICP-MS	MAE/(HCl/H_2O_2)	0.039	Pétursdóttir, Gunnlaugsdóttir, Jörundsdóttir, Mestrot, et al. (2012), Pétursdóttir, Gunnlaugsdóttir, Jörundsdóttir, Raab, et al. (2012)
		MAE/(HNO_3)	0.028	
		MAE/(NaOH/EtOH)	0.027	
	HPLC-HG-ICP-MS	MAE/(HCl/H_2O_2)	0.011	
		MAE/(HNO_3)	0.011	
		MAE/(NaOH/EtOH)	0.010	
	HPLC-ICP-MS	MAE/(HCl/H_2O_2)	<0.040	
		MAE/($MeOH/H_2O$)	ND	
		SON/(trifluoracetic acid/H_2O_2)	0.047	Baer et al. (2011)
	FI-HG-AAS	Shaking/(H_2O/HCl/HBr/hydrazine sulphate)	0.075	
	HR-ICP-MS	Shaking/(H_2O/HCl/HBr/hydrazine sulphate)	0.152	
	HPLC-HG-ICP-MS	MAE/(HCl/H_2O_2)	0.011	Pétursdóttir et al. (2014)
		MAE/(H_2O/MeOH)	0.012	
		SON and MAE/(trifluoracetic acid/H_2O_2)	0.011	
		Described in reference	0.036	
		MAE/(HNO_3)	0.011	
		MAE/(HNO_3/H_2O_2)	0.017	
		MAE/(H_2O)	0.011	
		SON/(H_2O)	0.010	
		MAE/(NaOH/EtOH)	0.010	
BCR 627	IEC/ICP-MS	MAE/(H_2O)	0.074	Leufroy et al. (2011)
		MAE/($MeOH/H_2O$)	0.192	
	IEC/ICP-MS	MAE/(MeOH)	0.100	Dufailly, Noel, Fremy, Beauchemin, and Guerin (2007)
	HG–AFS	SON/(HNO_3/Triton X-100)	0.070	Cava-montesinos et al. (2005)
	HPLC–ICP-MS	MAE/(EtOH NaOH)	0.015	Sloth et al. (2005)
	HPLC–ICP-MS	Matrix solid phase extraction/($MeOH/H_2O$)	0.080	Moreda-Piñeiro et al. (2008)
	IC–ICP-MS	MAE-enzymatic/(pronase/lipase)	ND[b]	Reyes et al. (2009)
	LC–ICP-MS	MAE/($MeOH/H_2O$)	0.325	Santos et al. (2013)

(*continued on next page*)

Table 3 (*continued*)

CRMs	Techniques	Extractions	iAs (mg kg^{-1})	References
SRM 1566b	HPLC-HG-AFS	Shaking/(H_3PO_4)	ND[b]	Geng et al. (2009)
	CT-HG AAS	Alkaline digestion/(NaOH)		
	HPLC–ICP-MS	MAE/(EtOH/NaOH)	0.004	Sloth et al. (2005)
	HPLC-ES-SRM	Shaking/(H_2O)	ND[b]	Nischwitz and Pergantis (2005)
	LC–ICP-MS	MAE/(MeOH/H_2O)	1.161	Santos et al. (2013)

MAE, microwave assisted extraction; SON, sonication.
[a] NM, not measured.
[b] ND, not detected.

Table 4
Total arsenic in seafood samples, concentrations are expressed as mg As kg^{-1} dry mass (mean ± SD, n = 3).

Samples	Species	Trade name	Origin	Total As
Fish	*Urophycis cirrata*	White fish	Brazil	35.2 ± 1.14
	Pagrus pagrus	Red porgy	Brazil	35.0 ± 0.16
	Merluccius hubbsi	Hake-1	Brazil	7.10 ± 0.04
	Merluccius gayi	Hake-2	Brazil	4.20 ± 0.11
	Phycis blennoides	Forkbeard	Spain	31.8 ± 1.27
	Sardina pilchardus	Sardine	Spain	7.42 ± 0.08
	Salmo sp.	Salmon-1	Spain	1.70 ± 0.09
	Salmo sp.	Salmon-2	Spain	1.77 ± 0.10
	Thunnus sp.	Tuna-1	Spain	1.44 ± 0.09
	Thunnus sp.	Tuna-2	Spain	1.71 ± 0.12
	Luvarus imperialis	Louvar	Spain	4.46 ± 0.08
	Xiphias gladius	Swordfish-1	Spain	5.10 ± 0.08
	Xiphias gladius	Swordfish-2	Spain	3.30 ± 0.21
	Xiphias gladius	Swordfish-3	Spain	2.90 ± 0.04
Crustaceans	*Aristeus antennatus*	Prawn-1	Spain	2.3 ± 0.07
	Aristaeopsis edwardsiana	Prawn-2	Spain	3.1 ± 0.08
	Crangon crangon	Shrimp	Spain	1.2 ± 0.05
Bivalves	*Tapes pullastra*	Clams-1	Spain	17.0 ± 1.40
	Tapes decussatus	Clams-2	Spain	12.2 ± 0.16
	Mytilus edulis	Mussel	Spain	12.9 ± 0.74
	Cerastoderma edule	Cockle	Spain	8.3 ± 0.02
	Ostrea sp.	Oyster	Spain	24.6 ± 0.30

samples. The samples were classified as fish (n = 14), crustaceans (n = 3) and bivalves (n = 5) and the values found for total As in seafood samples are reported in Table 4. The concentration of total As ranged from 1.2 to 35.2 mg kg^{-1} dry mass. Bivalves contained more total As than fish (with the exception of three fish samples). A mean of 10.2 mg kg^{-1} dry mass (dm) was found in fish, while in bivalves and crustaceans the mean were 15.0 and 2.2 mg kg^{-1}, respectively. These results are consistent with the literature (Baeyens et al., 2009; Fontcuberta et al., 2011; Leufroy et al., 2011; Moreda-Piñeiro et al., 2008; Sirot, Guérin, Volatier, & Leblanc, 2009). The 2004 EU SCOOP report (European Commission, 2004) and Sirot et al. (2009) highlighted the importance of geographical, seasonal and environmental factors in the large variation in arsenic levels in seafoods. Two Brazilian fish samples (whitefish and red porgy) and one Spanish fish sample (forkbeard) showed the highest levels of total As: 35.2 ± 1.14 mg kg^{-1}, 35.0 ± 0.16 mg kg^{-1} and 31.8 ± 1.27 mg kg^{-1} respectively. The levels of total As in oyster and mussel samples were 24.6 ± 0.30 mg kg^{-1} and 12.9 ± 0.74 mg kg^{-1}, respectively. Leufroy et al. (2011) found similar values in five different oyster samples (average of 20.4 mg kg^{-1} for total As) and ten different mussel samples (average of 11.3 mg kg^{-1} for total As). The Brazilian government, through the Ministry of Agriculture, Livestock and Food Supply (MAPA), established a reference value of 1 mg kg^{-1} for total As in fish (National Program for Residue & Contaminant Control, 2012). The values found in this work are above the values recommended by the Brazilian government. Although the seafood samples had high levels of total As, the dominant species was AB (approximately 66% for oyster and mussel, and 95% for fish, Table 2), which is considered non-toxic. In contrast, Zheng and Hintelmann (2004) found lower levels of AB in samples collected from the Moira Lake (less than 16% of total arsenic). Those data demonstrate the need to carry out speciation in seafood samples as the total amount of As does not provide enough information about the toxicity of the analysed sample.

3.4. Arsenic species in samples

A selection of 22 seafood samples including crustaceans, bivalves and fish, were analysed for their content of As species. The results are reported in Table 2.

AB was found the main arsenic species in all analysed samples as expected (Leufroy et al., 2011; Sirot et al., 2009) ranging from 48% to 95% of the total arsenic. DMA was also detected as minority compounds in mussels, clams and prawns, as reported in the literature (Cao et al., 2009; Cava-Montesinos, Nilles, Cervera, & Guardia, 2005; Leufroy et al., 2011; Moreda-Piñeiro et al., 2008; Sirot et al., 2009; Súñer et al., 2002). DMA was found in 73% of samples, and MA appeared in 36% of samples (prawns, shrimp, cockles and oysters). DMA was found at higher levels than MA in fish samples which is in agreement with other published studies (Cava-Montesinos et al., 2005; Leufroy et al., 2011; Sirot et al., 2009; Súñer et al., 2002). TMAO and AC were found in 50% and 18% of all samples respectively. As mentioned before, an interesting study was carried out by Zheng and Hintelmann (2004), which reported an unusual distribution of As species in fresh water fish samples. In this study, high concentration of DMA was found in a predatory fish sample and a high TETRA content was observed in the muscle tissue of pumpkinseed (34.9%) and largemouth bass (24.4%).

An unknown compound with a retention time of 279 s was found using the cationic column (UC-A, ranged from 0.6% to 27% of total arsenic) (Fig. 1), along with a second unknown compound (UC-B, ranged from 0.3% to 6% of the total arsenic) with a retention time of 360 s. These unknown cation species could be attributed to trimethylarsoniopropionate (TMAP) and tetramethylarsonium ion (TETRA), respectively, according to Kirby, Maher, Ellwood, and Krikowa (2004). However, it was not possible to check this attribution due to the lack of appropriate standards.

In terms of anionic species, two unknown compounds, UA-A and UA-B, with a retention time of 148 and 251 s respectively, were found as minor species in crustacean and bivalve samples (Fig. 1). These unknown peaks ranged from 0.4% to 0.9% and from 0.2% to 15% of the total arsenic, for UA-A and UA-B, respectively. These peaks could correspond to arsenosugar compounds such as dimethylarsinoylsugarglycol and dimethylarsinoylsugarphosphate, which were identified in fish and molluscs (Nischwitz & Pergantis, 2005). Due to the lack of appropriate standards, this attribution was not checked.

The inorganic arsenic was extracted, identified and quantified as As(V), and selectively separated from other arsenic compounds. It was found in 36% of all samples being always below 3.3% of the total arsenic. For fish samples, the inorganic arsenic content is in all cases below the limit of detection. (n = 14). This is illustrated in Fig. 2a, which shows that inorganic arsenic was not detected in red porgy extracts (continuous line), and also shows that the all the spiked iAs was successfully recovered as As(V) (dotted line). The extraction method not converted the other organoarsenic species into inorganic arsenic (iAs). Fig. 2b shows that the major arsenic compound in red porgy extracts was arsenobetaine. Low concentrations for iAs (<0.037 mg kg^{-1}) in fish have been reported in other studies which are in agreement with the results found in the present study (Fontcuberta et al., 2011; Larsen, Engman, Sloth, Hansen, & Jorhem, 2005; Leufroy et al., 2011). However, iAs was found in bivalves and crustaceans at concentrations of up to 0.35 mg kg^{-1}. In all samples analysed in this work, iAs accounted for less than 3.3% of the total arsenic and was below the limits allowed by Australia/New Zealand (Australia New Zealand Food Authority, 2013) and China (MHC, 2005). The highest concentration of iAs (0.35 ± 0.009 mg kg^{-1}) was found in the clam-1 sample, followed by cockle (0.27 ± 0.008 mg kg^{-1}). Chromatograms of the clam-1 extract from anion exchange (a) and cation exchange (b) are shown in Fig. 1. Inorganic arsenic was found in the clam-1 sample (Fig. 1a, continuous line), which was fortified with As(III) and As(V), and as can be seen, iAs was recovered successfully as As(V) (Fig. 1a, dotted line). The lowest concentration of iAs (0.033 ± 0.003 mg kg^{-1}) was found in shrimp, as previously observed (Baeyens et al., 2009; Leufroy et al., 2011; Sirot et al., 2009; Sloth, Larsen, & Julshamn, 2005).

The present results showed a wide variability in the arsenic species found in seafood samples, highlighting the need to carry out speciation to discern the toxic from the non-toxic species.

4. Conclusions

The differences found in the literature among the concentrations of iAs in several CRMs reinforce the need to develop reliable methodology to its determination. Therefore, a method for the determination of inorganic arsenic as well as for AB, DMA, MA, AC and TMAO species in seafood was proposed. Regarding the advantages of the proposed method, the conversion of As(III) to As(V) which allows the quantification of iAs as As(V) is the most notable factor. As(III) elutes near the void volume in the anion-exchange column and it could co-elute with other cationic species usually found in seafood (specially AB). Therefore, the oxidation of As(III) to As(V) allows the determination of iAs as As(V) which is well separated from other As species. Also it is remarkable that is not necessary to quantify two peaks to determine iAs, so errors are minimised. Thus, the present method allows an accurate quantification of iAs and could be a valuable tool for food control laboratories which assessing the iAs in seafood samples.

To assess the applicability of the method, total arsenic and arsenic species in different seafood samples, including fish, crustaceans and bivalves, were determined. AB was the predominant arsenic species in all samples. Inorganic arsenic content was below the detection limit in all fish samples, whereas it was found in all bivalves and crustacean samples (and CRMs) ranged from 0.02 to 0.71 mg As kg^{-1} of iAs.

For an accurate assessment of food safety more efforts will be needed such as validation and interlaboratory comparison exercise for iAs determination in seafood that, up to date, have shown unsatisfactory performances. Despite the lack of Brazilian and European legislation regulating the maximum levels of iAs in seafood, the present results have increased the availability of reliable results on inorganic arsenic in seafood and could be useful for EFSA in future dietary exposure to iAs and in further Directives on iAs in food commodities.

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5.4 Occurrence of inorganic arsenic in edible Shiitake (*Lentinula edodes*) products

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Occurrence of inorganic arsenic in edible Shiitake (*Lentinula edodes*) products

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ABSTRACT

The present study reports arsenic speciation analysis in edible Shiitake (*Lentinula edodes*) products. The study focused on the extraction, and accurate quantification of inorganic arsenic (iAs), the most toxic form of arsenic, which was selectively separated and determined using anion exchange LC–ICPMS. A wide variety of edible Shiitake products (fresh mushrooms, food supplements, canned and dehydrated) were purchased and analysed. A cultivated Shiitake grown under controlled conditions was also analysed. The extraction method showed satisfactory extraction efficiencies (>90%) and column recoveries (>85%) for all samples. Arsenic speciation revealed that iAs was the major As compound up to 1.38 mg As kg^{-1} dm (with a mean percentage of 84% of the total arsenic) and other organoarsenicals were found as minor species. Shiitake products had high proportions of iAs and therefore should not be ignored as potential contributors to dietary iAs exposure in populations with a high intake of Shiitake products.

1. Introduction

The consumption of wild edible mushrooms has increased worldwide during recent years. *Lentinula edodes* (Berk.) Pegler (also known by its Japanese name of Shiitake) is one of the five most cultivated edible mushrooms in the world, being particularly popular in China, Japan and other Asian countries (Chang & Miles, 2004; Kalač, 2013). Furthermore, it is a dietary source of protein, vitamin D, B complex vitamins and minerals. It is one of the best-known and best-characterised mushrooms, having been used in medicine for thousands of years. *L. edodes* mycelium extract and its purified fractions have many physiological properties including antitumour, antiviral, antioxidant, antifungal, hypoglycemic and immunomodulatory activity (Chang & Miles, 2004; Wasser, 2002).

Regarding the toxicological aspects of arsenic in food, inorganic arsenic (iAs, (arsenite or As(III) and arsenate or As(V)) is considered to be the most dangerous form due to its biological availability and physiological and toxicological effects (iAs is classified as a non-threshold, class 1 human carcinogen) (ATSDR Toxicological profile for arsenic, 2007). Other arsenic compounds, such as arsenobetaine (AB), are non-toxic and can be consumed without concern, while arsenosugars are potentially toxic (Feldmann & Krupp, 2011). Therefore, toxicological knowledge of the different arsenic species should be considered by legislators and regulators when establishing maximum arsenic levels in food directives.

The ability of some mushroom species to accumulate arsenic may represent a serious risk to consumer health (Dembitsky & Rezanka, 2003; Falandysz & Borovička, 2013; Kalač, 2010; Vetter, 2004). The arsenic content of mushrooms is regulated by genetic factors and natural conditions (type of soil, bedrock, habitat, environmental factors) (Falandysz & Borovička, 2013; Vetter, 2004). More than 50 different naturally occurring As-containing compounds have been identified, comprising both organic and inorganic forms (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2009). Some of these have been found in mushrooms, including methylarsonate (MA), dimethylarsinate (DMA), As(V), As(III), AB, arsenocholine (AC), trimethylarsine oxide (TMAO), tetramethylarsonium cation (TETRA) and arsenosugars (Koch, Wang, Reimer, & Cullen, 2000; Koch et al., 2013; Larsen, Hansen, & Gössler, 1998; Niedzielski, Mleczek, Magdziak, Siwulski, & Kozak, 2013; Smith, Koch, & Reimer, 2007; Soeroes et al., 2005; Šlejkovec, Byrne, Stijve, Goessler, & Irgolic, 1997).

The arsenic compounds in edible mushrooms are obviously of concern to the consumer and the regulatory authorities, but currently, no limits exist in the European Union (EU) on arsenic, either total or inorganic, in foods (European Union Regulation 1881/2006). On the other hand, China has a maximum allowable concentration of total arsenic in mushrooms of 0.5 and 1.0 mg As kg^{-1}, for

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fresh and dry mushrooms, respectively (MHC, 2003, 2005). Given this situation, the European Food Safety Authority (EFSA) (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2009) and the FAO/WHO Joint Expert Committee on Food Additives (JECFA) (FAO/WHO, Evaluation of certain contaminants in food, 2011) have evaluated dietary exposure to As. Both reported the urgent need for further data on arsenic species, particularly iAs data, in food commodities, in order to improve the background data for future risk assessment analysis. Furthermore, mushrooms were included among the foods that contribute to iAs exposure in the general European population (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2009). The report also highlighted the need for a robust validated analytical method for the determination of iAs in a range of food items. To this end, several proficiency tests (PTs) on iAs in different foodstuffs have been organised (Baer et al., 2011; de la Calle et al., 2011; de la Calle et al., 2012). Satisfactory performance was generally found for the determination of iAs in rice, wheat and vegetable food; and it was also emphasised that there is no reason not to consider the option of introducing possible maximum levels for iAs in rice, wheat, vegetable food and algae, in further discussions on risk management.

Due to the increasing focus on inorganic arsenic in food and given that mushroom consumption had increased considerably in recent years due to their nutritional properties, two PTs, using the same test item, IMEP-116 and IMEP-39, were organised by the Institute for Reference Materials and Measurements (IRMM) (Cordeiro et al., 2013). Thus, the total and inorganic arsenic content in mushrooms is a topic of current priority for the Directorate for Health and Consumers (DG SANCO) of the European Commission. The iAs concentration in the Shiitake test sample was quite high, at around 0.3 mg As kg^{-1}, accounting for 50% of the total As. Therefore, arsenic speciation data, particularly iAs data, for Shiitake samples are needed to estimate the health risk associated with dietary As exposure.

Although Shiitake has medicinal properties and is one of the most consumed and cultivated mushrooms, few studies of arsenic speciation appear in the literature (Wuilloud, Kannamkumarath, & Caruso, 2004). Thus, more studies on Shiitake are required to provide information about iAs levels, which would be useful in toxicological risk assessments. Therefore, the main goal of this study was to determine total arsenic and arsenic species in several edible Shiitake products. The study focused on the extraction, identification and accurate quantification of the toxic inorganic arsenic species. In addition, a preliminary study of Shiitake cultivation was performed in a small-scale mushroom facility in order to estimate the possible health risks of home-cultivated Shiitake grown on a commercial substrate. Fruiting bodies and substrate samples were investigated for total arsenic and arsenic species.

2. Materials and methods

2.1. Reagents and standards

All solutions were prepared with doubly deionised water obtained from Millipore water purification systems (Elix & Rios) (18.2 MΩ cm^{-1} resistivity and total organic carbon <30 μg L^{-1}). Nitric acid (69%, Panreac, Hiperpur) and hydrogen peroxide (31%, Merck, Selectipur) were used for the digestion and extraction procedures. Ammonium dihydrogen phosphate (Panreac, p.a.), ammonia solution (25%, Panreac, p.a.), pyridine (Scharlau, p.a.) and formic acid (98%, Panreac, p.a.) were used to prepare mobile phases.

External calibration standards for total As were prepared daily by dilution of a standard stock solution traceable to the National Institute of Standards and Technology (NIST), with a certified concentration of 1000 ± 5 mg As L^{-1} (Inorganic Ventures Standards). An arsenate standard solution of 1000 ± 5 mg As L^{-1} (Merck) was used for external quality control in total arsenic and arsenic speciation measurements.

Stock standard solutions (1000 mg As L^{-1}) for arsenic speciation were prepared as follows: As(III), from As_2O_3 (NIST, USA, Oxidimetric Primary Standard 83d, 99.99%) dissolved in 4 g L^{-1} NaOH (Merck, Suprapure); As(V), from $Na_2HAsO_4{\cdot}7H_2O$ (Carlo Erba) dissolved in water; MA, prepared from $(CH_3)AsO(ONa)_2{\cdot}6H_2O$ (Carlo Erba) dissolved in water; and DMA, prepared from $(CH_3)_2AsNaO_2{\cdot}3H_2O$ (Fluka) dissolved in water. AC from $(CH_3)_3As^+(CH_2)CH_2OHBr^-$ was supplied by the "Service Central d'Analyse" (CNRS Vernaison, France); and a certified reference material of AB from $(CH_3)_3As^+CH_2COO^-$ was supplied by National Metrology Institute of Japan (NMIJ, Japan) as NMIJ CRM 7901-a, standard solution. TMAO was prepared from $(CH_3)_3AsO$ (Argus Chemicals srl) dissolved in water. Arsenate, arsenite, DMA, MA, AC, TMAO and AB were standardised against As_2O_3 for our internal quality control. All stock solutions were kept at 4 °C, and further diluted solutions for the speciation analysis were prepared daily.

2.2. Samples and certified reference materials

Different types of Shiitake-based food commodities that are representative of all types of edible Shiitake products consumed in Spain, were purchased from markets, local supermarkets and retail stores in Barcelona, Spain, during 2012. A selection of edible Shiitake products was analysed: five fresh, four dehydrated, three canned and two food supplement samples. The three canned Shiitake are commercialised in glass vessels. According to the manufacturer, Shiitake food supplements contain both mycelium and primordia (young fruit body) cultivated into a biomass that is grown on a sterilised (autoclaved) substrate. Various brands were purchased and all samples were brought to the laboratory on the day of purchase and kept for no more than a day in the refrigerator until sample preparation, which was performed before the recommended time of consumption.

In addition, Shiitake was home-cultivated in a small-scale facility, from which mushrooms were collected as samples for further analysis, to expand the information reported in the study.

Two certified reference materials (CRMs) and a reference material (RM) were analysed during the study. NIST SRM 1570a spinach leaves was obtained from the NIST (Gaithersburg, MD, USA). WEPAL IPE-120 reference material *Agaricus bisporus* mushroom was produced by the Wageningen Evaluating Programs For Analytical Laboratories (WEPAL, Wageningen, The Netherlands). ERM-BC211 rice was obtained from the IRMM of the European Commission's Joint Research Centre (Geel, Belgium).

2.3. Apparatus and instrumentation

Mushroom samples were dried in an oven with natural convection (Digitronic, JPSelecta, Spain). The dried mushrooms were minced using a commercial mincer (Multiquick 5 Hand Processor, Spain) Braun). A microwave digestion system (Ethos Touch Control, Milestone), was used for the digestion and extraction procedures. An Agilent 7500ce inductively coupled plasma mass spectrometer (ICPMS) (Agilent Technologies, Germany) was used to determine total arsenic content. An Agilent 1200 Series LC system (Agilent Technologies, Germany) was used as the chromatographic system for arsenic speciation via coupling LC–ICPMS. The separations were performed on an anion-exchange column (Hamilton Company, USA) and cation-exchange column (Agilent Technologies, Germany) (Table 1). The outlet of the LC column

Table 1
Operating conditions of the LC–ICPMS system.

ICPMS parameters	
RF power	1550 W
Make up gas flow, Ar	0.32 L min^{-1}
Carrier gas flow, Ar	0.85 L min^{-1}
Spray chamber (type and temperature)	Scott-type and 15 °C
Sampler and skimmer cones	Nickel
Nebuliser	BURGENER Ari Mist HP
Sampling depth	8.0 mm
Cell exit	−36 V
Masses	*m/z* 75 (^{75}As), *m/z* 35 (^{35}Cl) and *m/z* 77 ($^{40}Ar^{37}Cl$)
Collision cell	OFF
Dwell time	2.0 s (*m/z* 75), 0.1 s (*m/z* 35 and *m/z* 77)
QP/OctP bias difference	3 V

Chromatographic conditions	Anionic exchange	Cationic exchange
Column	Hamilton PRP-X100 (250 mm × 4.1 mm, 10 μm)	Zorbax 300-SCX. (250 mm × 4.6 mm, 5 μm)
Pre column	Hamilton PRP-X100 (20 × 2.0 mm i.d., 10 μm)	Zorbax 300-SCX (12.5 mm × 4.6 i.d., 5 μm)
Mobile phase	20 mM $NH_4H_2PO_4$, pH = 5.8	20 mM pyridine, pH = 2.6
Flow rate	1.5 mL min^{-1}	1.5 mL min^{-1}
Injection volume	100 μL	50 μL
Column temperature	Room temperature 24 °C	Room temperature 24 °C
Pressure	145 bar	152 bar
Arsenic species	As(III), DMA, MA and As(V)	AB, AC and TMAO
Elution	Isocratic, 8 min	Isocratic, 9 min

was connected via polyether ether ketone capillary tubing to the nebuliser (Burgener Research Inc, Mississauga, Canada) of the ICPMS system (Table 1).

2.4. Cultivation of Shiitake

Cultivation of Shiitake was performed in a small-scale mushroom facility belonging to the University of Barcelona. Fruiting bodies of Shiitake were produced on a commercial pasteurised substrate inoculated with mycelium intended to be grown at homemade cultivation. The cultivation procedure followed the instructions supplied by the manufacturer. The mushrooms were grown under controlled conditions following the manufacturer's guidelines, and a large number of fruiting bodies were produced. The original substrate was submerged in tap water in a controlled chamber for 24 h. Then, the substrate was placed in a cool damp place at a temperature of 17–20 °C, under natural indoor light cycles. After a week the fungi began to fruit and all mushrooms were harvested. After the first harvest, the substrate was air dried for 20 days. After this time, the substrate was submerged in tap water for another 24 h and the whole process was repeated. This enabled a second Shiitake mushroom harvest.

The substrate was randomly sampled in triplicate three times during the cultivation study. Care was taken to collect substrate in which mycelium was not visible to the naked eye. The original substrate was first sampled before submersion and cultivation. A second sample was taken after the first cultivation (medium substrate) and a third sample after the second cultivation (final substrate). The tap water and the water remaining after substrate submersion (i.e. waste water), were also sampled during the cultivation study. Total arsenic and arsenic species were analysed by ICPMS and LC–ICPMS, respectively, in the three substrate samples and the tap and waste water samples.

2.5. Sample pretreatment

Fresh Shiitake mushrooms were cleaned by hand of substrate and foreign matter. The end of the stalk (in contact with the substrate) was removed using a stainless steel knife. Damaged or soiled parts were cut off with a knife and smaller particles were removed using a fine brush. Only the edible parts of the mushrooms were used for the analysis. Mushrooms were cut into small pieces that were then air-dried on filter paper and further dried in an oven at 40 °C for 24–48 h. The dried mushrooms were minced using a commercial mincer made of stainless steel until complete homogenisation. Care was taken to avoid contamination. Between samples, the mincer was washed once with soap and water, rinsed once with HNO_3 (about 10%), rinsed several times with deionised water, and then rinsed three times with doubly deionised water, before drying with cleaning wipes.

Shiitake food supplements, which are commercially available as tablets, were pulverized with an agate mortar, homogenised and stored over silica gel in a desiccator until analysis.

Canned Shiitake samples were drained and then dried in an oven at 40 °C for 24–48 h and finally minced using a commercial mincer until complete homogenisation. Powdered samples were stored over silica gel in a desiccator until analysis.

Dehydrated Shiitake samples were cut into small pieces and then minced using a commercial mincer until complete homogenisation and stored over silica gel in a desiccator until analysis.

Cultivated Shiitake were pretreated in the same way as the purchased fresh mushrooms. Substrate samples were pulverized, homogenised, and stored over silica gel in a desiccator until analysis of arsenic and arsenic species. Tap water and waste water were filtered through PET filters (Chromafil® PET, Macherey–Nagel, pore size 0.45 μm) and stored at 4 °C before analysis of total arsenic and arsenic species.

2.6. Moisture determination

Aliquots of 0.5 g samples were dried, in triplicate, at 102 ± 3 °C to constant weight in an oven. All the results in the study are expressed as dry mass.

2.7. Total arsenic determination

The total arsenic content of the mushroom samples, CRMs, RM and substrate samples was determined by ICPMS measurement after microwave digestion (Llorente-Mirandes, Calderón, López-Sánchez, Centrich, & Rubio, 2012). Helium gas was used in the collision cell to remove interferences in the ICPMS measurements. A solution of ^{9}Be, ^{103}Rh and ^{205}Tl was used as an internal standard. Each sample was digested and analysed in triplicate. The digestion blanks were also measured. Arsenic content in the samples was quantified by means of an external calibration curve for the standards. For quality control purposes, the standards of the calibration curve were run before and after each sample series. The detection (LOD) and quantification limits (LOQ) were estimated and were 0.006 and 0.021 mg As kg^{-1}, respectively.

2.8. Arsenic speciation analysis

The extraction of arsenic species was based on our previous studies (Llorente-Mirandes, Calderón, Centrich, Rubio, & López-Sánchez, 2014; Llorente-Mirandes et al., 2012) and was applied here to mushroom samples, CRMs, RM and substrate samples. Briefly, 0.25 g aliquots of the samples were weighed in PTFE vessels and then extracted by adding 10 mL of 0.2% (w/v) HNO_3 and 1% (w/v) H_2O_2 solution in a microwave system. This extraction method completely oxidises As(III) into As(V), without conversion of the other organoarsenicals into iAs. After extraction, arsenic speciation was carried out in extracts by LC–ICPMS (Llorente-Mirandes, Ruiz-Chancho, Barbero, Rubio, & López-Sánchez, 2010, 2011) using the conditions shown in Table 1. The total arsenic in the extracts

was determined by ICPMS (as described above). Arsenic species were quantified by external calibration curves. Extraction blanks were also analysed in each batch of samples. Each sample was extracted and analysed in triplicate. LOD and LOQ were estimated for each As species. The LODs for As(III), DMA, MA, As(V), AB, TMAO and AC were 0.0010, 0.0014, 0.0017, 0.0024, 0.0010, 0.0028 and 0.0018 mg As kg^{-1}, respectively. The LOQs for As(III), DMA, MA, As(V), AB, TMAO and AC were 0.0033, 0.0047, 0.0056, 0.0080, 0.0033, 0.0093 and 0.0060 mg As kg^{-1}, respectively.

3. Results and discussion

3.1. Quality assessment in the determination of total arsenic and arsenic species

3.1.1. Total arsenic

To evaluate the accuracy of total arsenic measurements a RM and two CRMs were analysed with every batch of samples. The present results in these CRMs showed good agreement with the certified values, as shown in Table 2. The percentage accuracy was 102% and 99% for NIST SRM 1570a and ERM-BC211, respectively.

3.1.2. Extraction efficiency

Extraction efficiencies (calculated as the ratio of total As in the extract to total As in the sample) were calculated. Several extraction solvents have been used for the speciation of arsenic in mushrooms. Extraction efficiencies appear to be highly variable, depending on the mushroom species and extraction solution, ranging from 7% to 129% (Koch et al., 2000; Larsen et al., 1998; Slekovec, Goessler, & Irgolic, 1999; Smith et al., 2007; Wuilloud et al., 2004; Šlejkovec et al., 1997). The present values ranged from 94% to 103% and extracted on average 98% of total arsenic (Table 3). These results indicated full extraction of the arsenic species that may exist in Shiitake mushrooms. The extraction efficiency of ERM-BC211, NIST SRM 1570a and WEPAL-IPE-120 was 98%, 93% and 99%, respectively (Table 2).

3.1.3. Column recovery

Column recovery (calculated as the ratio of the sum of the species eluted from the chromatographic columns to the total arsenic in the extract injected into the column) was calculated to guarantee the correctness of the chromatographic separation. This parameter, assessed in replicates with good reproducibility, allowed us to evaluate the quantification of the As species in mushroom samples. Values close to 100% usually indicate that all arsenic extracted was recovered from the analytical column. The present values obtained for column recoveries ranged between 87% and 104% and showed average column recoveries of 97% (Table 3). Satisfactory values were also obtained for the RMs: 102%, 92% and 94% for ERM-BC211, NIST SRM 1570a and WEPAL-IPE-120, respectively (Table 2).

3.1.4. Spiking experiments of inorganic arsenic

To assure the accurate identification and quantification of inorganic As species, three Shiitake samples were spiked by adding As(III) and As(V) standards to solid samples and then homogenised. The mixtures were left to stand for 30 min before extraction. Arsenate was the only inorganic species found in the spiked samples, showing the quantitative oxidation of As(III) to As(V) without conversion of the other organoarsenicals into iAs. The concentration of iAs was quantified as As(V) and determined via anion exchange LC–ICPMS. The recovery of iAs from fresh, cultivated and food supplement samples was: 93 ± 6, 97 ± 5 and 94 ± 5, respectively (mean% ± SD, $n = 3$). The results show that all of the iAs was recovered successfully (average recoveries of 95% for iAs in Shiitake samples). Furthermore, the ERM-BC211 rice material, which is certified in inorganic arsenic, was also spiked by adding As(III) and As(V) standards. The concentration of iAs was quantified as As(V) and the recovery of iAs was satisfactory: 102 ± 4%, $n = 3$.

3.1.5. Arsenic species in the reference materials

Arsenic speciation was performed on CRMs and the RM and the results are summarised in Table 2. To date, no CRMs are available for arsenic species in mushrooms. Therefore, the ERM-BC211 rice was used throughout the study to assess the accuracy and reliability of the As speciation results. The material was analysed and the results were in agreement with the certified values. The percentage

Table 2
Quality assessment of total arsenic and arsenic species in reference materials. Concentrations are expressed as mg As kg^{-1} dry mass (mean ± SD, $n = 3$).

Reference Material	Total As	Total extracted As	Arsenic species							Sum of As species	Extraction efficiency (%)	Column recovery (%)
			DMA	MA	iAs	AB	AC	TMAO	Unknown cation[d]			
ERM-BC211 rice	0.256 ± 0.009	0.252 ± 0.011	0.125 ± 0.005	0.011 ± 0.001	0.122 ± 0.006	<LOD	<LOD	<LOD	<LOD	0.258 ± 0.012	98	102
Certified value	0.260 ± 0.013[a]		0.119 ± 0.013[a]		0.124 ± 0.011[a]							
NIST SRM 1570a Spinach leaves	0.069 ± 0.005	0.064 ± 0.007	<LOD	<LOD	0.059 ± 0.005	<LOD	<LOD	<LOD	<LOD	0.059 ± 0.005	93	92
Certified value	0.068 ± 0.012[a]				0.054 ± 0.012[c]							
WEPAL-IPE-120 *Agaricus bisporus*	0.167 ± 0.012	0.166 ± 0.021	0.047 ± 0.004	<LOD	0.033 ± 0.001	0.067 ± 0.004	<LOD	<LOQ	0.009 ± 0.001	0.156 ± 0.010	99	94
Indicative value	0.137 ± 0.067[b]											

[a] Certified value: mean ± uncertainty.
[b] Indicative value: mean ± standard deviation.
[c] Reported value for iAs according to expert laboratories in IMEP-112: mean ± expanded uncertainty ($k = 2$) (de la Calle et al., 2012).
[d] Unknown cation arsenic species with a retention time of 380 s.

Table 3
Total arsenic and arsenic species in purchased edible Shiitake products. Concentrations are expressed as mg As kg^{-1} dry mass (mean ± SD, $n = 3$).

Type of Shiitake	Total As	Total extracted As	Arsenic species							Sum of As species	Extraction efficiency (%)	Column recovery (%)
			DMA	MA	iAs	Unknown anion[a]	AB	AC	TMAO			
Fresh-1	1.42 ± 0.06	1.41 ± 0.07	0.070 ± 0.004	0.025 ± 0.002	1.20 ± 0.03	<LOD	0.006 ± 0.001	<LOD	<LOQ	1.30 ± 0.037	99	92
Fresh-2	0.58 ± 0.02	0.57 ± 0.03	0.070 ± 0.003	0.009 ± 0.001	0.31 ± 0.01	0.067 ± 0.004	0.032 ± 0.003	<LOD	0.018 ± 0.002	0.51 ± 0.023	98	90
Fresh-3	0.11 ± 0.02	0.11 ± 0.01	<LOQ	<LOD	0.10 ± 0.01	<LOD	<LOD	<LOQ	<LOD	0.10 ± 0.010	96	95
Fresh-4	0.93 ± 0.01	0.93 ± 0.02	0.025 ± 0.001	0.021 ± 0.004	0.90 ± 0.04	<LOD	<LOQ	<LOD	<LOD	0.95 ± 0.045	99	102
Fresh-5	1.44 ± 0.04	1.40 ± 0.11	<LOQ	0.041 ± 0.004	1.38 ± 0.08	<LOD	<LOD	<LOD	<LOQ	1.42 ± 0.084	97	102
Canned-1	0.15 ± 0.01	0.15 ± 0.01	<LOQ	<LOQ	0.15 ± 0.01	<LOD	<LOD	<LOD	<LOD	0.15 ± 0.010	99	99
Canned-2	0.66 ± 0.07	0.62 ± 0.01	<LOQ	0.050 ± 0.004	0.58 ± 0.01	<LOD	<LOQ	<LOQ	<LOD	0.63 ± 0.014	93	102
Canned-3	0.17 ± 0.02	0.17 ± 0.01	<LOQ	<LOD	0.17 ± 0.01	<LOD	<LOD	<LOQ	<LOQ	0.17 ± 0.010	103	96
Food supplements-1	0.45 ± 0.01	0.44 ± 0.02	0.012 ± 0.001	0.012 ± 0.001	0.35 ± 0.02	<LOD	0.007 ± 0.001	<LOD	<LOD	0.38 ± 0.023	99	87
Food supplements-2	0.12 ± 0.01	0.12 ± 0.01	0.033 ± 0.001	<LOD	0.086 ± 0.011	<LOD	<LOD	<LOD	<LOD	0.12 ± 0.012	99	101
Dehydrated-1	0.14 ± 0.01	0.14 ± 0.01	0.009 ± 0.001	0.006 ± 0.001	0.12 ± 0.01	<LOD	<LOD	<LOD	<LOD	0.14 ± 0.012	100	96
Dehydrated-2	0.27 ± 0.02	0.25 ± 0.02	0.015 ± 0.001	0.010 ± 0.001	0.20 ± 0.01	<LOD	<LOD	<LOD	<LOD	0.23 ± 0.012	94	92
Dehydrated-3	0.29 ± 0.02	0.28 ± 0.01	0.020 ± 0.001	0.014 ± 0.001	0.22 ± 0.01	<LOD	<LOD	<LOD	<LOD	0.25 ± 0.012	96	89
Dehydrated-4	0.34 ± 0.03	0.33 ± 0.05	0.022 ± 0.001	0.014 ± 0.001	0.28 ± 0.02	<LOD	<LOD	<LOD	<LOD	0.32 ± 0.012	99	96

[a] Unknown anion arsenic species with a retention time of 255 s.

accuracy was 98% and 105% for iAs and DMA in ERM-BC211, respectively (Table 2).

The As speciation results in WEPAL-IPE-120 showed that AB was the major As species (40% of the total As). The inorganic arsenic content was 0.033 ± 0.001 mg As kg^{-1} (corresponding to 20% of the total As), while DMA accounted for 28% of the total As. No arsenic speciation studies on this RM mushroom have been found in the literature. However, studies on *Agaricus* sp. found that AB predominated in this mushroom genus (Koch et al., 2013; Smith et al., 2007; Soeroes et al., 2005; Šlejkovec et al., 1997), which is in agreement with the present results. Although WEPAL-IPE-120 (*A. bisporus*) is not certified for arsenic species, the sum of the As species (0.156 ± 0.010 mg As kg^{-1}) compared well with the indicative total As value of 0.137 ± 0.067 mg As kg^{-1}. An unknown compound was found by the cationic column with a retention time of 380 s and could be attributed to TETRA due to the matching of the retention times when using the same chromatographic conditions (Kirby, Maher, Ellwood, & Krikowa, 2004). However, it was not possible to check this attribution due to the lack of appropriate standards.

Regarding As species in the NIST SRM 1570a, inorganic arsenic was the major compound at 0.059 ± 0.005 mg As kg^{-1}, which was in agreement with the reference value assigned by expert laboratories in the proficiency test IMEP-112: 0.054 ± 0.012 mg As kg^{-1} (de la Calle et al., 2012).

3.1.6. External quality control

This method was tested with participation as an expert laboratory in two recent proficiency tests organised by the European Union Reference Laboratory for Heavy Metals in Feed and Food (EURL-HM) and the International Measurement Evaluation Program (IMEP) from the IRMM, IMEP-116 and IMEP-39, Determination of total Cd, Pb, As, Hg and inorganic As in mushrooms (Cordeiro et al., 2013). Satisfactory results were obtained compared with the assigned value for iAs, which demonstrates the validity and reliability of the present method. Therefore, this method could be recommended for the quantification of inorganic arsenic in edible mushrooms.

3.2. Total arsenic content in purchased Shiitake

The total arsenic content in the purchased edible Shiitake products is shown in Table 3 and ranged from 0.11 to 1.44 mg As kg^{-1} dry mass (dm). The mean arsenic concentration of 14 samples was 0.51 mg As kg^{-1} dm. Total arsenic was highest in fresh samples ($n = 5$): 0.90 ± 0.57 mg As kg^{-1} dm (mean ± SD) with wide variability between the samples. The total arsenic content for dehydrated ($n = 4$) and canned ($n = 3$) samples was 0.26 ± 0.08 and 0.33 ± 0.29 mg As kg^{-1} dm, respectively. Two food supplements of different brands were analysed and the total arsenic content was 0.45 ± 0.01 and 0.12 ± 0.01 mg As kg^{-1} dm. Four of the fresh Shiitake samples exceeded the limit of 0.5 mg As kg^{-1} established by China for fresh mushrooms (MHC, 2003, 2005). However, none of the dehydrated Shiitake exceeded the limit of 1.0 mg As kg^{-1} established by China for dry mushrooms (MHC, 2003, 2005).

The present arsenic results are in the usual range found in mushrooms from unpolluted areas (0.5–5 mg As kg^{-1}, Kalač, 2010). However, arsenic content appears to be highly variable, with significant differences according to the soil arsenic concentration as well as the ability of mushroom species to accumulate arsenic (Falandysz & Borovička, 2013; Kalač, 2010). To date, there are few studies of arsenic content in Shiitake in the literature. Several Shiitake purchased in Brazil contained As in concentrations ranging between 0.083 and 0.210 mg As kg^{-1} dm (Maihara, Moura, Catharino, Castro, & Figueira, 2008). Another study reported an arsenic content of 1.3 mg As kg^{-1} dm in a Shiitake sample (Wuilloud

et al., 2004). According to Haldimann and co-authors (1995), the As content in five Shiitake mushrooms varied from 0.04 to 0.07 mg As kg^{-1} dm. The available results on arsenic in Shiitake-based food are limited and conflicting. Given the number of samples analysed in the present study and the small amount of data available in the literature, the present values of arsenic content cannot be generalised to indicate the concentrations commonly present in Shiitake mushrooms.

3.3. Arsenic species in purchased Shiitake

The arsenic speciation results for the purchased edible Shiitake products are shown in Table 3. Inorganic arsenic was the predominant As compound in all Shiitake products and ranged from 0.086 to 1.38 mg As kg^{-1} dm, with a mean value of 0.43 mg As kg^{-1} dm. Inorganic arsenic accounted for 53–99% of the total arsenic with a mean percentage of 84% of the total arsenic, whereas DMA, MA, AB, and TMAO accounted for a few percent of the total arsenic. DMA accounted for 2.7–28%, MA accounted for 1.6–7.6% and AB accounted for 0.4–5.5% of the total arsenic. TMAO was only quantified in one sample, accounting for 3.1% of total arsenic, and AC was below the LOQ in all samples. An unknown compound separated by the anionic column was found in one sample of fresh Shiitake, with a retention time of 255 s. This unknown anionic arsenic species could be a phosphate arsenosugar. This hypothesis is supported by the fact that the retention time of phosphate arsenosugar, present in *Fucus serratus* extract, matches the retention time of the present unknown peak, when using the same chromatographic conditions (Madsen, Goessler, Pedersen, & Francesconi, 2000). However, due to the lack of appropriate standards, this identification was not checked.

The finding that almost all the arsenic in the present edible Shiitake products was present as inorganic As is shown in Fig. 1. An example of this behaviour is illustrated in an anion exchange chromatogram of fresh Shiitake extract in which iAs was identified as the main arsenic species; DMA was also clearly detected and traces of MA and cationic species were also present.

To date and to our knowledge, few studies on arsenic speciation in Shiitake are present in the literature. A study on inorganic arsenic content in Hong Kong foods found an iAs value ranging from 0.036 to 0.053 mg As kg^{-1} dm in dehydrated Shiitake samples (Wong, Chung, Chan, Ho, & Xiao, 2013). Our results on iAs in dehydrated samples ($n = 4$) are consistent with this study, with a mean value of 0.21 mg As kg^{-1} dm corresponding to 79% of the total arsenic. Wuilloud and colleagues (2004) analysed Shiitake samples by size-exclusion liquid chromatography (SEC) coupled to UV and ICPMS for detection (SEC-UV–ICPMS). In their study arsenic was found to be associated mainly with a molecular weight (MW) fraction of 4.4–4.9 kDa for all extraction solvents. The authors concluded that the arsenic species are mainly in a form that is not associated with proteins or other high MW compounds, which is consistent with the present results.

Different proportions of arsenic species have been reported in the literature depending on the mushroom species (Dembitsky & Rezanka, 2003; Falandysz & Borovička, 2013; Kalač, 2010). Gonzálvez, Llorens, Cervera, Armenta and de la Guardia (2009) reported that iAs species were the major compounds in several of the studied mushrooms and that the iAs concentration ranged from 0.14 to 0.89 mg As kg^{-1}, similar to the present results. However, a high iAs content was found in *Lycoperdon* sp. mushroom samples on a gold mine site contaminated with arsenic (Koch et al., 2000). Slekovec and co-authors (1999) reported that iAs was the predominant As compound, with the sum of arsenite and arsenate up to 35.5 mg As kg^{-1} dm in *Thelephora terrestris*. A recent study also found high levels of iAs of up to 27.1 and 40.5 mg As kg^{-1} dm for As(III) and As(V), respectively, for *Xerocomus badius* from different sample collection places (Niedzielski et al., 2013). Arsenic species content could depend on the environment; the site of sample collection is an important factor that influences both the concentration and form of As present in mushroom fruiting bodies. However, it is not entirely clear whether mushrooms accumulate inorganic arsenic from the soil, or produce it through biotransformations.

The occurrence of inorganic arsenic in food is a complex subject, because foods that are usually high in arsenic, such as seafood and fish (Fontcuberta et al., 2011) or algae (Llorente-Mirandes et al., 2010, 2011), often have a low iAs content, whereas iAs can be the major arsenic species in other foods with a lower total arsenic content, such as rice (Llorente-Mirandes et al., 2012) and cereal based-food (Llorente-Mirandes et al., 2014). Despite the increased focus in the European Commission (EC) on iAs in food commodities, no maximum levels have been set for iAs to date. However, there are ongoing discussions in the EC and CODEX Alimentarius on the potential future regulation of inorganic arsenic in rice and rice-based products. A maximum level of 0.2 mg As kg^{-1} has been proposed, but this has not been implemented in the legislation (CODEX, 2012). On the other hand, Australia and New Zealand have established different limits for iAs: 1 mg As kg^{-1} for seaweed and molluscs and 2 mg As kg^{-1} for crustaceans and fish (ANFZA (Australia New Zealand Food Authority) Food Standards Code. 2011). China has maximum limits for inorganic arsenic for different foodstuffs such as rice (MHC, 2005). According to our present results, edible Shiitake products contained in all cases high percentages of toxic inorganic arsenic (accounting for 84% of the total As). These iAs concentrations were higher than those usually found in cereal-based products (Llorente-Mirandes et al., 2014), fish, vegetable foods and meat (Fontcuberta et al., 2011) and similar to those of other widely consumed foods such as rice and rice products (Llorente-Mirandes et al., 2012), and in some cases were even higher (up to 1.38 mg As kg^{-1} dm, Table 3). Although it is true that the quantity and frequency of Shiitake intake are relatively low compared to that of rice or cereal-based food in the European population, it should not be ignored as a potential contributor to dietary iAs exposure. Nevertheless, more data on As speciation in edible Shiitake products are needed in order to accurately estimate the dietary exposure to inorganic As in such populations. There is also lack of data on bioaccessibility of iAs species in edible Shiitake products, although in a recent study, high rates of As bioaccessibility from several mushrooms are reported (Koch et al., 2013). The consideration of bioaccessibility and arsenic speciation data into the exposure assessment can further refine and improve the risk assessment process.

3.4. Cultivated Shiitake

As well as dehydrated, fresh, canned and food supplements, another way to consume Shiitake is through its cultivation in commercial substrate inoculated with mycelium intended to be grown at home. Therefore, to investigate the distribution of arsenic compounds and the potential health risks involved in the consumption of cultivated Shiitake, a preliminary cultivation study was performed.

For this, Shiitake was cultivated according to the instructions supplied by the manufacturer. Tap and waste water solutions and substrate samples were analysed before and after each harvest. The first and second harvest produced a considerable number of mushrooms of different sizes. Differences in the total yield were found between harvests: 319 g and 222 g (wet mass) for the first and second harvest, respectively. The total arsenic concentrations and arsenic species in the substrate, water and mushroom samples over the two harvest periods are summarised in Table 4.

The total arsenic in the waste water samples collected after each substrate submersion was 3.5 and 4.6 μg As L^{-1} for the first

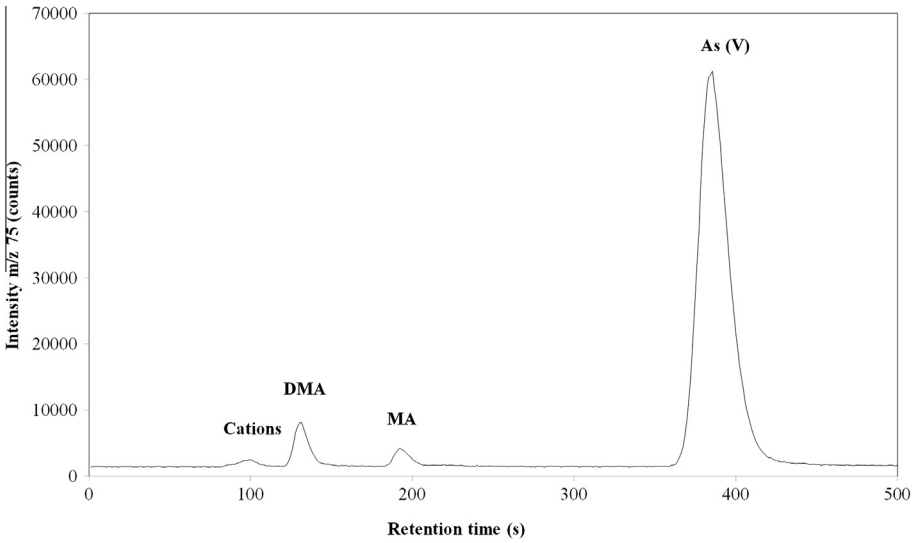

Fig. 1. Chromatogram from anion exchange by LC–ICPMS of fresh Shiitake extract.

Table 4
Total arsenic and arsenic species in cultivated Shiitake, substrate samples, and tap and waste water. Concentrations are expressed as mg As kg^{-1} dry mass (mean ± SD, n = 3) for Shiitake and substrate samples. Concentrations are expressed as μg As L^{-1} for tap and waste water (mean ± SD, n = 3).

Harvest	Sample	Total As	Total extracted As	Arsenic species								Sum of As species	Extraction efficiency (%)	Column recovery (%)
				As(III)	DMA	MA	As(V)	AB	AC	TMAO	Unknown cation [b]			
First	Mushroom-1	0.39 ± 0.02	0.38 ± 0.02	<LOD	0.026 ± 0.002	0.034 ± 0.002	0.33 ± 0.01	<LOQ	<LOD	<LOQ	0.014 ± 0.001	0.40 ± 0.015	99	105
	Original substrate	0.14 ± 0.01	0.13 ± 0.01	<LOD	0.004 ± 0.001	<LOQ	0.12 ± 0.02	<LOD	<LOD	<LOD	<LOD	0.12 ± 0.021	92	93
	Medium substrate	0.12 ± 0.02	0.12 ± 0.01	<LOD	0.005 ± 0.001	<LOQ	0.11 ± 0.01	<LOD	<LOD	<LOD	<LOD	0.12 ± 0.011	98	96
	Tap water-1	0.85 ± 0.04	n.e[a]	<LOD	<LOD	<LOD	0.82 ± 0.05	<LOD	<LOD	<LOD	<LOD	0.82 ± 0.050	–	–
	Waste water-1	3.5 ± 0.30	n.e[a]	1.06 ± 0.09	0.17 ± 0.01	0.19 ± 0.02	2.03 ± 0.15	<LOD	<LOD	<LOD	<LOD	3.44 ± 0.27	–	–
Second	Mushroom-2	0.42 ± 0.03	0.42 ± 0.01	<LOD	0.022 ± 0.001	0.012 ± 0.001	0.38 ± 0.02	<LOQ	<LOD	<LOQ	0.013 ± 0.002	0.43 ± 0.024	99	102
	Final substrate	0.15 ± 0.01	0.15 ± 0.02	<LOD	0.007 ± 0.001	<LOD	0.13 ± 0.01	<LOD	<LOD	<LOD	<LOD	0.14 ± 0.011	97	91
	Tap water-2	0.86 ± 0.03	n.e[a]	0.79 ± 0.04	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.79 ± 0.040	–	–
	Waste water-2	4.6 ± 0.60	n.e[a]	0.73 ± 0.04	0.31 ± 0.03	0.24 ± 0.02	2.85 ± 0.20	<LOD	<LOD	<LOD	<LOD	4.12 ± 0.29	–	–

[a] No extraction procedure was applied to water samples.
[b] Unknown cation arsenic species with a retention time of 380 s.

and second harvest, respectively. Inorganic arsenic (as the sum of arsenite and arsenate) was the major compound, corresponding to 88% and 78% of the total As in the first and second, respectively. Furthermore, DMA and MA were determined as minor species in both cases, probably extracted from the mycelium and/or substrate.

Substrate samples were collected throughout the cultivation study and the total As content was 0.14, 0.12 and 0.15 mg As kg^{-1} -dm for the initial, medium and final substrate, respectively. The major arsenic compound in the three substrate samples was iAs and DMA was also quantified as a minor species. The results showed that the arsenic content of the substrate, either total or species, remained unchanged during the cultivation study.

In terms of fruiting bodies, the total arsenic content in the first and second harvest was 0.39 and 0.42 mg As kg^{-1} dm respectively (Table 4), which is consistent with the range obtained in the present study for all commercial edible Shiitake (0.11–1.44 mg As kg^{-1} dm) (Table 3) and also within the range reported in the literature (Maihara et al., 2008; Wuilloud et al., 2004). The arsenic concentrations of the fruiting bodies did not differ significantly between the first and second harvest. The distribution of arsenic species in Shiitake was similar to that of the purchased mushrooms and revealed that iAs was the major As compound with a concentration of 0.33 mg As kg^{-1} dm (accounting for 85% of the total As) and 0.38 mg As kg^{-1} dm (accounting for 90% of the total As) in the first and second harvest, respectively. These results are consistent with the range found in commercial edible samples (0.086–1.38 mg As kg^{-1} of iAs) (Table 3). Other arsenic compounds were found as minor species and similar distributions were found in each harvest: DMA 6.7% and 5.2%, MA 8.7% and 2.9% of the total As for the first and second harvest, respectively. AB and TMAO were below the LOQ and AC was below the LOD. Although MA was not found in the initial substrate, it was detected in both mushroom samples. Furthermore, an unknown

compound was found by the cationic column with a retention time of 380 s. This unknown cationic arsenic species could be attributed to TETRA due to the matching of the retention times when using the same chromatographic conditions (Kirby et al., 2004). However, it was not possible to check this attribution due to the lack of appropriate standards. This arsenic species was not found in any of the substrate samples and is shown in Table 4 as 'Unknown cation'.

Few studies on arsenic species in cultivated mushrooms are available in the literature. Smith and co-authors cultivated *A. bisporus* (Smith et al., 2007), which was grown in compost amended with either arsenic-contaminated mine waste or an arsenate solution. Surprisingly, AB was found in mushrooms and was absent from compost not inoculated with *A. bisporus*. The authors hypothesised that the biosynthesis of AB was a product of fungal, not microbial, arsenic metabolism. In another study of cultivated *A. bisporus* (Soeroes et al., 2005) the results showed that mycelia were capable of taking up As(V) of the contaminated substrate. Arsenic speciation revealed that the majority of the incorporated arsenic in the treated *A. bisporus* was present as inorganic arsenic, highlighting the potential health risk posed by its consumption.

According to the present results, toxic inorganic arsenic was the main arsenic species found in both the cultivated and purchased Shiitake products. However, it is not entirely clear whether Shiitake mushrooms accumulate inorganic arsenic from the substrate, or produce it through biotransformations. Therefore, more studies on the cultivation of Shiitake grown on different commercial substrates and under different cultivation conditions are needed to investigate the uptake and distribution of arsenic in mushroom fruiting bodies.

4. Conclusions

Total arsenic and arsenic species were determined in several edible Shiitake products as well as in home-cultivated fruiting bodies. Arsenic speciation analysis showed that inorganic arsenic was the predominant arsenic compound in all samples, accounting for 84% of the total arsenic. Moreover, other arsenic species such as DMA, MA, AB, and TMAO were found as minor compounds. Despite the low intake of Shiitake products in the European population, the found inorganic arsenic contents could contribute to iAs exposure and therefore Shiitake products should not be ignored as possible source of iAs.

The analytical method used may contribute to increase the availability of reliable results on inorganic arsenic in edible mushrooms. Furthermore, the present results may be useful in ongoing discussions in the European Commission and the CODEX Alimentarius for establishing and implementing future maximum levels of inorganic arsenic in food commodities.

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5.5 Arsenic speciation in commercial edible mushrooms and mushroom supplements by HPLC-ICPMS. Assessment of inorganic arsenic exposure

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Arsenic speciation in commercial edible mushrooms and mushroom supplements by HPLC-ICPMS. Assessment of inorganic arsenic exposure

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ABSTRACT

Inorganic arsenic (iAs) is classified as a nonthreshold, class 1 human carcinogen. Some edible mushrooms are able to accumulate large amounts of arsenic which may represent a potential risk to consumer health. The present study reports arsenic and arsenic speciation analysis in several edible mushrooms and mushroom supplements marketed in Spain. Arsenic species such as iAs, DMA, MA, AB, AC, TMAO and some unknown species were determined. Total and inorganic arsenic ranged from 0.053 to 2.8 and from 0.021 to 0.61 mg As kg^{-1}, respectively. Consumption of these mushrooms would lead to a daily exposure to iAs within the range of 0.01 to 0.33 μg iAs day^{-1}. For an adult, this exposure corresponds to values ranging from 0.0002 to 0.0056 μg kg^{-1} bw day^{-1} depending on the mushroom ingested, which is well below the exposure risk range stated by the EFSA (0.3–8 μg kg^{-1} bw day^{-1}) and JECFA (2–7 μg kg^{-1} bw day^{-1}). Therefore, the regular consumption of these mushrooms or at the dosages indicated for mushroom supplements would not contribute significantly to dietary exposure to iAs and it can be concluded that there is no extra toxicological risk.

Keywords: Inorganic arsenic; Arsenic speciation; HPLC-ICPMS; Mushrooms; Risk assessment; Dietary exposure.

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1. INTRODUCTION

Edible mushrooms are a popular and beneficial food, and are considered a delicacy in many countries, especially in China, Japan and other Asian countries as well as in central and eastern Europe (Kalač, 2010). Some mushrooms have medicinal properties (Wasser, 2003) and are able to accumulate large amounts of certain elements in their fruiting bodies that are essential to fungi and their consumers, but may also accumulate hazardous elements like arsenic (As) (ATSDR, 2007). Typical arsenic levels in terrestrial foods are usually below <0.3 mg kg^{-1} while the usual content of arsenic in mushrooms from unpolluted areas and accumulating species is 0.5–5 mg kg^{-1} dry matter (Kalač, 2010). In addition, some mushroom species are able to accumulate high concentrations of arsenic present in their growth substrates, representing a potential risk to consumer health (Dembitsky and Rezanka, 2003; Falandysz and Borovicka, 2013; Kalač, 2010; Vetter, 2004). The arsenic content of mushrooms is regulated by different factors, both genetic, i.e. species of mushroom, and environmental, i.e. sampling zone and arsenic content in soil. The taxonomic position of mushrooms seems to be the most important factor in arsenic uptake and content (Vetter, 2004).

Arsenic is a widespread metalloid in the environment and its toxic effects greatly depend on its species and chemical form. More than 50 different naturally occurring As-containing compounds have been identified, comprising both organic and inorganic forms (EFSA, 2009). Regarding the toxicological aspects of arsenic in food, living organisms are particularly vulnerable to inorganic arsenic (iAs,(arsenite or As(III) and arsenate or As(V)), which is considered to be the most dangerous form due to its biological availability and physiological and toxicological effects (iAs is classified as a nonthreshold, class 1 human carcinogen) (ATSDR, 2007). On the other hand, arsenobetaine (AB), which is usually found in fish and seafood, is considered non-toxic and can be consumed without concern (Borak and Hosgood, 2007). Meanwhile, not much is known about the toxicity of arsenosugars, which are commonly found in algae and could be considered as potentially toxic (Feldmann and Krupp, 2011) since they are biotransformed by humans into toxic organoarsenicals (Sharma and Sohn, 2009). In addition, lipid-soluble As compounds (arsenolipids) have been reported as major compounds of arsenic in fatty fish and their toxicity is not yet known (Feldmann and Krupp, 2011; Francesconi, 2010).

Among the regulations proposing maximum levels of arsenic in food, few establish specific levels for iAs. Very recently, the European Union published Regulation (EU) No 2015/1006 (Commission Regulation 2015/1006) amending Annex to Regulation (EC) No 1881/2006 (Commission Regulation 1881/2006) regarding the maximum levels of iAs in rice and rice-based products but not for other foodstuffs. The only country that considers arsenic in mushrooms is China, which has a maximum level for total arsenic in mushrooms of 0.5 mg As kg^{-1} for edible fungi and their products (MHC, 2012). The toxic effects of inorganic arsenic forms led the Joint Commission FAO/WHO in 1989 to set a provisional tolerable weekly intake (PTWI) for inorganic arsenic of 15 μg kg^{-1} of body weight (equivalent to 2.1 μg kg^{-1} bw per day) (WHO, 1989). Recently, the European Food Safety Authority (EFSA) (EFSA, 2009 and 2014) and the FAO/WHO Joint Expert Committee on Food Additives (JECFA) (WHO, 2011)

evaluated dietary exposure to iAs. Both recommended that the dietary exposure to iAs should be reduced and reported the urgent need for further data on arsenic species, particularly iAs data, in food commodities, in order to improve the background data for future risk assessment analysis. Both concluded that the PTWI parameter was no longer appropriate and should no longer be used and it was thus withdrawn. The EFSA and JECFA evaluations provided estimates of toxicological intake limits for iAs as a Benchmark Dose Level (BMDL) of 0.3–8 μg kg^{-1} bw day^{-1} for a 1% increased risk of cancers of the lung, skin and bladder as well as skin lesions (EFSA $BMDL_{01}$) (EFSA, 2009) and 2–7 μg kg^{-1} bw day^{-1} for a 0.5% increased risk of lung cancer (JECFA $BMDL_{0,5}$) (WHO, 2011). The organisations also highlighted the need to develop validated methods for specific and selective determination of iAs in various types of foodstuffs.

Dietary supplements have beneficial health effects and are popular due to their easy accessibility, therapeutic efficacy and relatively low cost. Some dietary supplements are made from edible mushrooms and may contain elevated concentrations of arsenic, contributing to the overall exposure to this contaminant. Although the consumption of mushrooms and their supplements has increased considerably in recent years due to their nutritional properties, few studies of arsenic speciation in edible mushrooms marketed in Catalonia (Spain) appear in the literature. Several arsenic compounds have been reported, with their occurrence depending on the mushroom species (Dembitsky and Rezanka, 2003; Falandysz and Borovicka, 2013; Kalač, 2010). Accurate data on arsenic species content, especially iAs data, in mushrooms are essential both in primary research and subsequent assessment of health risks. Therefore, more studies on edible mushrooms are required to provide information about iAs levels, which would be useful when assessing their toxicological implications.

The main goal of this study was to determine total arsenic and arsenic species in twelve fresh mushrooms and four mushroom supplements commercially marketed in Catalonia (Spain). The content of total arsenic and arsenic species was determined by inductively coupled plasma mass spectrometry (ICPMS) and by both anion and cation exchange chromatography coupled to ICPMS (HPLC-ICPMS). The study focused on the extraction and accurate quantification of the toxic inorganic arsenic species to evaluate the contribution to the overall exposure of inorganic arsenic by these mushrooms, and thereby to assess the potential health risks involved with their consumption.

2. MATERIALS AND METHODS

2.1. Reagents and standards

Analytical-grade reagents were used throughout the study. All solutions were prepared with doubly deionised water obtained from a Millipore water purification system (Elix & Rios) (18.2 MΩ cm^{-1} resistivity and total organic carbon <30 μg L^{-1}). All stock solutions were kept at 4 °C, and further diluted solutions were prepared daily as required. Details of specific reagents and standards can be found in the Supplementary material.

2.2 Samples and certified reference materials

Edible mushrooms were purchased from local markets in Barcelona, Spain, during 2013. A selection of twelve mushroom species was analysed (Table 1). All samples were brought to the laboratory on the day of purchase and kept for no more than a day in the refrigerator until sample preparation. Only the edible parts of the mushrooms were used for the analysis. Specific information on sample pretreatment of the assayed mushrooms can be found in the Supplementary material. In addition, four mushroom supplements were purchased from local shops (Table 1). According to the manufacturer, mushroom food supplements contain both mycelium and primordia (young fruit body) cultivated into a biomass that is grown on a sterilised (autoclaved) substrate.

Two rice certified reference materials (CRMs) and a mushroom reference material (RM) were analysed during the study. NMIJ CRM 7503-a White Rice Flour was purchased from the National Metrology Institute of Japan (NMIJ, Japan). ERM-BC211 rice was obtained from the IRMM of the European Commission's Joint Research Centre (Geel, Belgium). WEPAL IPE-120 reference material *Agaricus bisporus* mushroom was produced by the Wageningen Evaluating Programs For Analytical Laboratories (WEPAL, Wageningen, The Netherlands).

2.3 Apparatus and instrumentation

Mushroom samples were dried in an oven with natural convection (Digitronic, JPSelecta, Spain). The dried mushrooms were minced using a commercial mincer (Multiquick 5 Hand Processor, Braun, Spain). A microwave digestion system (Ethos Touch Control, Milestone) was used for the digestion and extraction procedures. An Agilent 7500ce ICPMS (Agilent Technologies, Germany) was used to determine total arsenic content. An Agilent 1200 Series HPLC system (Agilent Technologies, Germany) was used as the chromatographic system for arsenic speciation via coupling HPLC-ICPMS. The separations were performed on a Hamilton PRP-X100 anion-exchange column (AEC) (Hamilton Company, USA) and Zorbax-SCX300 cation-exchange column (CEC) (Agilent Technologies, Germany). The outlet of the HPLC column was connected via polyetherketone capillary tubing to the Burgener Ari Mist HP nebuliser (Burgener Research Inc, Mississauga, Canada) of the ICPMS system.

2.4. Moisture determination

Aliquots of 0.5 g samples were dried, in triplicate, at 102 ± 3 ºC to constant weight in an oven. All results shown in Table 1 and Table 2 are expressed as dry mass. The average moisture content was 92% and 9% for fresh mushrooms and mushroom supplements, respectively.

2.5 Total arsenic determination

The total arsenic content of the mushroom samples, CRMs and RM was determined by ICPMS measurement after microwave digestion (Llorente-Mirandes, et al., 2014). Helium gas was used in the collision cell to remove interference in the ICPMS measurements. A solution of ^{103}Rh was used as an internal standard. The digestion blanks were also measured. Arsenic content in the samples was quantified by means of an external calibration curve for the standards. The standards of the calibration curve were run before and after each sample series considering as acceptable a 90–110% recovery. In order to further control the stability of our measurements, a quality control standard solution and a CRM solution were measured after every eight samples. The detection (LOD) and quantification limits (LOQ) were estimated and were 6.5 and 21.6 µg As kg^{-1}, respectively.

2.6 Arsenic speciation analysis

The extraction of arsenic species was based on our previous study (Llorente-Mirandes et al., 2014). This extraction method completely oxidises As(III) into As(V), thereby allowing the determination of total inorganic arsenic (sum of As(III) + As(V)) as As(V), and furthermore, without conversion of organoarsenic compounds into inorganic arsenic. After extraction, arsenic speciation was carried out in extracts by both AEC and CEC coupled to ICPMS (HPLC-ICPMS) using the conditions previously reported (Llorente-Mirandes et al., 2014). Chromatographic peaks were identified on the basis of their retention times by comparison with standards. Unknown compounds were quantified using the calibration curve for the closest eluting known compound. Arsenic species were quantified by external calibration curves. Extraction blanks were also analysed in each batch of samples. In each speciation run, a quality control standard solution and a CRM solution were measured every ten samples and also at the end of the sequence to control the stability of the instrument sensitivity (considering acceptable a 90–110% recovery). LOD and LOQ were estimated for each As species. The LODs for As(III), DMA, MA, As(V), AB, TMAO and AC were 1.0, 1.4, 1.7, 2.4, 1.0, 1.5 and 1.3 µg As kg^{-1}, respectively. The LOQs for As(III), DMA, MA, As(V), AB, TMAO and AC were 3.3, 4.7, 5.6, 8.0, 3.3, 4.9 and 4.3 µg As kg^{-1}, respectively.

3. RESULTS AND DISCUSSION

3.1 Assessment of quality assurance

3.1.1 Total arsenic

To assess the accuracy of total arsenic measurements, two certified reference materials were analysed alongside the assayed samples: ERM-BC211 with a certified value of 0.260 ± 0.013 mg As kg^{-1}, obtaining 0.256 ± 0.008 mg As kg^{-1} (n = 3, mean ± standard error), and NMIJ CRM 7503-a with a certified value of 0.098 ± 0.007 mg As kg^{-1}, obtaining 0.097 ± 0.004 mg As kg^{-1} (n = 3, mean ± standard error). Moreover, the measured value (0.159 ± 0.010 mg As kg^{-1})

in the WEPAL IPE-120 reference material was consistent with the indicative value (0.137 ± 0.067 mg As kg^{-1}).

3.1.2 Arsenic species in the reference materials

To date, no CRMs are available for arsenic species in mushrooms. Therefore, two rice CRMs, NMIJ CRM 7503-a and ERM-BC211, were used throughout the study to assess the accuracy of the As speciation results. Measured values (mean value ± SD, n=3) in NMIJ CRM 7503-a rice were in agreement with the certified values: 0.0136 ± 0.0008 vs 0.0133 ± 0.0009 mg As kg^{-1} for DMA, respectively and 0.0835 ± 0.004 vs 0.0841 ± 0.003 mg As kg^{-1} for iAs, respectively (certified value expressed as sum of the certified values for As(III) and As(V) ± the square sum of their uncertainties). Satisfactory values were also obtained in ERM-BC211 in comparison with the certified values: 0.121 ± 0.005 vs 0.119 ± 0.013 mg As kg^{-1} for DMA, respectively and 0.123 ± 0.005 vs 0.124 ± 0.011 mg As kg^{-1} for iAs, respectively. Moreover, a mushroom RM (WEPAL-IPE-120) obtained from a proficiency test was analysed, and showed that AB was the major As species (0.065 ± 0.004 mg As kg^{-1}), and DMA and iAs were also found: 0.044 ± 0.005 and 0.035 ± 0.003 mg As kg^{-1}, respectively, consistent with our previous study (Llorente-Mirandes et al., 2014). Furthermore, the sum of the As species (0.152 ± 0.010 mg As kg^{-1}) matched well with the indicative total As value of 0.137 ± 0.067 mg As kg^{-1}.

3.1.3 Spiking experiments of inorganic arsenic

Given the lack of a mushroom CRM with an inorganic arsenic certified value, spiking experiments were also performed to assess the accuracy of the quantification of inorganic As species. Five edible mushrooms were spiked by adding As(III) and As(V) standards to solid samples which were then homogenised. The mixtures were left to stand for 30 min before extraction. Arsenite was not found in the spiked samples and arsenate was the only inorganic species, showing the quantitative oxidation of As(III) to As(V). Therefore, the concentration of iAs was quantified as As(V) and determined via anion exchange HPLC-ICPMS. The recovery of iAs from *L. deliciosus, M. oreades, M. procera, A. bisporus* and *L. lepidum* was: 96 ± 5, 93 ± 4, 93 ± 5, 97 ± 4, and 98 ± 4, respectively (mean % ± SD, n = 3). The results show that all of the iAs was recovered successfully, with an average recovery of 95% for iAs (n=5), without conversion of the other organoarsenicals into iAs. Furthermore, the ERM-BC211 rice material, which is certified in inorganic arsenic, was also spiked by adding As(III) and As(V) standards and the recovery of iAs was satisfactory: 96 ± 6%, n=3.

3.1.4 Mass balance study

A mass balance study was performed to evaluate the arsenic speciation method. For this, extraction efficiency and column recovery were calculated for each assayed mushroom and CRM. Extraction efficiencies were calculated as the ratio of total As in the extract to total As in the sample and ranged from 78 to 102%. On average 93% of total arsenic was extracted (Table 2), indicating full extraction of the arsenic species present in the analysed mushrooms. Furthermore, high extraction efficiencies were obtained for RM: 99%, 97% and 96%, for ERM-BC211, NMIJ CRM 7503a and WEPAL-IPE-120, respectively. Column recovery was

calculated as the ratio of the sum of the species eluted from the chromatographic columns to the total arsenic in the extract and ranged between 80% and 106%. The average column recovery was 98% (Table 2), indicating that all arsenic extracted was recovered from the analytical column and that an accurate quantification of As species was performed. Furthermore, satisfactory values were also obtained for the RMs: 100%, 103% and 99% for ERM-BC211, NMIJ CRM 7503a and WEPAL-IPE-120, respectively. These results indicate a satisfactory mass balance study by means of evaluation of extraction efficiencies and column recoveries.

3.2 Total arsenic content

The total arsenic (tAs) content in the purchased edible mushrooms is shown in Table 1 and ranged from 0.053 to 2.83 mg As kg^{-1} dry mass (dm). The mean arsenic concentration of all assayed mushrooms was 0.68 ± 0.97 mg As kg^{-1} dm (mean ± SD, n=16) with much variability depending on the mushroom species. The total arsenic content for fresh mushrooms (n=12) and mushroom supplements (n=4) was 0.86 ± 1.07 and 0.14 ± 0.03 mg As kg^{-1} dm, respectively. Sorting by genus, arsenic content in *Craterellus* was 0.10 ± 0.07 for the four species of this genus and ranged from 0.053 to 0.200 mg As kg^{-1} dm. Meanwhile, greater variability was found in the genus *Pleurotus*, in which arsenic levels ranged from 0.057 to 0.57 mg As kg^{-1} dm (n=3, two fresh mushrooms and a mushroom supplement) with a mean value of 0.25 ± 0.28 mg As kg^{-1} dm.

Table 1. Total arsenic (tAs) in fresh edible mushroom and mushroom supplements, concentrations are expressed as mg As kg^{-1} dry mass (mean ± SD, n = 3).

Mushroom species	Trade name	Total arsenic (tAs)
Fresh mushrooms		
Craterellus cornucopioides	Horn of Plenty, Trumpet of the dead, Black Chanterelle	0.200 ± 0.020
Craterellus cibarius	Chanterelle, Girole	0.053 ± 0.005
Craterellus tubaeformis	Trumpet Chantarelle	0.065 ± 0.005
Craterellus lutescens	Golden Chantarelle	0.081 ± 0.004
Pleurotus ostreatus	Oyster Mushroom	0.57 ± 0.04
Pleurotus eryngii	Umbel Oyster Mushroom, King oyster mushroom, Boletus of the steppes	0.057 ± 0.008
Leccinum lepidum	---	0.64 ± 0.07
Marasmius oreades	Fairy ring mushroom, Scotch bonnet	2.37 ± 0.13
Boletus edulis	Penny bun, Porcino, Cep, King bolete	0.147 ± 0.020
Agaricus bisporus	Champignon mushroom, Button Mushroom	0.79 ± 0.01
Lactarius deliciosus	Saffron milk cap, Red pine mushroom	2.56 ± 0.19
Macrolepiota procera	Parasol Mushroom	2.83 ± 0.09
Mushroom supplements		
Pleurotus ostreatus	Oyster Mushroom	0.132 ± 0.010
Coriolus versicolor [a]	Turkey tail	0.107 ± 0.011
Ganoderma lucidum	Reishi, Lingzhi, Lacquered Bracket	0.154 ± 0.020
Grifola frondosa	Maitake, Hen of the Woods, Sheep's head	0.187 ± 0.010

[a] Mushroom identification as stated on the product label. *Coriolus versicolor* is now known as *Trametes versicolor*

The present results are similar to those reported in previous studies on the same wild and cultivated species growing on uncontaminated soils or substrates and with no evidence of significant variation (Falandysz and Borovicka, 2013; Kalač, 2010; Melgar et al., 2014; Nearing et al., 2014). High variability in arsenic content has been reported in the literature depending on the species of mushroom and on the sampling site or the type of soil. Low levels of tAs below 0.050 µg As kg^{-1} dm have been reported in 13 species of common edible mushrooms (Vetter, 2004). Meanwhile, other species are considered as arsenic bioaccumulators independent of their habitat and high arsenic contents of up to 146 mg As kg^{-1} dw have been reported (Vetter, 2004). Extreme levels of 1420 mg As kg^{-1} dw have been reported for *Laccaria amethystea* in polluted areas (Larsen et al., 1998).

Few studies have been performed on mushroom supplements. The present results are consistent with a study in which 21 mushroom supplements were analysed with a mean value of 0.40 ± 0.33 and ranging from 0.05 to 1.50 mg As kg^{-1} (Melgar et al., 2014). Furthermore, higher arsenic contents, ranging from 0.58 to 5.00 mg As kg^{-1}, have been reported in a study of dietary supplements based on herbs, other botanicals and algae purchased on the Danish market (Hedegaard et al., 2013).

3.3 Arsenic species

The arsenic speciation results of edible mushrooms and mushroom supplements are shown in Table 2, respectively. Nine different arsenic species were determined in the aqueous/nitric acid extracts from the anion and cation exchange chromatographic analysis (HPLC-ICPMS). There was much variability in their proportions, depending on the species of mushroom. Inorganic arsenic, AB and DMA were the predominant arsenic compounds in the assayed mushrooms. iAs ranged from 0.021 to 0.613 mg As kg^{-1} dm, with a mean value of 0.147 ± 0.175 mg As kg^{-1} dm (mean ± SD, n=16) and accounted for 44.9% of the tAs. Meanwhile, AB accounted for 0.7 to 52.8% of the tAs with a mean percentage of 19.4%. DMA accounted for 1.9 to 41.7% of the tAs, with a mean value of 18.4%. Other arsenic compounds such as MA, AC and TMAO accounted for a few percent of the total arsenic; MA accounted for 1.1 to 29.7% (n=9), AC accounted for 0.4 to 4.8% (n=4) and TMAO accounted for 0.8 to 16.4% (n=11).

Table 2. Total arsenic, total extracted arsenic and arsenic species in fresh edible mushrooms and mushroom supplements (mg As kg^{-1} dry mass (mean ± SD, n = 3).

Mushroom species	Total As	Total Extracted As	Arsenic species									Sum of As species	EE (%)	CR (%)
			DMA	MA	As (V)	UA [a]	AB	TMAO	AC	UC-A [b]	UC-B [c]			
Craterellus cornucopioides	0.200 ± 0.020	0.171 ± 0.013	0.016 ± 0.002	0.005 ± 0.0001	0.112 ± 0.004	<LOD	0.004 ± 0.001	0.005 ± 0.001	<LOD	<LOD	<LOD	0.142 ± 0.005	86	83
Craterellus cibarius	0.053 ± 0.005	0.047 ± 0.003	0.011 ± 0.002	<LOD	0.027 ± 0.001	<LOD	0.009 ± 0.001	<LOD	<LOD	<LOD	<LOD	0.047 ± 0.002	88	100
Craterellus tubaeformis	0.065 ± 0.005	0.059 ± 0.003	0.009 ± 0.001	0.009 ± 0.001	0.032 ± 0.002	<LOD	0.011 ± 0.001	<LOD	<LOD	<LOD	<LOD	0.061 ± 0.003	90	104
Craterellus lutescens	0.081 ± 0.004	0.072 ± 0.011	0.016 ± 0.002	0.007 ± 0.0001	0.037 ± 0.007	<LOD	0.004 ± 0.001	0.005 ± 0.001	<LOD	<LOD	<LOD	0.069 ± 0.007	89	96
Pleurotus ostreatus	0.57 ± 0.04	0.56 ± 0.01	0.017 ± 0.001	0.068 ± 0.002	0.48 ± 0.04	<LOD	0.004 ± 0.001	0.005 ± 0.001	<LOD	<LOD	<LOD	0.57 ± 0.04	98	103
Pleurotus eryngii	0.057 ± 0.008	0.051 ± 0.004	0.007 ± 0.001	0.011 ± 0.001	0.021 ± 0.001	<LOD	0.008 ± 0.001	0.005 ± 0.001	<LOD	<LOD	<LOD	0.052 ± 0.002	89	102
Leccinum lepidum	0.64 ± 0.07	0.65 ± 0.04	0.27 ± 0.02	<LOD	0.347 ± 0.02	<LOD	0.012 ± 0.002	0.007 ± 0.001	<LOD	<LOD	<LOD	0.63 ± 0.03	102	97
Marasmius oreades	2.37 ± 0.13	1.96 ± 0.10	0.044 ± 0.005	0.043 ± 0.001	0.613 ± 0.05	0.039 ± 0.005	1.08 ± 0.16	0.069 ± 0.007	0.027 ± 0.004	0.004 ± 0.001	0.016 ± 0.002	1.94 ± 0.17	83	99
Boletus edulis	0.147 ± 0.020	0.144 ± 0.007	0.017 ± 0.001	0.023 ± 0.002	0.028 ± 0.001	<LOD	0.011 ± 0.001	0.024 ± 0.002	0.007 ± 0.001	0.004 ± 0.001	0.0015 ± 0.001	0.116 ± 0.004	98	80
Agaricus bisporus	0.79 ± 0.01	0.72 ± 0.04	0.216 ± 0.003	<LOD	0.069 ± 0.004	<LOD	0.413 ± 0.01	0.017 ± 0.001	<LOD	0.004 ± 0.001	0.003 ± 0.001	0.72 ± 0.01	92	100
Lactarius deliciosus	2.56 ± 0.19	1.99 ± 0.31	0.096 ± 0.009	0.76 ± 0.06	0.099 ± 0.009	<LOD	0.893 ± 0.04	0.0213 ± 0.003	0.011 ± 0.001	0.008 ± 0.001	0.077 ± 0.006	1.97 ± 0.07	78	99
Macrolepiota procera	2.83 ± 0.09	2.80 ± 0.13	0.87 ± 0.04	0.032 ± 0.005	0.085 ± 0.009	0.048 ± 0.003	1.493 ± 0.07	0.173 ± 0.02	0.045± 0.002	0.049 ± 0.002	0.031 ± 0.002	2.82 ± 0.08	99	101
Pleurotus ostreatus [d]	0.132 ± 0.010	0.132 ± 0.010	0.030 ± 0.003	<LOD	0.096 ± 0.008	<LOD	0.003 ± 0.001	0.004 ± 0.001	<LOD	<LOD	<LOD	0.133 ± 0.009	100	101
Coriolus versicolor [d]	0.107 ± 0.011	0.104 ± 0.010	0.018 ± 0.001	<LOD	0.085 ± 0.008	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.103 ± 0.008	98	99
Ganoderma lucidum [d]	0.154 ± 0.020	0.143 ± 0.019	0.030 ± 0.003	<LOD	0.121 ± 0.01	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.151 ± 0.010	93	106
Grifola frondosa [d]	0.187 ± 0.010	0.187 ± 0.009	0.075 ± 0.005	<LOD	0.096 ± 0.015	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.171 ± 0.016	100	91

[a] UA-B is an unknown anion arsenic species with a retention time of 251 s.
[b] UC-A is an unknown cation arsenic species with a retention time of 304 s.
[c] UC-B is an unknown cation arsenic species with a retention time of 389 s.
[d] Mushroom supplements. *Coriolus versicolor* is now known as *Trametes versicolor*

The arsenic speciation of fresh market mushrooms differed according to the mushroom species (Table 2), consistent with the literature (Dembitsky and Rezanka, 2003; Falandysz and Borovicka, 2013; Kalač, 2010; Melgar et al., 2014; Nearing et al., 2014; Kuehnelt and Goessler, 2003). Arsenic species content could also depend on the environment; the site of sample collection and the type of soil are important factors that influence both the concentration and form of As present in mushrooms. The differences between the speciation patterns of market bought mushroom (n=12) are highlighted in Figure 1, which shows the proportions of arsenic compounds found in the respective mushroom extracts. Two of the most widely consumed and cultivated edible mushrooms in the world are *Agaricus bisporus* and *Pleurotus* spp. (particularly *P. ostreatus*) (Kalač, 2013), being particularly popular in China, Japan and other Asian countries. Two species of the genus *Pleurotus* were analysed and their iAs content was 83.7% and 36.6% of the total arsenic, respectively, for *P. ostreatus* and *P. eryngii* (Figure 1). This is illustrated in Figure 2a, which shows an anion exchange chromatogram of fresh *P. ostreatus* extract in which iAs was identified as the main arsenic species; MA and DMA were also clearly detected. Furthermore, traces of AB and TMAO were also present as shown in the cation exchange chromatogram (Figure 2b). These results contrast with the predominance of DMA and with the absence of iAs in the same mushroom, *P. ostreatus* (Nearing et al., 2014). Regarding *A. bisporus*, the distribution of arsenic compounds is shown in the chromatograms of Figure 3a (AEC-HPLC-ICPMS) and Figure 3b (CEC-HPLC-ICPMS). AB was the major compound; DMA, iAs and TMAO were found in smaller proportions. Several studies have reported that AB predominates in the genus *Agaricus* sp. (Koch et al., 2013; Nearing et al., 2014; Šlejkovec et al., 1997; Smith et al., 2007), which is consistent with the present results (Figure 1) and is shown in the corresponding chromatograms (Figure 3a and 3b).

The genus of *Craterellus*, commonly known as Chanterelles, includes *C. cornucopioides, C. cibarius, C. tubaeformis,* and *C. lutescens*, and contained a large proportion of inorganic arsenic (45.5% to 56.0% of the tAs, Figure 1), which is consistent with previously reported values (Nearing et al., 2014), as well as DMA, MA, AB and other compounds in smaller proportions. Meanwhile, *M. oreades, L. deliciosus* and *M. procera* contained predominantly AB as well as other organoarsenic compounds while iAs was a minority compound, corresponding to 26% in *M. oreades* and below 4% in the other two mushrooms. In *B. edulis* similar levels of iAs, MA, DMA were found and AB and AC were also detected as minor compounds. Furthermore, a high proportion of TMAO, which is unusual in mushrooms, was found, consistent with previous reports (Nearing et al., 2014). *L. lepidum* contained a high proportion of iAs and DMA, corresponding to 54.2% and 41.7%, respectively, consistent with the compounds previously reported for the genus *Leccinum* (Koch et al., 2000).

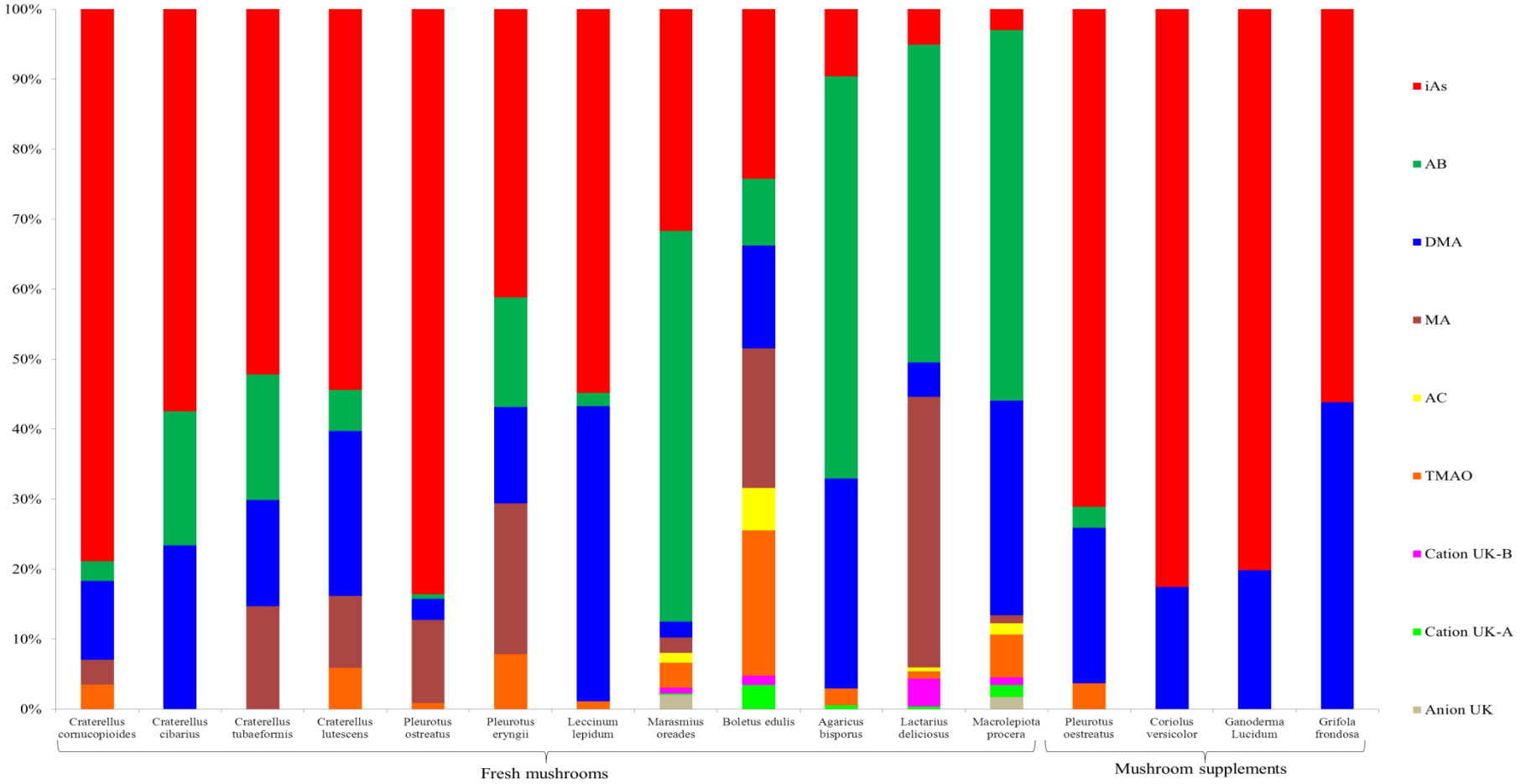

Figure 1. Proportions of arsenic species in extracts with respect to the sum of arsenic species, for each fresh mushroom and mushroom supplement. Cation UK-A and cation UK-B are peaks found in the CEC-HPLC-ICPMS chromatograms that do not match any available standards. Anion UK is a peak found in the AEC-HPLC-ICPMS chromatogram that does not match any available standards

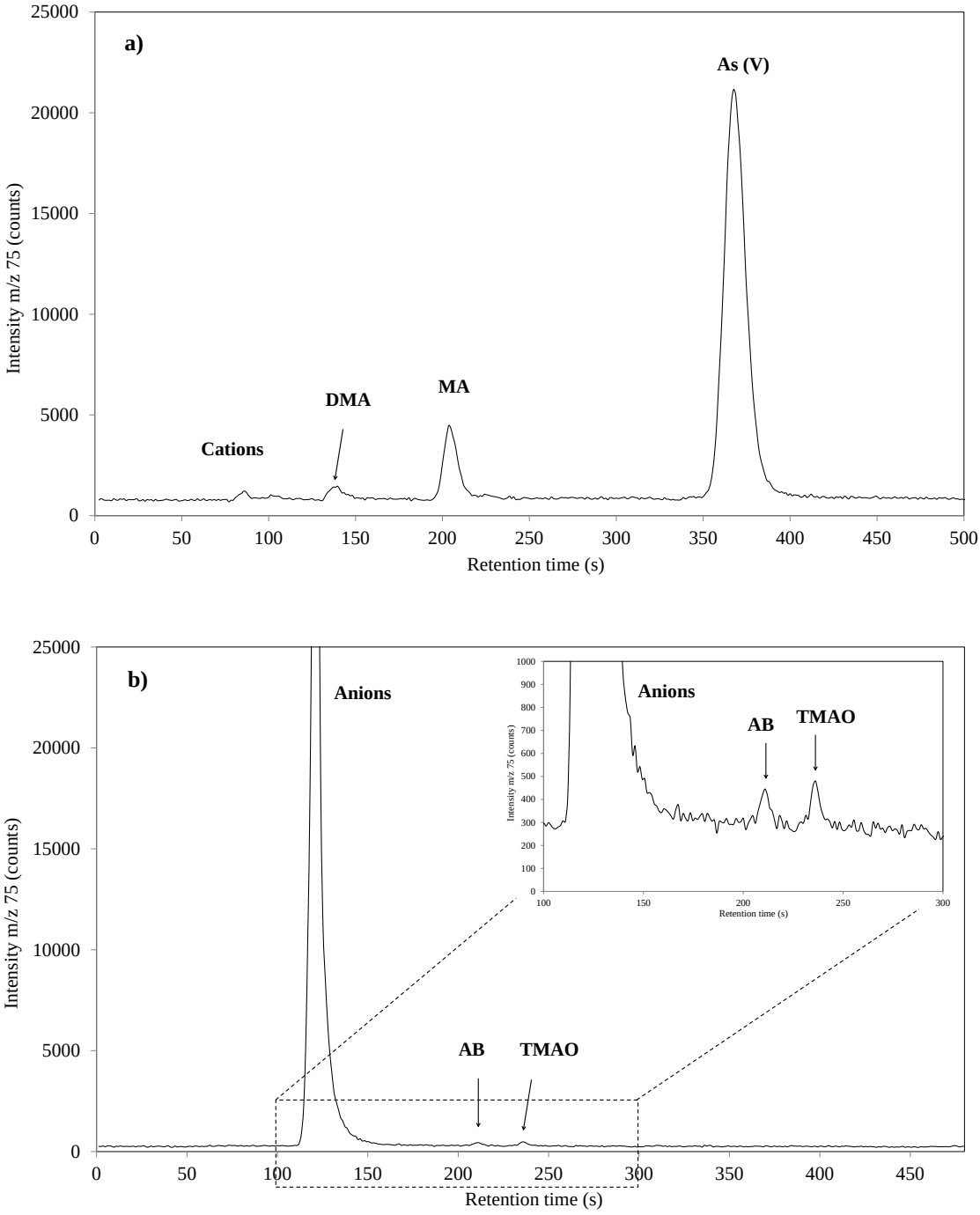

Figure 2. Chromatogram of *Pleurotus ostreatus* extract (Oyster mushroom) by anion exchange (a) and cation exchange (b) by HPLC–ICPMS

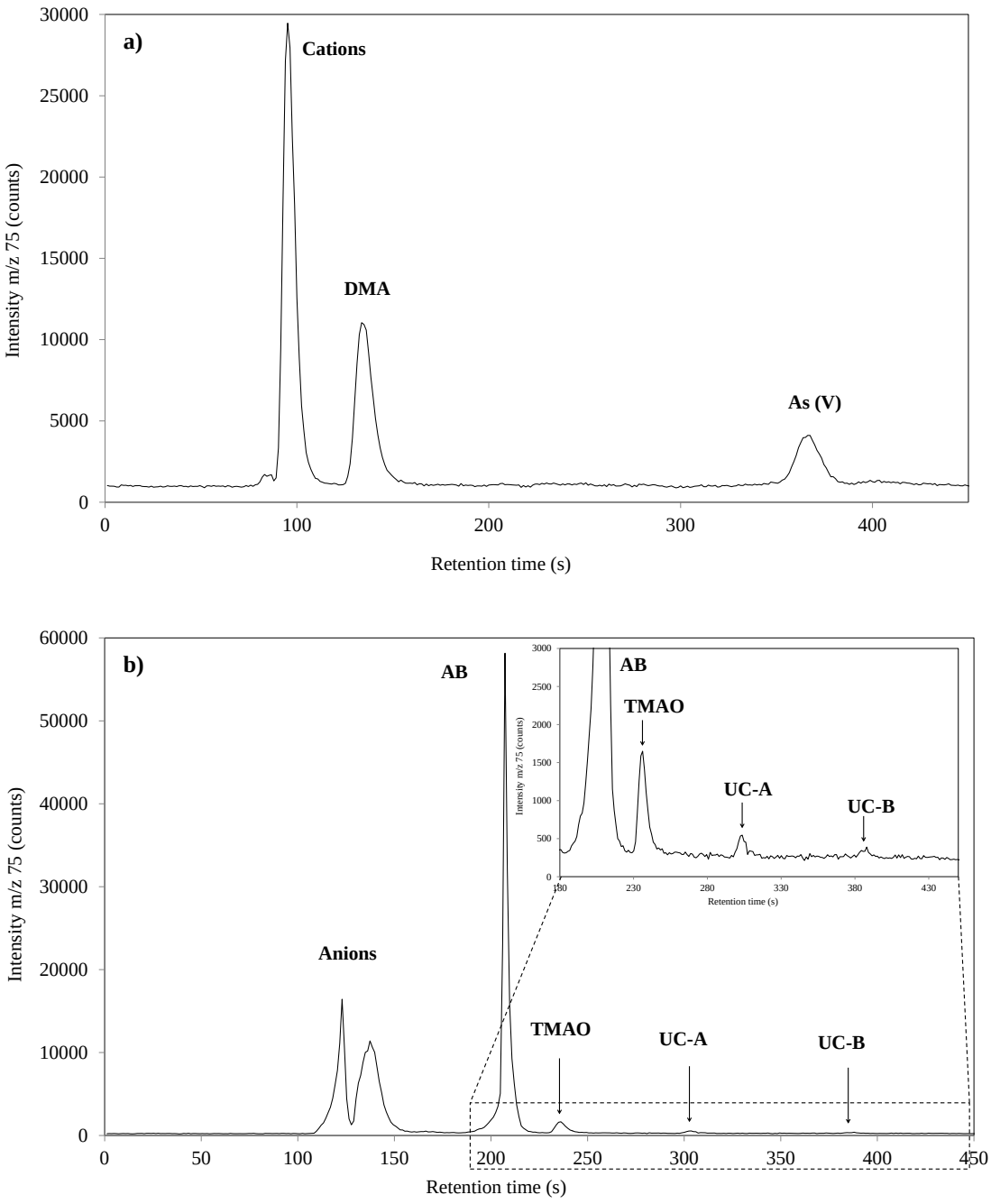

Figure 3. Chromatogram of *Agaricus bisporus* extract (Button mushroom) by anion exchange (a) and cation exchange (b) by HPLC–ICPMS

Two unknown cationic species were separated and determined by CEC-HPLC-ICPMS in some mushroom samples, accounting for 0.2 to 2.7% (n=5) and 0.4 to 3.0% (n=5) of the tAs, respectively for UC-A and UC-B. These species were well separated from the other cationic species (Figure 3b). UC-A eluted at a retention time of 304 s and could be attributed to trimethylarsonium propionate (TMAP, $(CH_3)_3As^+CH_2CH_2COO^-$) due to the matching of the retention time when using the same chromatographic conditions (Kirby et al., 2004). UC-B eluted at a retention time of 389 s and is suspected to be tetramethylarsonium ion (TETRA,

$(CH_3)_4As^+$) due to the matching of the retention time (Kirby et al., 2004). However, it was not possible to verify these identifications due to the lack of appropriate standards and need to be verified with detailed spiking and molecular mass spectrometry experiments with an authentic standard. Furthermore, an unknown anionic compound (UA) was separated and determined by AEC-HPLC-ICPMS in *M. oreades* and *M. procera* mushrooms, accounting for 1.6 and 1.7% of the tAs. This species was correctly separated from other anionic species eluting at the retention time of 251 s. This unknown anionic arsenic species could be a phosphate arsenosugar, as suggested by the fact that the retention time of this compound matches the retention time of the unknown peak, when using the same chromatographic conditions (Madsen et al., 2000). However, due to the lack of appropriate standards, this identification was not checked.

The distribution of arsenic compounds in mushroom supplements (n=4) is shown in Figure 1. All food supplements contain high proportions of inorganic arsenic (51.4% to 79.7% of the tAs) as well as DMA. Furthermore, *P. ostreatus* supplement also contained AB and TMAO. To date, no previous data are available for arsenic species in these mushroom supplements; however the present speciation pattern is similar to those reported for *Lentinula edodes* (Shiitake) supplements, with iAs and DMA being the predominant compounds (Llorente-Mirandes et al., 2014). Moreover, high iAs levels (up to 3.17 mg As kg^{-1}) have been reported in a study of dietary supplements based on herbs, other botanicals and algae, indicating that their consumption could contribute significantly to the dietary exposure to iAs (Hedegaard et al., 2013).

Few arsenic speciation data have been reported in the literature for the assayed mushrooms. Since some of the present results differ from those reported previously, and were based on a single sample, generalizations about arsenic species for these mushroom could not be made in the present study. It should be borne in mind that the speciation pattern could depend on the environment, the site of sample collection as well as on the species of mushroom, and therefore mushrooms should be sampled and analysed individually from each site and study.

3.4 Relationship between arsenic species and total arsenic

The iAs content ranged from 0.021 to 0.613 mg As kg^{-1} dm (Table 2) and was consistent with previous studies on edible mushroom from non-contaminated sites (Gonzálvez et al., 2009; Kuehnelt et al., 1997; Nearing et al., 2014). Different proportions of iAs were found in the assayed samples depending on the species of mushroom, ranging from 3.0 to 83.7% of the tAs, with a mean value of 36.4% and 70.6% for fresh mushrooms and mushroom supplements, respectively (Table 3). Furthermore, iAs made up the majority of the sum of the extracted arsenic in some of the species (Figure 1): *C. cornucopioides, C. cibarius, C. tubaeformis, C. lutescens, P. ostreatus, P. eryngii* and *L. lepidum*.

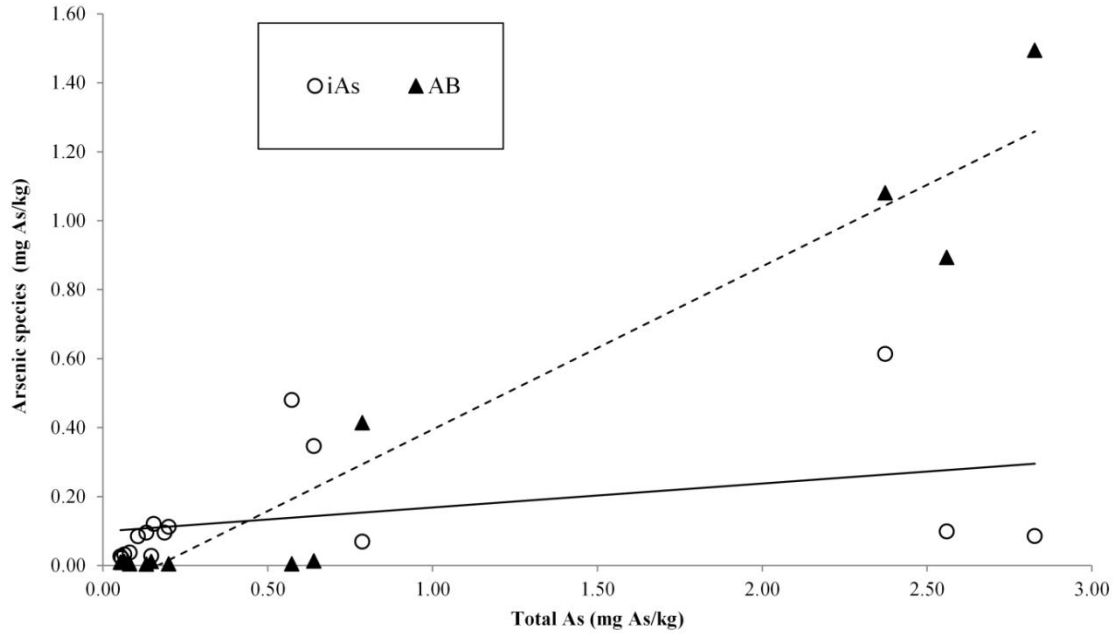

Figure 4. Relationship between arsenic species and total arsenic for fresh mushrooms and mushroom supplements. Inorganic arsenic is represented by circles and arsenobetaine by triangles

The correlation between iAs and tAs as well as AB and tAs for the analysed mushrooms is shown in Figure 4. The concentration of AB in the mushrooms containing this compound (n=13) was positively correlated with total arsenic ($y = 0.4731x - 0.0785$, $R^2 = 0.9298$). Meanwhile, there was no relationship between iAs and tAs for any of the assayed mushrooms. The regression analysis confirmed this fact ($y = 0.0695x + 0.0992$, $R^2 = 0.1489$), highlighting that iAs content is independent of tAs and depends on the species of mushroom. On the other hand, other foodstuffs such as rice showed high correlations between iAs species and tAs (Llorente-Mirandes et al., 2012; Meharg et al., 2009). Furthermore, rice may be classified into two populations, depending on the form of As in the grain: iAs-type and DMA-type (Zavala et al., 2008). This did not seem to be the case for the assayed mushrooms. Given the number of samples analysed in the present study and the small amount of speciation data available in the literature, the present relationship between iAs and tAs content cannot be extrapolated to all mushrooms.

3.5 Assessment of inorganic arsenic exposure

To assess dietary exposure to arsenic and the toxicological implications of the ingestion of fresh mushrooms and mushroom supplements, it is necessary to known the contribution of iAs in the diet of these foods. The maximum exposure to inorganic arsenic from the consumption of the mushrooms in the present study was calculated by multiplying the daily consumption (g/day wet mass of uncooked food) with the iAs content (mg As kg^{-1} wet mass) of each sample (Table 3). Daily consumption of edible mushrooms was obtained from the latest data published by the Spanish Agency for Food Safety and Nutrition (AESAN, 2011). It is

estimated that the average daily consumption of mushrooms per person is approximately 5.61 g/person/day. Meanwhile, the daily consumption of mushroom supplements was derived from the recommended number of pills per day as stated on the product label (2 to 6 pills per day; 4 per day was used for calculations) and the mass of the pills. The daily exposure to inorganic arsenic ranged from 0.01 to 0.33 μg iAs day^{-1} depending on the mushroom sample (Table 3). The highest exposure came from the four mushroom supplements and *M. oreades* and *P. ostreatus*, and the lowest from *C. cibarius*, *P. eryngii*, and *B. edulis.*

Table 3. Percentage of inorganic arsenic in mushrooms, recommended dose, and estimated inorganic arsenic exposure.

Mushroom species	Inorganic arsenic (%)	Daily consumption (g $person^{-1}$ day^{-1})	iAs daily intake (μg As day^{-1})
Craterellus cornucopioides	56.0	5.61	0.06
Craterellus cibarius	50.0	5.61	0.01
Craterellus tubaeformis	49.0	5.61	0.01
Craterellus lutescens	45.9	5.61	0.02
Pleurotus ostreatus	83.7	5.61	0.25
Pleurotus eryngii	37.2	5.61	0.01
Leccinum lepidum	54.2	5.61	0.15
Marasmius oreades	25.8	5.61	0.26
Boletus edulis	19.1	5.61	0.02
Agaricus bisporus	8.8	5.61	0.04
Lactarius deliciosus	3.9	5.61	0.05
Macrolepiota procera	3.0	5.61	0.05
Pleurotus ostreatus [c]	72.5	3.05	0.27
Coriolus versicolor [c]	79.4	3.04	0.23
Ganoderma lucidum [c]	78.6	3.04	0.33
Grifola frondosa [c]	51.2	3.04	0.26

[a] Daily consumption of fresh mushroom correspond to the average daily consumption for the Spanish population according to the Spanish Agency for Food Safety and Nutrition (AESAN, 2011).
[b] Daily consumption of mushroom supplements is calculated based on recommended intake of pills per day as stated on the product label multiplied by the mass of the pills.
[c] Mushroom supplements. *Coriolus versicolor* is now known as *Trametes versicolor.*

EFSA estimated that the exposure to inorganic arsenic from food and water across several European countries ranges from 0.09 to 0.38 μg kg^{-1} bw day^{-1} for the average adult consumer (EFSA, 2014). It also established a range of benchmark doses between 0.3 and 8 μg kg^{-1} bw day^{-1} as a reference point for risk characterisation of iAs (European Food Safety Authority, 2009). In 2010, JECFA recommended a narrower range of between 2 and 7 μg kg^{-1} bw day^{-1} (WHO, 2011). Taking these parameters as a reference and for an adult of 60 kg body weight, the consumption of the analysed samples would lead to an exposure ranging from 0.0002 to 0.0056 μg kg^{-1} bw day^{-1} from this dietary source alone, depending on the mushroom ingested. The contribution to the present exposure to iAs is quite low relative to the estimated exposure for the average consumer in European countries (EFSA, 2014) and is considerably lower than the limits recommended by EFSA and JECFA. Even considering the worst case

scenario (0.0056 μg kg^{-1} bw day^{-1}), the present exposure corresponds to 2% and 0.3% of the lower value of the EFSA and JECFA BMDL ranges, respectively. These percentages are very small, and illustrate that the contribution to dietary inorganic arsenic exposure stemming from the consumption of these mushrooms and mushroom supplements is too small to be considered a toxicological risk. Nevertheless, this fact cannot be generalised to other wild or cultivated mushrooms or food supplements. Besides, it should be taken into account that other foodstuffs could be a source of iAs that have not been considered in this study and that might increase the daily exposure to iAs. Furthermore, special attention should be paid to the consumption of bioaccumulator mushroom species or wild mushrooms growing in contaminated areas.

The levels calculated are the maximum daily intake values because the estimates presupposed that all the iAs present in the mushrooms eventually reaches the blood stream. However, there are many cooking treatments that could reduce the content of arsenic ingested via mushrooms. Our previous results showed that tAs decreased by 9% and 11% in griddled *A. bisporus* and *P. ostreatus*, respectively with respect to raw mushroom (Llorente-Mirandes et al., 2016). Boiling, meanwhile, decreased tAs content by 53% and 71% in *A. bisporus* and *P. ostreatus*, respectively producing high differences with respect to the tAs content of the raw mushroom assayed. To date, there are no data on arsenic content in the other mushroom species following cooking treatment; however several studies have been published on other foods in which high percentages of arsenic were released from food into the cooking water e.g. seaweeds (García Sartal et al., 2012), rice (Fontcuberta et al., 2011) and pasta samples (Cubadda et al., 2003). In addition, the bioaccessibility of arsenic should be assessed to refine and improve the toxicological risk process. High bioaccessibility of total arsenic was found in our previous study in raw *A. bisporus* and *P. ostreatus* (Llorente-Mirandes et al., 2016). For instance, bioaccessibility of tAs in raw *A. bisporus* was 83% and 86% for gastric (G) and gastrointestinal (GI) fractions, respectively; meanwhile in raw *P. ostreatus* was 88% and 94% for G and GI fractions, respectively. Even when a cooking process led to a decrease in tAs content in these mushrooms, the bioaccessibility of tAs remained high. Bioaccessibility values in cooked *A. bisporus* varied between 88% and 100%, meanwhile in cooked in *P. ostreatus* values varied between 83% and 94%. Furthermore, Koch et al. (2013) reported that bioaccessibility of total arsenic in raw mushrooms ranged from 20% to 91% in G extracts and from 22% to 94% in GI extracts depending on the species of mushroom. Therefore, the real levels of iAs that are bioaccessible for potential consumers of these mushrooms are likely to be lower than the values reported in this study.

4. CONCLUSIONS

Total arsenic levels ranged from 0.053 to 2.8 mg As kg^{-1} dm and were within the usual range in mushrooms analysed in unpolluted areas. The speciation pattern was highly variable depending on the species of mushroom, i.e. inorganic arsenic, DMA and AB were found in high proportions while MA, AC, TMAO and three unknowns were found in smaller proportions. The toxic inorganic arsenic content ranged from 0.021 to 0.61 mg As kg^{-1} dm with an average content of 0.147 mg As kg^{-1} dm for mushrooms and mushroom supplements. Different

proportions of iAs were found depending on the species of mushroom, ranging from 3.0 to 83.7% of the total arsenic. There was a positive correlation between AB and tAs in mushrooms, but there was no significant correlation between iAs and their tAs content.

Consumption of these mushrooms and the recommended dose of mushroom supplements would lead to a daily exposure to iAs within the range from 0.0002 to 0.0056 μg kg^{-1} bw day^{-1} from this dietary source alone, depending on the mushroom ingested, which is well below the exposure risk range stated by EFSA and JECFA. Furthermore, although the consumption of fresh mushrooms and mushroom supplements has increased in recent years, they cannot be considered a staple food and their inclusion in the diet remains at low levels; therefore, it could be concluded that there is no health risk associated with regular consumption of these mushrooms or at the dosages indicated for mushroom supplements.

Finally, current knowledge of the speciation and bioaccessibility of arsenic in edible mushrooms is limited, so more studies are needed in order to assess the toxicological risk involved with their consumption. The present results increase the availability of reliable arsenic species data in edible mushrooms and could be useful for further studies on dietary exposure to inorganic arsenic.

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SUPPLEMENTARY MATERIAL

Reagents and standards

All solutions were prepared with doubly deionised water obtained from Millipore water purification systems (Elix & Rios) (18.2 MΩ cm^{-1} resistivity and total organic carbon <30 μg L^{-1}). Nitric acid (69%, Panreac, Hiperpur) and hydrogen peroxide (31%, Merck, Selectipur) were used for the digestion and extraction procedures. Ammonium dihydrogen phosphate (Panreac, p.a.), ammonia solution (25%, Panreac, p.a.), pyridine (Scharlau, p.a.) and formic acid (98%, Panreac, p.a.) were used to prepare mobile phases.

External calibration standards were prepared daily by dilution of a standard stock solution with a certified concentration of 1000 ± 5 mg As L^{-1} (Inorganic Ventures Standards, arsenic in 2% (v/v) HNO_3) traceable to the National Institute of Standards and Technology (NIST). A standard solution of arsenate with a certified concentration of 1000 ± 5 mg As L^{-1} (Merck, Certipur®, H_3AsO_4 in 2% (v/v) HNO_3) traceable to the NIST was used as internal quality control in total arsenic and arsenic speciation measurements.

Stock standard solutions (1000 mg As L^{-1}) for arsenic speciation were prepared as follows: As(III), from As_2O_3 (NIST, USA, Oxidimetric Primary Standard 83d, 99.99%) dissolved in 4 g L^{-1} NaOH (Merck, Suprapure); As(V), from $Na_2HAsO_4 \cdot 7H_2O$ (Carlo Erba) dissolved in water; MA, prepared from $(CH_3)AsO(ONa)_2 \cdot 6H_2O$ (Carlo Erba) dissolved in water; and DMA, prepared from $(CH_3)_2AsNaO_2 \cdot 3H_2O$ (Fluka) dissolved in water. AC from $(CH_3)_3As^+(CH_2)CH_2OHBr^-$ was supplied by the ‘‘Service Central d’Analyse” (CNRS Vernaison, France); and a certified reference material of AB from $(CH_3)_3As^+CH_2COO^-$ was supplied by National Metrology Institute of Japan (NMIJ, Japan) as NMIJ CRM 7901-a, standard solution. TMAO was prepared from $(CH_3)_3AsO$ (Argus Chemicals srl) dissolved in water. Arsenate, arsenite, DMA, MA, AC, TMAO and AB were standardised against As_2O_3 for our internal quality control. All stock solutions were kept at 4 °C, and further diluted solutions for the speciation analysis were prepared daily.

Sample pretreatment

Fresh mushrooms were cleaned by hand of substrate and foreign matter. The end of the stalk (in contact with the substrate) was removed using a stainless steel knife. Damaged or soiled parts were cut off with a knife and smaller particles were removed using a fine brush. Only the edible parts of the mushrooms were used for the analysis. Mushrooms were cut into small pieces that were then air-dried on filter paper and further dried in an oven at 40ºC for 24–48 hours. The dried mushrooms were minced using a commercial mincer made of stainless steel until complete homogenization. Care was taken to avoid cross-contamination. Between samples, the mincer was washed once with soap and water, rinsed once with HNO_3 (approx. 10%), rinsed several times with deionised water, and then rinsed three times with doubly deionised water, before drying with cleaning wipes.

Mushroom food supplements, which are commercially available as pills, were pulverized with an agate mortar, homogenised and stored over silica gel in a desiccator until analysis.

5.6 Assessment of arsenic bioaccessibility in raw and cooked edible mushrooms by a PBET method

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Assessment of arsenic bioaccessibility in raw and cooked edible mushrooms by a PBET method

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ABSTRACT

The present study reports arsenic analysis in *Lentinula edodes*, *Agaricus bisporus* and *Pleurotus ostreatus* before and after being cooked. Furthermore, arsenic in raw and cooked mushroom was determined in the gastric and gastrointestinal bioaccessible fractions obtained after simulating human digestion by means of an *in vitro* physiologically based extraction test (PBET). Several certified reference materials (SRM 1568a, SRM 1570a, CRM 7503-a, BC211 and IPE-120) were analysed to evaluate the proposed methods. Total arsenic content was 1393, 181 and 335 μg As kg^{-1} for *L. edodes*, *A. bisporus* and *P. ostreatus*, respectively, and decreased by between 53% and 71% in boiled mushroom and less than 11% in griddled mushroom. High bioaccessibility was observed in raw, boiled and griddled mushroom, ranging from 74% to 89% and from 80% to 100% for gastric and gastrointestinal extracts, respectively, suggesting the need to consider the potential health risk of consumption of the mushrooms analysed.

1. Introduction

Food and drinking water are the principal routes of exposure to arsenic (As) for humans (IARC, International Agency for Cancer Research, 2012; WHO, World Health Organization, 2011). Regarding the toxicological aspects of arsenic in food, inorganic arsenic (iAs: arsenite or As(III) and arsenate or As(V)) is considered to be the most dangerous form due to its biological availability and physiological and toxicological effects (iAs is classified as a non-threshold, class 1 human carcinogen) (ATSDR Toxicological profile for arsenic, 2007). On the other hand, organic arsenic forms are mainly considered to be non-toxic (i.e. arsenobetaine) or potentially toxic (e.g. arsenosugars or arsenolipids) (Feldmann & Krupp, 2011).

The European Food Safety Authority (2009, 2014) and the Joint FAO/WHO Expert Committee on Food Additives (JEFCA) (FAO/WHO, Evaluation of certain contaminants in food, 2011) have recently shown an interest in the content of arsenic in food, especially inorganic arsenic, and have evaluated dietary exposure to arsenic. Mushrooms as well as other foods were included among the foodstuffs that contribute to arsenic exposure in the general European population (European Food Safety Authority, 2009). Among the regulations proposing maximum levels of arsenic tolerated in food, few establish specific levels for iAs. Very recently, the European Union published Regulation (EU) 2015/1006 (European Commission, 2015) amending Annex to Regulation (EC) No. 1881/2006 (European Commission, 2006) regarding the maximum levels of iAs in rice and rice-based products but not for other foodstuffs.

The capacity of some mushroom species to accumulate arsenic may represent a serious risk to consumer health (Falandysz & Borovicka, 2013; Kalač, 2010; Vetter, 2004); nonetheless, the consumption of edible mushrooms has increased considerably worldwide in recent years due to their nutritional properties. The most widely cultivated edible mushrooms in the world are *Agaricus bisporus* (also known as the button mushroom, white mushroom, brown mushroom or portobello mushroom), *Lentinula edodes* (often called by its Japanese name of shiitake) and *Pleurotus* spp. (particularly *P. ostreatus*, known as the oyster mushroom or hiratake mushroom) (Kalač, 2013), and they are particularly popular in China, Japan and other Asian countries.

Due to the increasing mushroom consumption, the Directorate General for Health and Consumers (DG SANCO) of the European Commission requested the European Union Reference Laboratory for Heavy Metals in Feed and Food (EURL-HM) to test the analytical capabilities of National Reference Laboratories (NRLs) to determine heavy metals in mushrooms. Two proficiency tests were organised via the International Measurement Evaluation Programme (IMEP) on behalf of the EURL-HM using the same test item (shiitake mushroom): IMEP-116 and IMEP-39 (Cordeiro et al., 2015), highlighting

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the fact that arsenic content in mushrooms is currently a priority issue for the DG SANCO of the European Commission.

A complete food safety assessment should always evaluate the intake of arsenic from food on the basis of the product as ingested by the consumer. In the context of human health risk assessment, bioavailability refers to the fraction of the substance that reaches the systemic circulation (blood) from the gastrointestinal (GI) tract (bioavailable fraction) and which is available to promote its action in the exposed organism (Reeder, Schoonen, & Lanzirotti, 2006). A first step in bioavailability assessment is the study of bioaccessibility, which indicates the maximum fraction of a trace element or other substance in food that is theoretically released from its matrix in the GI tract (bioaccessible fraction), and thus becomes available for intestinal absorption (i.e. enters the blood stream) (Oomen et al., 2002). Both *in vitro* and *in vivo* methods for evaluating bioavailability have been proposed. The *in vitro* methods provide an effective approximation to *in vivo* situations and offer the advantages of good reproducibility, simplicity, rapidity, ease of control, low cost and high precision, as it is possible to control conditions better than with *in vivo* tests (Moreda-Piñeiro et al., 2011). The inclusion of bioaccessibility data when assessing exposure can further refine and improve the risk assessment process. In addition, the fact that food is generally consumed in processed form, after a preservation treatment or cooking, must be taken into account since it has been reported that cooking affects the concentration of arsenic content as well as arsenic species distribution (Devesa, Vélez, & Montoro, 2008; Moreda-Piñeiro et al., 2011).

A limited number of arsenic bioaccessibility studies has been conducted, mostly concerning conventional food items; fish and shellfish (Koch et al., 2007; Moreda-Piñeiro et al., 2012), edible seaweeds (García Sartal, Barciela-Alonso, & Bermejo-Barrera, 2012; García-Sartal et al., 2011; Koch et al., 2007; Laparra, Vélez, Montoro, Barberá, & Farré, 2003), rice (Laparra, Vélez, Barberá, Farré, & Montoro, 2005), vegetables (Calatayud, Bralatei, Feldmann, & Devesa, 2013; Juhasz et al., 2008) and country foods (food obtained by hunting and gathering) from contaminated sites in Canada (Koch et al., 2013). There is thus a lack of data on the bioaccessibility of arsenic in edible mushrooms. Only one recent study has been found which reported high As bioaccessibility rates in several raw mushrooms (Koch et al., 2013). However, great variability of arsenic bioaccessibility has been reported between different mushrooms samples, suggesting that generalisations about arsenic cannot be made at this point. This highlights the importance of performing more bioaccessibility studies of arsenic in mushrooms to refine and improve the risk assessment process.

To date and to the best of our knowledge, no studies have been published on the bioaccessibility of arsenic in cooked edible mushrooms. Therefore, for the first time, the present preliminary study focused on two objectives to assess the potential health risks involved in the consumption of mushrooms. The first was to assure the reliability of analytical methods by establishing analytical parameters. The second was to determine arsenic content and bioaccessibility by an *in vitro* PBET method in three edible mushrooms, *A. bisporus*, *L. edodes*, *P. ostreatus*, before and after being boiled or griddled.

2. Materials and methods

2.1. Reagents, standards and certified reference materials

All solutions were prepared with doubly deionised water obtained from Millipore water purification systems (Elix & Rios) (18.2 MΩ cm^{-1} resistivity and total organic carbon <30 μg L^{-1}). Nitric acid (69%, Panreac, Hiperpur) and hydrogen peroxide (31%, Merck, Selectipur) were used for the microwave digestion procedure. Pepsin (Panreac), citric acid (Fluka), maleic acid (99%, Aldrich), DL-lactic acid (Sigma–Aldrich), hydrochloric acid (37%, Panreac, Hiperpur) and glacial acetic acid (100%, Merck, pro-analysis) were used for the gastric solution. Sodium hydrogen carbonate (Merck), porcine bile salts (Sigma–Aldrich), amylase (Sigma–Aldrich) and pancreatin (Sigma–Aldrich) were used for the gastrointestinal solution.

External calibration standards were prepared daily by dilution of a standard stock solution with a certified concentration of 1000 ± 5 mg As L^{-1} (Inorganic Ventures Standards, arsenic in 2% (v/v) HNO_3) traceable to the National Institute of Standards and Technology (NIST). A standard solution of arsenate with a certified concentration of 1000 ± 5 mg As L^{-1} (Merck, Certipur®, H_3AsO_4 in 2% (v/v) HNO_3) traceable to the NIST was used as internal quality control in arsenic measurements.

Four certified reference materials (CRMs) and a reference material (RM) were analysed during the study. SRM 1570a spinach leaves and SRM 1568a rice flour were obtained from the NIST (NIST, Gaithersburg, MD, USA). WEPAL IPE-120 reference material *A. bisporus* mushroom was produced by the Wageningen Evaluating Programs for Analytical Laboratories (WEPAL, Wageningen, the Netherlands). ERM-BC211 rice was obtained from the IRMM of the European Commission's Joint Research Centre (Geel, Belgium). NMIJ CRM 7503-a white rice flour was purchased from the National Metrology Institute of Japan (NMIJ, Japan). All CRMs were used as provided, without further grinding.

2.2. Apparatus and instrumentation

An inductively coupled plasma mass spectrometry (ICPMS) Agilent 7500ce (Agilent Technologies, Germany) was used to determine arsenic content. A microwave digestion system (Ethos Touch Control, Milestone) was used for the digestion procedure. All mushroom samples were minced using a commercial mincer (Multiquick 5 Hand Processor, Braun, Spain). A thermo-agitator Bath Clifton NE5-28D (Fischer Scientific) (37 °C ± 0.1) was used for the physiologically based extraction test (PBET) of the samples and CRMs.

2.3. Samples and sample pretreatment

L. edodes, *A. bisporus* and *P. ostreatus* mushrooms were obtained from a local market in Barcelona (Spain) in 2014. All samples were brought to the laboratory on the day of purchase and kept for no more than one day in the refrigerator until sample pretreatment. Mushrooms were manually cleaned of substrate and foreign matter. The end of the stalk (in contact with the substrate) was removed using a stainless steel knife. Damaged or soiled parts were cut off with a knife and smaller particles were removed using a fine brush. Mushrooms were cut into small pieces before each cooking procedure. Only the edible parts of the mushrooms were used for cooking tests. Each edible mushroom species was manually homogenised and divided into three portions, which were subjected to different cooking treatments. The first one, the raw product, was directly minced until complete homogenisation and the other two subsamples were cooked, i.e. griddled or boiled. After being cooked, mushrooms were minced using a commercial mincer made of stainless steel until complete homogenisation. Care was taken to avoid contamination. Between samples, the mincer was washed once with soap and rinsed several times with deionised water, and then rinsed three times with doubly deionised water, before drying with cleaning wipes. All samples were stored in freezer bags at −4 °C until analysis was performed (up to 24 h).

2.4. Cooking procedures of mushroom samples

Around 100 g of mushroom was boiled in approximately 700 mL of doubly deionised water for 10 min. Once the mushroom

samples had been boiled, the cooking water was separated for further analysis. Furthermore, around 100 g of mushroom was also cooked on a griddle for 10 min.

2.5. Moisture determination

Aliquots of 0.5 g of sample both raw and cooked were dried, in triplicate, at 102 ± 3 °C to constant weight in an oven. All the results in the study are expressed as dry mass.

2.6. Acid digestion for arsenic determination

Microwave acid digestion of raw, griddled and boiled mushroom samples and CRMs was performed as described in detail elsewhere (Llorente-Mirandes, Ruiz-Chancho, Barbero, Rubio, & Lopez-Sanchez, 2010). The digested samples were diluted with water to 25 mL. The digestion blanks were also measured. The digested samples were kept at 4 °C until analysis of arsenic content by ICPMS (24–48 h).

2.7. In-vitro physiologically based extraction test (PBET) method

The PBET method was adapted from the previously described method (Funes-Collado, Rubio, & López-Sánchez, 2015). The test was carried out in two stages; gastric (G) and gastro + intestinal (GI). Solution aliquots were separated at each stage for analysis, yielding two solutions per sample; the G solution and the GI solution. The reagent blanks were also analysed in each batch of samples.

The gastric step was carried out in triplicate using 5 g of raw, griddled or boiled mushroom sample in a 100 mL stoppered glass flask to which 50 mL of freshly prepared gastric solution was added. The gastric solution contained 1.25 g L^{-1} pepsin, 0.50 g L^{-1} citric acid, 0.50 g L^{-1} maleic acid, 420 μl L^{-1} DL-lactic acid and 500 μl L^{-1} acetic acid dissolved in water, and the pH was adjusted to 1.3 with concentrated hydrochloric acid. After 15 min, the pH value was checked and if necessary readjusted to pH 1.3. Flasks were covered and incubated at 37 °C with orbital–horizontal shaking at 150 rpm for 60 min. Then, flasks were placed in an ice-water bath to stop the enzymatic digestion. At the end of the gastric phase mixing, a 5 mL aliquot was collected from the solution for analysis (G sample). Prior to the intestinal digestion step, the pH of the gastric digests was raised to pH 7 by dropwise addition of saturated $NaHCO_3$ solution. Then, 2 mL of the intestinal solution (0.4 g L^{-1} pancreatin, 0.1 g L^{-1} amylase and 1.5 g L^{-1} porcine bile salts) was added and incubation at 37 °C continued for an additional 3 h at 37 °C. The enzymatic reaction was stopped by immersing the flasks in an ice-water bath. Following mixing, a 10 mL aliquot (GI sample) was collected.

All G and GI extracts were transferred to polypropylene centrifuge tubes and centrifuged at 3500 rpm for 12 min to separate the soluble fraction. The supernatants were filtered through PET filters (Chromafil PET, Macherey–Nagel, pore size 0.45 μm) prior to performing analysis. The extracts were kept at 4 °C until analysis.

2.8. Arsenic determination by ICPMS

Arsenic content was determined in raw, griddled and boiled mushroom samples, in cooking water and in gastric and gastrointestinal fractions by ICPMS. Operating conditions are listed in Table 1. Helium gas was used in the collision cell to remove polyatomic interferences (i.e. $^{40}Ar^{35}Cl$) in the ICPMS measurements. A 20 μg L^{-1} solution of ^{9}Be, ^{103}Rh and ^{205}Tl was used as an internal standard. Arsenic content in the digested samples was quantified by means of an external calibration curve prepared in 2% HNO_3 for the standards. Arsenic content in G and GI fractions was quantified by a standard addition curve in order to minimise matrix effects.

Table 1
ICPMS operating parameters.

Tuning parameters	
General	
RF power	1550 W
RF matching	1.76 V
Peristaltic pump speed	0.1 rps
Stabilization delay	30 s
Sampler and skimmer cones	Nickel
Nebuliser	BURGENER Ari Mist HP
Number of replicates	3
Spray chamber (type and temperature)	Scott-type and 15 °C
Gas flows	
Carrier gas flow, Ar	0.75 L min^{-1}
Make up gas flow, Ar	0.39 L min^{-1}
Torch alignment	
Sampling depth	7.5 mm
Ion lenses	
Extract 1	0 V
Extract 2	−130 V
Omega Bias-ce	−18 V
Omega Lens-ce	0.8 V
Cell entrance	−26 V
QP Focus	−15 V
Cell exit	−36 V
Quadrupole and octopole parameters	
QP/OctP bias difference	2 V
Reaction cell	
Collision cell	ON
He gas	3.6 mL min^{-1}
Mass-to-ratio	
As	*m*/*z* 75
Be, Rh and Tl (internal standard)	*m*/*z* 9, *m*/*z* 103 and *m*/*z* 205, respectively

2.9. Quality assurance – quality control

A rigorous quality control (QC) programme was conducted throughout sample analysis. For ICPMS measurements, acceptance criterion was $R^2 \geqslant 0.9990$ for every calibration curves (i.e. both for total arsenic and for bioaccessible arsenic methods). The residual errors at each calibration point were checked, accepting a residual error of ⩽15% for the lowest calibration level and ⩽10% for the others. QC standard solutions, prepared from a different stock standard source at two concentrations levels, were measured after every 5 samples. Data were accepted only when QC samples were 90–110% of the expected value. Additionally, the standards of the calibration curve were run before and after each sample series applying the same criteria. Each sample was digested (Section 2.6), extracted (Section 2.7) and analysed (Section 2.8) in triplicate. Reagent blanks of total arsenic and PBET methods were also analysed in each batch of samples and there were no outliers for blank controls. To assess the accuracy of arsenic measurements, several CRMs were analysed during sample analyses.

2.10. Statistical analysis

A Student's *t*-test (2 tails) was applied to compare measured total arsenic and certified values in the CRMs. A 95% confidence level was adopted for all comparisons.

The results for analysed samples were analysed statistically by a one-factor analysis of variance (ANOVA). All the assays were performed at least in triplicate. A significance level of *p*-value <0.05

was adopted for all comparisons. A p-value of less than 0.05 indicates a statistically significant difference among variances at a 95% confidence level.

3. Results and discussion

In order to compare the results obtained from the analysis of raw, griddled or boiled mushroom, all results were expressed in the same units, as micrograms per kilogram of mushroom, dry mass (dm). To do this, humidity was calculated (Section 2.5) for each cooking process and for each mushroom species. Furthermore, arsenic content in the water used for boiling samples was expressed as micrograms per kilogram of mushroom, dry mass, for comparison purposes. In the following discussion of results, the term "gastric phase (G)" will be used to indicate the bioaccessibility extraction phase representing the stomach, and "gastric + intestinal phase (GI)" will be used for the phase that included both sequential stomach and intestine steps, where results were obtained from the extract produced at the end of the sequence.

3.1. Analytical quality control study

3.1.1. Limits of detection and quantification

Limits of detection (LODs) and limits of quantification (LOQs) were calculated as three times the standard deviation (3σ) and ten times the standard deviation signal (10σ) of ten blanks, respectively, for the total arsenic method and the PBET method (G and GI fractions). The instrumental limits were converted to sample limits by correcting by the sample weight and extraction dilution factor. LODs were 5.2, 9.8 and 11 μg As kg^{-1} dry mass for total arsenic, G and GI fractions, respectively. LOQs were 17, 33 and 36 μg As kg^{-1} dry mass for total arsenic, G and GI fractions, respectively.

3.1.2. Accuracy of arsenic determination

To assess the accuracy of the total arsenic method, five CRMs were analysed during the study (Table 2). A statistical test was applied to compare determined total arsenic and certified values. The student's t-test indicated that there were no statistically significant differences between the determined and the certified values. Therefore, the accuracy of the total arsenic method was satisfactorily assessed. Moreover, the measured value (170.2 ± 8.2 μg As kg^{-1}) in the WEPAL IPE-120 reference material was in agreement with the indicative value (137 ± 67 μg As kg^{-1}).

Table 2
Accuracy and repeatability values of the total arsenic method. Total arsenic content is expressed as μg As kg^{-1} dry mass (mean ± SD, $n = 6$). Repeatability is expressed as RSD (%, $n = 6$). CRMs were analysed within a day and by the same analyst.

Certified reference materials	Matrix	Accuracy		Repeatability (RSD %)
		Certified value	Measured value	
NIST SRM 1568a	Rice	290 ± 30[a]	286.7 ± 6.1	2.1
NIST SRM 1570a	Spinach leaves	68 ± 12[a]	68.5 ± 4.1	5.9
NMIJ CRM 7503-a	Rice	98 ± 7[a]	97.8 ± 3.8	3.9
ERM-BC211	Rice	260 ± 13[a]	256.1 ± 6.7	2.6
WEPAL IPE-120	Mushroom (*Agaricus bisporus*)	137 ± 67[b]	170.2 ± 8.2	4.8

[a] Certified value: mean ± uncertainty.
[b] Indicative value: mean ± standard deviation.

3.1.3. Bioaccessibility of arsenic in reference materials

To assure the qaulity of the results, an accurate evaluation of the *in vitro* assays is required prior to application of the approach to specific studies. At present, validation of these approaches is incomplete due to the lack of suitable CRMs. Although several CRMs have been used in bioaccessibility studies (Moreda-Piñeiro et al., 2011), to date, no CRMs are commercially available for bioaccessible arsenic content. Therefore, to evaluate the PBET method two of the RMs available for total arsenic (WEPAL IPE-120 *A. bisporus* and ERM-BC211 rice) were extracted six times by the same analyst to control the bioaccessibility fractions. The results are shown in Table 3. For our internal QC, arsenic content in G and GI fractions was checked throughout the study and the results for real samples were only accepted when RM values were 85–115% of the established value (Table 3).

The bioaccessibility (BA%) of arsenic was calculated as a percentage using the following equation:

$$\text{BA}\ (\%) = \frac{[\text{As in G or GI fraction}]}{[\text{As in sample}]} \times 100 \quad (1)$$

where BA (%) is the percentage of bioaccessibility; [As in G or GI fraction] is the As concentration in gastric or gastrointestinal phase after PBET extraction; and [As in sample] is the total As concentration after the microwave-assisted acid digestion procedure. In both RMs, there were significant differences ($p < 0.05$) between the bioaccessible concentrations obtained for G and GI fractions, meaning that an increase was observed in bioaccessibility when comparing G fractions versus GI fractions as expected. Data on As bioaccessibility have previously been reported for several CRMs (Leufroy, Noël, Beauchemin, & Guérin, 2012; Moreda-Piñeiro et al., 2011). However, to date, no As bioaccessibility results have been found in the literature on ERM-BC211 and WEPAL IPE-120 materials, and therefore the present results cannot be compared. More results on arsenic bioaccessibility in food CRMs are needed to compare different *in vitro* methods and also to establish the suitability of the same *in vitro* method by different laboratories. Furthermore, the chemical form in which arsenic is present in the matrix could influence its bioaccessibility (Leufroy et al., 2012; Moreda-Piñeiro et al., 2011). This assumption obviously requires more research to be confirmed.

3.1.4. Repeatability

The repeatability (%) of the methods employed was assessed analysing several CRMs, and it was calculated from the data presented in the accuracy and bioaccessibility sections, 3.1.2 and 3.1.3 section, respectively. In each case, the relative standard deviation (RSD) was calculated of six replicates ($n = 6$) obtained in one day and by the same analyst. Repeatability values were calculated for the total arsenic method (Table 2) and were below 6% for all CRMs, showing excellent repeatability. For within-day repeatability of the PBET method RSD values were below 6% and 9% for the G and GI fractions, respectively (Table 3). As expected, for both CRMs, higher values were obtained for gastrointestinal extracts than for gastric extracts, probably due to the complexity of the GI matrix components, which produced high variability between replicates.

For real samples, each one was digested, extracted by the PBET method and analysed in triplicate. Replicates had acceptable repeatability with a RSD ($n = 3$) usually below 6% for the total arsenic method in all analysed samples (Table 4). Furthermore, acceptable repeatability was obtained with a RSD ($n = 3$) usually below 8% in bioaccessibility extracts of the G or GI fraction (except for two bioaccessibility extracts, 10.4% and 12.2%) (Table 5). The repeatability values obtained here for G and GI fractions in all samples were in the range previously reported in a study of bioaccessibility in mushrooms (Koch et al., 2013).

Table 3
Quality control results of bioaccessibility study. Bioaccessible arsenic in gastric (G) and gastrointestinal (GI) fractions and repeatability values. Concentrations are expressed as μg As kg^{-1} dry mass (mean ± SD, $n = 6$). Repeatability is expressed as RSD (%, $n = 6$) and the CRMs were analysed within a day and by the same analyst.

CRM	Matrix	Bioaccessibility[a]				Repeatability (RSD %, $n = 6$)	
		As in G fraction	BA (%) in G fraction[b]	As in GI fraction	BA (%) in GI fraction[b]	G fraction	GI fraction
ERM-BC211	Rice	249.9 ± 7.5	98 ± 4	268 ± 16	105 ± 7	3.0	6.0
WEPAL IPE-120	Mushroom (*Agaricus bisporus*)	149.4 ± 8.5	88 ± 7	177 ± 15	104 ± 10	5.7	8.4

[a] Acceptance criterion: values accepted only when results were 85–115% of the established value.
[b] Bioaccessibility = [(As in bioaccessible fraction, G or GI)/(As in sample)] × 100.

3.2. Arsenic contents in edible mushrooms

3.2.1. Arsenic in raw edible mushrooms

Total arsenic content in raw mushroom is shown in Table 4. The present results are in the usual range found in mushrooms from unpolluted areas, from 500 to 5000 μg As kg^{-1} (Kalač, 2010). However, significant differences in arsenic concentration (p-value < 0.05) were observed depending on the type of mushroom species analysed. Arsenic concentration in raw *A. bisporus* and *P. ostreatus* was below the maximum allowable concentration of 500 μg As kg^{-1} established by China for edible mushrooms (MHC, 2012). In contrast, arsenic content in raw *L. edodes* exceeded this maximum limit. Furthermore, it has recently been reported that toxic inorganic arsenic was the predominant arsenic species in *L. edodes* (Cordeiro et al., 2015; Llorente-Mirandes, Barbero, Rubio, & López-Sánchez, 2014), suggesting that this mushroom could be a potential contributor to dietary iAs exposure in populations with a high intake of *L. edodes* products.

The arsenic content of mushrooms is regulated by different factors, both environmental, i.e. sampling zone and arsenic content in soil, and genetic, i.e. the ability of mushroom species to accumulate arsenic (Vetter, 2004). High variability in arsenic contents has been reported in the literature (Falandysz & Borovicka, 2013; Kalač, 2010). Some mushroom species can accumulate high amounts of arsenic and this phenomenon seems to be independent of their habitats (Vetter, 2004). For example, for *Laccaria amethystina*, which is an arsenic accumulator, high arsenic contents have been reported. A mean concentration was above 59,000 μg As kg^{-1} dm, with a maximum value of 146,900 μg As kg^{-1} dm. Meanwhile, a study analysed 37 common edible mushroom species and the arsenic contents were below 50 μg As kg^{-1} dm in 13 species (Vetter, 2004).

From the present results, *L. edodes* had the highest total arsenic content, which was within the range found in our previous study of this mushroom (range from 110 to 1440 μg As kg^{-1} dm) (Llorente-Mirandes et al., 2014). The few studies on arsenic content in *L. edodes* found in the literature show high variability in As content, e.g. one study reported high As content, at 1300 μg As kg^{-1} dm (Wuilloud, Kannamkumarath, & Caruso, 2004), while other authors have reported low arsenic content in Brazilian shiitake, ranging from 12 to 210 μg As kg^{-1} dm (Maihara, Moura, Catharino, Castro, & Figueira, 2008).

A. bisporus is the most commonly consumed mushroom worldwide and consequently several authors have analysed this mushroom. Furthermore, it has been reported that some species of the genus *Agaricus* have the capacity to accumulate arsenic. For example, in samples gathered from different habitats in Hungary, the maximum concentrations found were about 13,000–18,000 μg As kg^{-1} dm (Vetter, 2004). In another study of edible mushrooms collected in Italy, high variability in arsenic content was reported for the *Agaricus* genus, ranging from 210 to 5000 μg As kg^{-1} dm (Cocchi, Vescovi, Petrini, & Petrini, 2006). Meanwhile, we observed low As content in our study, which is in agreement with the results obtained in another study on *A. bisporus*, in which total As ranged from 97 to 163 μg As kg^{-1} dm (Maihara et al., 2008).

The As content in *P. ostreatus* was within the range found in cultivated mushrooms, especially in wood-rotting fungi such as *Pleurotus* sp., which are generally in the range of 90 to 500 μg As kg^{-1} dm (Vetter, 2004) or even lower, as in the case of a study of Brazilian mushrooms in which several *Pleurotus* sp. samples were analysed and low As content was found, ranging from 9 to 73 μg As kg^{-1} dm (Maihara et al., 2008).

Table 4
Concentration of total arsenic in raw, griddled and boiled mushrooms and in boiling water. Concentrations expressed as μg As kg^{-1} dry mass (mean ± SD, $n = 3$).

Sample	Total arsenic			
	Raw	Griddled	Boiled	Boiling water
Lentinula edodes	1393 ± 61	1316 ± 45	568 ± 23	879 ± 27
Agaricus bisporus	185.0 ± 9.0	167.7 ± 7.7	86.2 ± 1.8	103.4 ± 6.1
Pleurotus ostreatus	335 ± 19	298.7 ± 6.2	98.3 ± 3.1	242.1 ± 9.6

Table 5
Bioaccessible arsenic in gastric (G) and gastrointestinal (GI) fractions of PBET method expressed as μg As kg^{-1} dry mass (mean ± SD, $n = 3$).

Sample	Cooking treatment	Bioaccessible arsenic	
		Total As in G fraction	Total As in GI fraction
Lentinula edodes	Raw	1028 ± 12	1346 ± 26
	Griddled	1008 ± 57	1057 ± 45
	Boiled	437 ± 25	516 ± 39
Agaricus bisporus	Raw	154.0 ± 8.2	159.2 ± 4.1
	Griddled	147 ± 15	168 ± 20
	Boiled	77.1 ± 2.7	81.5 ± 4.7
Pleurotus ostreatus	Raw	295 ± 15	313.5 ± 6.0
	Griddled	250.1 ± 7.7	269 ± 16
	Boiled	81.8 ± 4.1	92.3 ± 2.9

3.2.2. Arsenic in cooked edible mushrooms

Mushrooms are generally consumed after a cooking treatment, e.g. boiled, griddled, baked or grilled, which may alter the concentration of arsenic (Devesa et al., 2008). Therefore, the effect of griddling or boiling on the arsenic content was evaluated for each of the mushroom species analysed, and the arsenic results are shown in Table 4. The effect of cooking, i.e. griddling or boiling, on arsenic content was different for each of the mushroom species analysed.

Griddling produced significant differences ($p < 0.05$) in *P. ostreatus*, where arsenic decreased by around 11% in griddled mushroom with respect to raw mushroom. However, griddling did not produce significant differences ($p > 0.05$) in *L. edodes* and *A. bisporus* with respect to the arsenic content of the raw product.

Boiling, meanwhile, decreased arsenic content by between 53% and 71% in all mushroom species analysed, producing significant differences ($p < 0.05$) in all mushrooms with respect to the arsenic

content of the raw mushroom assayed. Samples of the water used to boil mushrooms were analysed and the arsenic concentrations obtained are shown in Table 4 (water results expressed as dry mass of mushroom weight). The results obtained suggest that a high percentage of arsenic was leached into the boiling water during the cooking treatment. Therefore, for an overall and accurate study of risk assessment, the effect of food processing for each type of mushroom should be considered. Even though boiling *L. edodes* caused a significant reduction ($p < 0.05$) in arsenic with respect to the raw sample, both griddled and boiled shiitake exceeded the limit of 500 μg As kg^{-1} established by China for mushrooms (MHC, 2012).

To date and to the best of our knowledge, no data on arsenic content in these mushrooms subjected to cooking treatments have been reported in the literature, therefore the results obtained in this study cannot be compared. However, our results are in agreement with other arsenic studies on cooking foods. For example, it has been reported that boiling food decreases arsenic content substantially (Devesa et al., 2008), and several studies have been published on foods in which high percentages of arsenic were released from food into the cooking water, e.g. seaweeds (García Sartal et al., 2012; García-Sartal et al., 2011; Laparra et al., 2003), rice (Raab, Baskaran, Feldmann, & Meharg, 2009) and pasta samples with a significant decrease in arsenic (about 60%) after a cooking process (Cubadda, Raggi, Zanasi, & Carcea, 2003).

3.3. Bioaccessible arsenic in mushrooms

3.3.1. Bioaccessible arsenic in raw edible mushrooms

The arsenic content in the G and GI bioaccessible fractions in raw mushroom is shown in Table 5. Significant differences were found in arsenic concentrations in G and also in GI extracts (p-value < 0.05) depending on the type of mushroom species analysed, because of the difference in the contents in the initial raw samples.

The bioaccessibility of arsenic (BA, %) was calculated as a percentage using the equation shown above, and results varied between 74% and 88% for the G fraction (Fig. 1a) and 86% and 97% for the GI fraction (Fig. 1b). In raw mushroom, an increase in bioaccessibility was observed when comparing G fractions versus GI fractions (Fig. 1a and b). This finding seems to be quite obvious since these are the consecutive steps of the PBET method. In the G step, part of the arsenic was solubilised and when the extraction time was extended to the intestinal phase, an increased bioaccessibility value was observed. This increase was statistically significant ($p < 0.05$) when BA values for the G and GI fractions from all raw mushrooms were considered together, and also for BA values for *L. edodes*. However, the same was not observed for *A. bisporus* and *P. ostreatus* ($p > 0.05$), in which no significant differences were found between BA values in the G and GI fractions.

To the best of our knowledge, only one study on bioaccessible arsenic content in raw mushrooms exists in the literature (Koch et al., 2013), in which it was found that BA values in several raw mushrooms ranged from 20% to 91% in G extracts and from 22% to 94% in GI extracts. The results obtained in the present study for *A. bisporus* are in agreement with this study, which reported BA values higher than 58% in both G and GI extracts in *Agaricus* sp. The same authors reported an increase in the bioaccessibility of arsenic in the seven mushroom species analysed when comparing G and GI values (Koch et al., 2013).

3.3.2. Bioaccessible arsenic in cooked edible mushrooms

The arsenic content in the G and GI bioaccessible fractions in griddled and boiled mushroom is shown in Table 5. The effect of cooking on the arsenic content in the G and GI fractions was different for each of the mushroom species analysed.

For *L. edodes*, no significant differences were observed ($p > 0.05$) between As content in the G fraction of griddled mushroom and the G fraction obtained from raw mushroom, whereas boiling produced significant differences ($p < 0.05$) with respect to raw mushroom. This was to be expected because of the difference in As content in the initial sample (raw, griddled or boiled). However, for GI fractions, both griddling and boiling treatments produced significant differences ($p < 0.05$) in *L. edodes* compared to arsenic content in the GI fraction of raw mushroom.

Griddling *A. bisporus* did not produce significant differences ($p > 0.05$) in As content in the G or GI fractions with respect to the As content in G or GI fractions in the raw mushroom. However, the As content in G or GI fractions were significant lower ($p < 0.05$) in boiled *A. bisporus* than in the G or GI fractions obtained from the raw mushroom.

In the case of *P. ostreatus*, the As content in the G or GI fractions of both griddled and boiled mushroom was significantly lower ($p < 0.05$) than in the G or GI fractions obtained from raw mushroom.

Bioaccessibility (BA, %) of arsenic in both the G and GI fractions was calculated as a percentage using the equation shown above (1), and the results are shown in Fig. 1a and b. No significant differences ($p > 0.05$) were observed in the gastric fraction between values in raw mushroom and after being cooked (griddled or boiled) for any of the mushroom species analysed (Fig. 1a). However, bioaccessibility presented a different behaviour in the GI fraction from each of the assayed mushroom species (Fig. 1b). A significant decrease ($p < 0.05$) was observed in *L. edodes* after being griddled but not after being boiled with respect to BA in the GI fraction from raw mushroom. A significant ($p < 0.05$) increase in BA in the GI fraction was observed after griddling and boiling *A. bisporus*. In *P. ostreatus*, no significant differences ($p > 0.05$) were observed in BA of the GI fraction between BA in raw mushroom and after being cooked (griddled or boiled).

Bioaccessibility of arsenic in griddled and boiled mushroom varied between 77% and 89% and 80% and 100% for G and GI fractions, respectively. As observed in raw mushroom, an increase was detected when comparing G fractions versus GI fractions in cooked mushroom. This increase was statistically significant ($p < 0.05$) when BA% values of G and GI fractions from cooked mushroom were considered together. Considering all mushroom species and all cooking treatments, mean values were 83% and 92% for G and GI fractions, respectively. When all gastric values were compared to gastrointestinal values for each mushroom species and for all types of cooking treatment (raw, griddling and boiling), significant ($p < 0.05$) differences between G and GI fractions were observed. Higher bioaccessibility values of As were found in GI fractions compared to G fractions, indicating that the GI step plays an important role in the solubilisation of arsenic. Therefore, in order not to underestimate the bioaccessibility of arsenic, an intestinal phase should be included in future bioaccessibility studies of mushrooms to ensure an accurate estimation of bioaccessible arsenic.

To date, no previous data are available for bioaccessibility of arsenic in these mushrooms subjected to a cooking treatment, and therefore the results obtained in this study cannot be compared. The bioaccessibility of an element depends not only on the matrix, but also on the chemical form of the analyte and the model used (Leufroy et al., 2012; Moreda-Piñeiro et al., 2011). Furthermore, it should be borne in mind that cooking not only affects bioaccessible arsenic content but could also modify and transform some arsenic species present in the raw product. Therefore, more studies on arsenic speciation in bioaccessible fractions (G and GI) in raw and cooked mushroom should be performed to improve the risk assessment process.

It might be useful to determine whether the high As bioaccessibility values obtained by the *in vitro* PBET method are in agreement

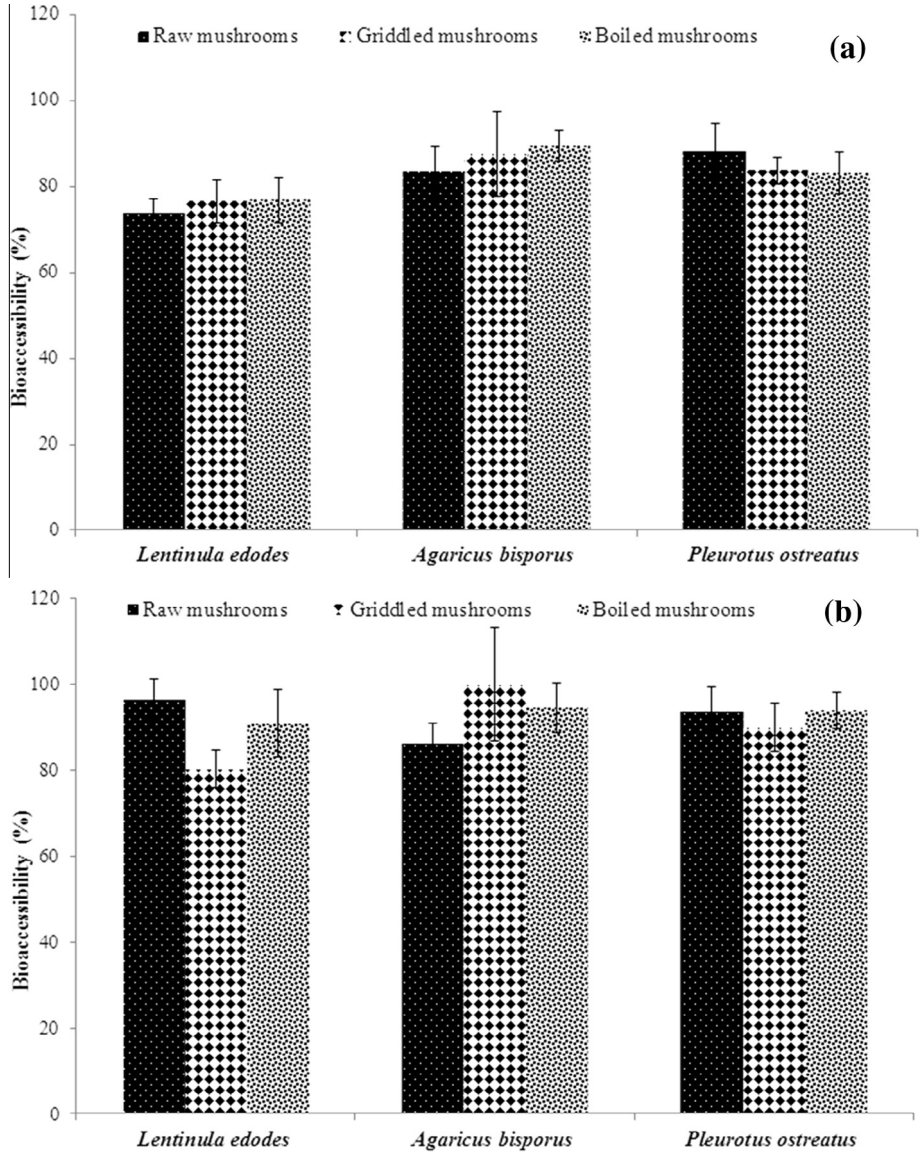

Fig. 1. Bioaccessibility (%) of arsenic in the gastric (a) and gastrointestinal (b) fractions in raw, griddled and boiled mushroom determined by a PBET method. Error bars denote combined standard uncertainty.

with the high bioavailability As values obtained by *in vivo* assays. Few studies on this subject in some foods have been found in the literature (He & Zheng, 2010; Juhasz et al., 2006, 2008). In general, a high variability in As bioavailability has been reported, depending on the different types of food which have been studied. Thus, bioavailability values using an *in vivo* swine model were 33% and 88% for different varieties of rice (Juhasz et al., 2006) and from 50% to 100% in vegetables (Juhasz et al., 2008). Unfortunately, data for arsenic bioavailability in the assayed edible mushrooms have not been reported, therefore the relationship between the present *in vitro* bioaccessibility results and *in vivo* bioavailability cannot be established.

3.4. Mass balance of cooking procedure

To evaluate the cooking procedure, a mass balance approach was performed for each mushroom sample. Arsenic concentrations were determined in raw and boiled mushroom, as well as in the water used to cook each type of mushroom (Table 4). For the mass balance of the cooking procedure, the sum of arsenic concentrations in both fractions (boiled mushroom and boiling water) was statistically compared with the arsenic content in the raw mushroom. ANOVA *p*-values were 0.2876, 0.5057 and 0.6552 for *L. edodes*, *A. bisporus*, *P. ostreatus*, respectively and were higher than $p > 0.05$ (at 95% confidence interval), indicating that there was no statistically significant difference between variance values. Therefore, the arsenic concentration in raw mushroom and the sum of arsenic concentrations in boiled mushroom and water were statistically equal.

4. Conclusions

For the first time, a study of arsenic bioaccessibility in raw and cooked mushroom using a PBET method is reported, enabling assessment of the potential health risk involved in consumption of the most commonly consumed mushrooms worldwide. Detection and quantification limits, repeatability and accuracy of both total arsenic and PBET methods were satisfactory assessed by analysing several CRMs.

Boiling mushrooms decreased arsenic content which is released into the cooking water. This indicates that for further reliable and

accurate studies of risk assessment, mushrooms must be analysed in the same form as ingested by the consumer. Special care is required in the case of *L. edodes*, where total arsenic in raw, griddled and boiled mushroom exceeded the maximum limit established by Chinese legislation.

Even when a cooking process led to a decrease in As content, the bioaccessibility of arsenic remained high, with values of 83% and 92% for the G and GI fractions, respectively. Therefore, a GI phase should be included in further studies so as not to underestimate the bioaccessible arsenic and to ensure the highest conservative estimation.

Further studies on the bioaccessibility of arsenic species in mushrooms which consider the effect of cooking should be conducted in order to improve the risk assessment process. Analytical tools for validation and quality control purposes, such as a Certified Reference Material with a bioaccessible arsenic content, should also be available. Lastly, it should be noted that more studies on *in vivo* bioavailability measurements are required to demonstrate the suitability of and validate *in vitro* bioaccessibility methods.

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PART IV: DISCUSSION OF THE RESULTS

Chapter 6

Development, quality assessment and validation parameters of established methods

Chapter 6 is focused on the development, quality control assessment and validation parameters of established methods. A global discussion of the most interesting results in these fields is presented. Therefore, the aim of the **Chapter 6** is to discuss the method development as well as to summarize the assessment of internal and external quality control (QC) parameters and the evaluation of validated methods.

First, the selection and development of methods applied in the thesis for total arsenic, arsenic species and bioaccessible arsenic determination are discussed following the steps of the analytical process. In analytical speciation analysis, reliable results are only obtained if all steps involved in the analytical process pursue species integrity, including the distribution accounted in the original matrix. Generally, there are three steps in speciation analysis: (1) extraction (2) separation and (3) measurement and quantification of As species. Each step needs to be tested and optimized to obtain reliable results. Sample extraction procedures, separation of arsenic species and use of standards, and also the analytical technique used for arsenic species determination in food samples are discussed here. Furthermore, the suitability of an *in vitro* physiologically based extraction test (PBET) to evaluate arsenic bioaccessibility in the gastric and gastrointestinal fractions is also discussed.

Second, quality control (QC) and quality assurance (QA) evaluation are still not widely implemented in methods for elemental speciation. Nevertheless, noticeable efforts have been made in recent years to develop analytical strategies to support the reliability of results in this field of analysis. A comprehensive scheme of QA in analytical chemistry laboratories would include the following elements: validation of analytical methods; use of CRMs; routine application of internal QC; and participation in PT [141]. Method validation is an essential component of the measures that a laboratory should implement to allow it to produce reliable analytical data and demonstrate whether the method fits for a particular analytical purpose. Furthermore, a maximum limit of iAs in rice and rice products is recently established in EU legislation [107]. Therefore, if food control laboratories should now be ready to determine iAs in rice, they need suitable and robust methods for accomplish the present legislation. To this end, the validation of methods as well as participation in PT and the analysis of CRMs should be performed, as mandated by the ISO/IEC 17025 standard for laboratory accreditation purposes [138]. Hence, all QC parameters evaluated in the methods proposed in this thesis are discussed in this chapter. For this, an overall discussion of the internal QC studies, validated parameters and external QC are shown in the following paragraphs with the aim to evaluate the methods developed and to provide accurate and reliable speciation data.

All analytical methods applied in the thesis are summarised in **Table 6.** Developed methods are named A to F to facilitate further discussion and evaluation related to these methods. Thereby, the method for the determination of total arsenic is named method A, methods for arsenic speciation analysis are called B, C, D and E; and method for estimate arsenic bioaccessibility is method F (**Table 6)**. A summary of internal QC parameters evaluated, validation parameters and external QC assessment for all developed methods are presented and discussed in the following paragraphs.

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Table 6. Methods evaluated for the determination of arsenic species, total arsenic and arsenic bioaccessible.

Method	Sample weight (dry mass)	Reagents	Apparatus	Time and temperature conditions	Sample treatment	Measurement of As
Total arsenic						
A	0.10-0.50g	8 mL HNO_3 + 2 mL H_2O_2	Microwave digestion	60 min, max. T= 190ºC	Dilution in water up to 20/25 mL.	ICPMS [b]
Arsenic species						
B	0.10-0.30g	10 mL of 0.2% HNO_3/ 1% H_2O_2 solution (w/v)	Microwave-assisted extraction	45 min, max. T= 90ºC		
C	0.10-0.30g	10 mL of 0.2% HNO_3 solution (w/v)	Microwave-assisted extraction	45 min, max. T= 90ºC	Centrifugation at 3500 rpm (10 min) and filtration (PET filters, pore size 0.45 µm).	
D	0.10-0.30g	10 mL of H_2O solution	End-over-end shaker	30 rpm for 16 h at room temperature		Anion and cation exchange chromatography coupled to ICPMS [a]
E	0.2g	20 mL of a MeOH/H_2O solution (1:1, v/v)	Microwave-assisted extraction	10 min at 40W	Centrifugation at 2500 rpm (10 min). Evaporation of MeOH under an IR lamp (T<40 °C, 4h). Dilution of extract in water up to 20 mL and filtration through a nylon membrane (0.2µm). Clean-up with a C18 cartridge.	
Arsenic bioaccessible						
F	0.50g	Gastric and Gastrointestinal solutions [b]	Thermo-agitator with orbital–horizontal shaker	1h at 37ºC + 3h at 37ºC	Centrifugation at 3500 rpm (10 min) and filtration (PET filters, pore size 0.45 µm)	ICPMS [b]

[a] Operating conditions of the HPLC–ICPMS system are reported in **Article IV**. [b] Reagents used in the PBET method and operating conditions of ICPMS system are reported in **Article IX.**

6.1 Total arsenic determination

A discussion of the selection and development of the applied method for total arsenic (tAs) determination (method A) is presented as follows. Furthermore, as a method quality assessment, the main QC parameters are discussed and evaluated in the following paragraphs.

6.1.1 Method development

Our research group has developed a method for tAs determination based on sample digestion with a closed-vessel microwave system and arsenic measurement by ICPMS due to its extremely high sensitivity and wide dynamic range, adequate for determination of trace and ultra-trace levels of arsenic. The method was satisfactory applied in plants [142, 143] and seaweeds [144] showing the reliability of the approach. Thus, in the present thesis this method was selected to be evaluated and to be applied in food samples. Thereby, in all studies presented in **Chapters 4 and 5**, tAs content was determined by ICP-MS after closed-vessel microwave digestion (Method A, **Table 6**). Some modifications in the relation of HNO_3 and H_2O_2 (4:1, 3:1 or 2:1), in the % of added HNO_3, in sample weight and in the final dilution in water were made depending on the analysed samples. ICPMS operating conditions for measuring arsenic content were reported in **Article IX.**

6.1.2 Analytical quality assurance

To obtain reliable results in any field, it requires the use of validated analytical methods with well-established performance and the implementation of quality control (QC) activities covering all steps of the analytical process. Therefore, a rigorous quality assurance study was conducted in all research studies performed to develop an analytical method for total arsenic. For this the following items were assessed: internal QC evaluation, validation parameters and external QC evaluation.

Internal Quality Control

Several QC parameters were evaluated in the method for total arsenic determination: type of calibration, use of internal QC; use of internal standard; check instrumental drift; analysis of blanks; analysis of CRMs; check possible interferences; sample replicates, among others. The main internal QC parameters evaluated in the present thesis for total arsenic determination (method A) are summarised in **Table 7** and the quality criteria to evaluate this internal QC are shown in **Table 8.**

Table 7. Evaluation of internal quality control assessment in the developed method (A) for total arsenic determination.

Publication	Food commodity	Internal quality control					
		Calibration	Instrumental drift	Blanks	Internal STD	CRMs	Replicates
Article II	Rice	✓	✓	✓	✓	✓	✓
Article III	Cereal-based foods	✓	✓	✓	✓	✓	✓
Article IV	*L. edodes* products	✓	✓	✓	✓	✓	✓
Article V	Edible mushrooms	✓	✓	✓	✓	✓	✓
Article VI	Marine seaweeds	✓	✓	✓	✓	✓	✓
Article VII	Edible seaweeds	✓	✓	✓	✓	✓	✓
Article VIII	Fish and shellfish	✓	✓	✓	✓	✓	✓
Article IX	Mushrooms	✓	✓	✓	✓	✓	✓

Table 8. Summary of criteria to evaluate internal quality control parameters in the developed method (A) for total arsenic determination.

Quality control parameter	Evaluation conditions	Frequency	Quality criteria
Calibration	NIST traceable standards. External calibration curve	Before and at the end of the each sample series	Quantification of tAs calibration standards with other standard or against a primary standard
Instrumental drift	QC standard solutions at two concentrations levels	Post-calibration, every ten samples and at the end of the run	Values within 90 and 110% of the expected value
Blanks	Reagent blanks	One for each batch of samples	Blank values<LOQ
Internal standard	^{72}Ge or ^{103}Rh in standard mode	Added on-line to sample and standard solutions	Values within 80 and 120% of the target value
CRMs	CRM (or RMs) of the same matrix of samples	One for each batch of samples	Values within 85 and 115% of the certified value
Replicates	Samples preparation was performed in triplicate.	All samples analysed in triplicates	Acceptable if RSD between replicates <10%

First, before each ICPMS run, the instrument was tuned daily to maximise ion signals and to ensure sufficiently low levels of oxides (CeO^{+}/Ce^{+} < 1.2%), doubly-charged ions (Ce^{2+}/Ce^{+} < 2%), by analysing a commercial solution containing 10 μg L^{-1} of lithium, yttrium, cerium, thallium and cobalt in 2% (v/v) nitric acid. Total arsenic was determined by ICPMS measuring mass at m/z 75. One of the main poly-atomic ions, $[^{40}Ar^{35}Cl]^{+}$, has nearly the same mass-to-charge ratio (m/z 75) as monoisotopic arsenic, and could interfere with its determination in samples with significant amounts of chlorine. One of the commonly used techniques to reduce this interference in quadrupole ICPMS is the well-known mathematical correction equation. However, this correction formula may introduce errors in the case of a routine method of analysis if sample matrix components vary, and can be particularly unreliable for low concentrations of arsenic. Only ICP-MS equipped with a high-resolution mass analyser, i.e. a sector field system, would be able to separate these two signals. Other alternative to minimise chlorine interference is to use an ICP-QQQ because it allows for easy monitoring and better control of possible interferences compared to normal single quadrupole instruments. The theory is that the $^{40}Ar^{35}Cl$ interference should be eliminated by using O_2 in the reaction cell which converts As^{+} to AsO^{+} product ion. $^{40}Ar^{35}Cl^{+}$ don't react and remain at mass 75. Arsenic was measured in the second quadrupole as $^{75}As^{16}O^{+}$ on m/z 91 which rejects all masses except target AsO^{+} product ion at 91. This instrumentation is highly expensive than conventional single quadrupole instruments but its use is beneficial for optimization of selective HG and to perform HG-ICPMS or HPLC-HG-ICPMS analysis. The fact is that both ICPMS used in the thesis, UB and Public Health Agency of Barcelona (ASPB), are quadrupole ICPMS. Therefore, although a loss of sensitivity compared to mode no gas, helium was used as a collision gas to remove $^{40}Ar^{35}Cl$ interference in the octopole reaction system (ORS).

For tAs determination, commercially available standards were prepared daily by dilution of a standard stock solution with a certified concentration of 1001 ± 5 mg As L^{-1} (Inorganic Ventures Standards) and traceable to the National Institute of Standards and Technology (NIST). Total arsenic content in the digested samples (method A) and also in the extracts of speciation methods B, C, D and E was quantified by means of an external calibration curve prepared in 2% HNO_3 for the commercially available standards. Curves ranged from 0.125 to 5 μg As L^{-1}) for low tAs content or from 5 to 100 μg As L^{-1} for high tAs content. In general, a solution of 20 μg As L^{-1} of ^{9}Be, ^{103}Rh and ^{205}Tl was used as an internal standard to monitor instrumental drift and matrix effects. In case of arsenic measurements, ^{103}Rh is normally used. However, a solution of ^{72}Ge was also used as an internal standard in the optimization of the method for arsenic species in cereal-based food (**Article III**). In this case, the final solutions (standards and samples) were prepared with 2% isopropyl alcohol (or 40% if introduced within the online internal standard) to minimise the effects of the dissolved carbon on arsenic response [145]. Each sample preparation was performed and analysed in triplicate to eliminate batch-specific error and to monitor repeatability. To verify the lack of contamination in the reagents or during the preparation of samples, blanks were analysed together with samples. To assess instrumental response and run quality, QC standard solutions from different source of standards (As standard solution of 1000 ± 5 mg As L^{-1}, Merck) were measured after

every some samples. Furthermore, several CRMs were analysed throughout the routine sample analyses to evaluate the methods

Evaluation of validation parameters

Several validation parameters were evaluated in studies presented above in **Chapters 4 and 5**. The validation parameters were established as specified elsewhere [146]. A summary of parameters evaluated in the developed methods for total arsenic is shown in **Table 9**. The main parameters evaluated are LOD/LOQ, precision and accuracy. There are several possible conceptual approaches to estimate LOD and LOQ, each providing a somewhat different definition of the limits and different approaches to estimate them. Detection and quantification limits were calculated as three times the standard deviation and ten times the standard deviation signal of ten digestion blanks (**Table 9)**. Precision and accuracy were evaluated analysing CRMs. Several materials from different food matrices were analysed among the research studies selecting in each case the most similar with the assayed samples. As noted before, the presence of AB in fish and marine species could be a problem in the determination of tAs as its chemical decomposition is very difficult and tAs content can be easily underestimated. Arsenobetaine is difficult to digest and require digestion temperatures of around 280°C when microwave digestion is used. To evaluate this fact, several seafood CRMs were analysed (**Article VIII**) and our results in marine CRMs are summarised in (**Figure 10**). No underestimation of tAs was observed probably due to the high temperatures reached in the plasma of ICPMS which would eliminate the abovementioned problem. Furthermore, various terrestrial food CRMs were also analysed to expand the evaluation of accuracy to other food matrix (**Figure 11**). As can be observed in **Figures 10 and 11,** average measured values (blue bars) were in agreement with the certified values (red bars) performing a satisfactory assessment of accuracy.

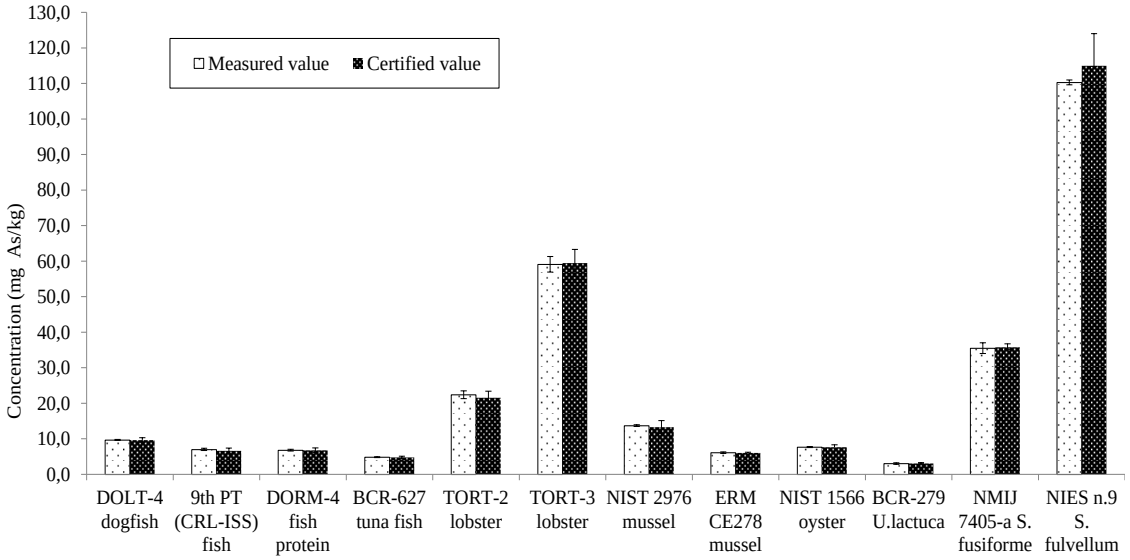


Figure 10. Accuracy assessment of the total arsenic method by analysis of seafood CRMs. Average concentrations are expressed as mg As kg^{-1} (mean ± U). Error bars denote combined standard uncertainty.

Table 9. Summary of validation parameters evaluated in the developed method (A) for total arsenic determination.

Publication	Food commodity	Validation parameters									
		LOD	**LOQ**	**Linearity**	**Repeatability**	**Intermediate Precision**	**Trueness**	**Accuracy**	**Selectivity**	**Uncertainty**	**External QC**
Article II	Rice	✓	✓	**n.e**	✓	✓	**n.e**	✓	**n.e**	**n.e**	✓
Article III	Cereal-based foods	✓	✓	✓	**n.e**	**n.e**	**n.e**	✓	**n.e**	**n.e**	✓
Article IV	*L. edodes* products	✓	✓	**n.e**	**n.e**	**n.e**	**n.e**	✓	**n.e**	**n.e**	✓
Article V	Mushrooms	✓	✓	**n.e**	**n.e**	**n.e**	**n.e**	✓	**n.e**	**n.e**	**n.e**
Article VI	Marine seaweeds	✓	✓	✓	**n.e**	**n.e**	**n.e**	✓	**n.e**	**n.e**	**n.e**
Article VII	Edible seaweeds	✓	✓	✓	**n.e**	**n.e**	**n.e**	✓	**n.e**	**n.e**	**n.e**
Article VIII	Fish and shellfish	✓	✓	**n.e**	✓	**n.e**	**n.e**	✓	**n.e**	**n.e**	**n.e**
Article IX	Mushrooms	✓	✓	**n.e**	✓	**n.e**	**n.e**	✓	**n.e**	**n.e**	**n.e**

[a] Details of method A are shown in **Table 6.**

n.e means not evaluated

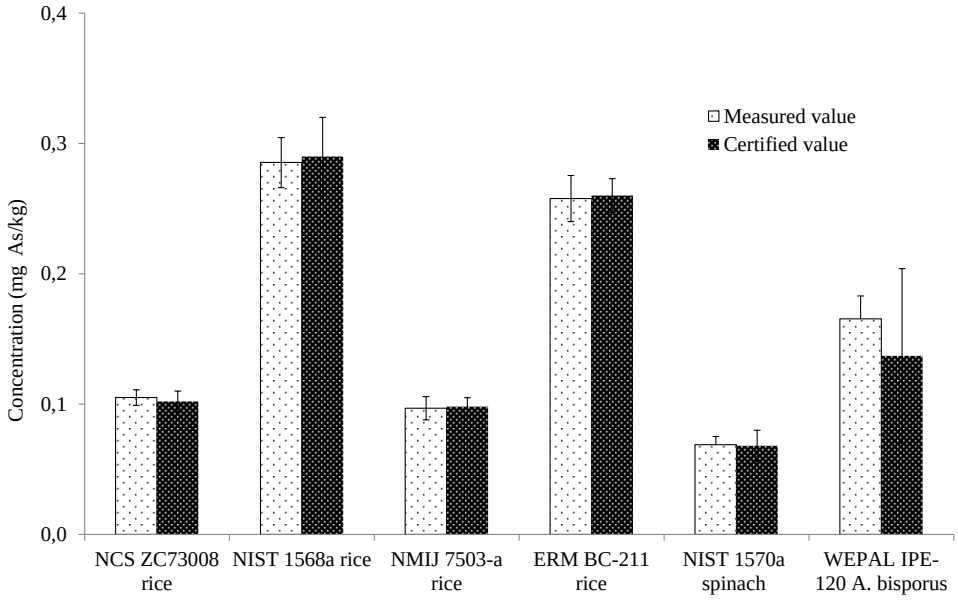


Figure 11. Accuracy assessment of the total arsenic method by analysis of terrestrial food CRMs. Average concentrations are expressed as mg As kg^{-1} (mean ± U). Error bars denote combined standard uncertainty.

External Quality Control

As external quality control, the proposed method for tAs determination (method A) was tested in several proficiency tests (PTs) as well as in certification studies of CRMs and results are summarised in **Table 10.**

To test the reliability of the method, we participated in various PTs organised by the International Measurement Evaluation Program (IMEP) from the Institute for Reference Materials and Measurements (IRMM) of the Joint Research Centre (JRC). The general aim of these PTs is to: "judge the state of the art of analytical capability for the determination of total and inorganic arsenic in several foodstuffs with a view to future discussions on the need for possible regulatory measures and future discussions on risk management and the possibility of introducing maximum levels for iAs in the European Union". In some cases, the test material was certified for tAs (a CRM is used in some PT) but in other cases the material is home-made produced by IRMM an tAs content was unknown. Unfortunately, the test materials were not certified for iAs, so it were sent to some expert laboratories in the field to determine iAs and/or tAs contents. Thereby, our research group participated as expert laboratory analysing total arsenic and inorganic arsenic in the test samples with the aim to assign a reference values for this measurands.

Table 10. Summary of external QC assessment for total arsenic method (method A): participation in PTs and certification of CRMs.

External QC	Description	Sample	Sample Origin	Total arsenic (mg As kg^{-1})		References
				Measured value (mean value ± SD)	Assigned or Certified value (mean value ± U)	
Proficiency tests						
IMEP-107	*Proficiency test*	Rice	Provided by Aberdeen University and produced by IRMM	0.164 ± 0.009	0.172 ± 0.018	[147, 148]
IMEP-30/109	*Proficiency test*	Fish (NRC DOLT-4)	NRC CRM DOLT-4	9.10 ± 0.11	9.66 ± 0.62 [a]	Article X; [149, 150]
IMEP-112	*Proficiency test*	Wheat	Provided by Istituto di Sanita and produced by IRMM	0.157 ± 0.005	0.177 ± 12	
IMEP-112	*Proficiency test*	Vegetable	NIST SRM 1570a spinach leaves	0.0685 ± 0.0041	0.068 ± 0.012	Article XI; [151]
IMEP-112	*Proficiency test*	Algae	Produced by IRMM	53.1 ± 1.2	58.3 ± 7.0	
IMEP-116/39	*Proficiency test*	Mushroom	Provided by University of Barcelona and produced by IRMM	0.612 ± 0.011	0.646 ± 0.048	Article XII; [152, 153]
IMEP-118	*Proficiency test*	Peas in brine. Drained product	Produced by IRMM	0.118 ± 0.006	0.117 ± 0.018	[154]
IMEP-118	*Proficiency test*	Peas in brine. Solid/liquid composite	Produced by IRMM	0.122 ± 0.007	0.121 ± 0.014	
FAPAS 07151	*Proficiency test*	Rice	Produced by FAPAS	0.465 ± 0.019	0.476 ± 0.085	[155]
Certification of CRMs						
ERM-BC211	*Certification studies*	Rice	CRM produced by IRMM	0.251 ± 0.010	0.260 ± 0.013 [a]	
ERM-BC211	*Stability testing 2013*	Rice	CRM produced by IRMM	0.254 ± 0.021	0.260 ± 0.013 [a]	[156]
ERM-BC211	*Stability testing 2014*	Rice	CRM produced by IRMM	0.260 ± 0.020	0.260 ± 0.013 [a]	
BCR-627	*Stability testing*	Tuna fish tissue	CRM produced by IRMM	4.8 ± 0.1	4.8 ± 0.3 [a]	[157, 158]
ERM-AC626	*Confirmation measurements*	Arsenobetaine solution	CRM produced by IRMM	100.8 ± 0.7	Unpublished results	Unpublished report
ERM-CD200	*Long Term Stability Study*	Bladderwrack seaweed	CRM produced by IRMM	53.6 ± 1.2	55 ± 4 [a]	[159]

[a] Certified values

Therefore, our laboratory was selected to perform tAs analysis in several IMEPs and tAs results are shown in **Table 10**. Specific details such as objectives, analyte, assigned values, and results of participants (z-score), comments and main conclusions of each IMEP are summarised in **Article I.** Further information of IMEP-30/109, IMEP-112 and IMEP-116/39 can be found in **Articles X, XI and XII**, respectively and in specific reports (**Table 10**). In each IMEP study, an accurate quality assessment was performed to assure the reliability of our results. This includes internal QC and evaluation of precision and accuracy by analysis of some CRMs. Several types of food samples were analysed in these PTs covering a wide range of food commodities: rice, fish, wheat, vegetable, algae, mushroom, and peas in brine (drained product and Solid/liquid composite). In general, obtained results in these proficiency tests were considered acceptable and showed the ability of the developed tAs method to provide reliable and accurate results.

Furthermore, the method was tested participating in a PT organised by the Central Science Laboratory-Food Analysis Performance Assessment Scheme (CSL-FAPAS). The result obtained for tAs content was in agreement with the assigned value (**Table 10)** and % of recovery compared with an assigned reference value was acceptable: within ± 15%.

Additionally, method A was employed in some certification studies of CRMs. Accurate results were obtained compared to certified value in the all studies of ERM-BC211 rice (**Table 10)**. Besides, acceptable tAs results were obtained in the stability studies of BCR-627 and ERM-CD 200 further demonstrating its validity and reliability. Furthermore, we participated in the confirmation measurements for the production of an arsenobetaine CRM (ERM-AC626). The material is in development and production steps by IRMM and therefore our results cannot be presented here.

6.2 Arsenic species determination

A discussion of the selection and development of a method for arsenic speciation analysis is presented in **section 6.2.1**. Furthermore, as a method quality assessment, the main QC parameters are discussed and evaluated in the following paragraphs (**section 6.2.2**).

6.2.1 Method development

Our research group has been working on elemental speciation for several years. In case of arsenic speciation some procedures, methods and techniques have been recently evaluated. Among them, a separation technique coupled to an element-selective analyser was the most applied approach for arsenic speciation purposes. In this way, our research group developed a method for arsenic speciation analysis based on a soft extraction to avoid changes in the distribution of arsenic species and arsenic measurement by HPLC-ICPMS. A suitable performance of separation conditions for arsenic species was achieved [144] and arsenic species were analysed by both anion and cation exchange chromatography. The method was satisfactory applied in some samples, plants were extracted with water, water/methanol (9 + 1, v/v), and water/methanol (1 + 1, v/v) [142, 143] and seaweeds with water [144] showing the suitability of the developed approach.

Due to the separation of arsenic species was satisfactory achieved in our previous studies [144], the same chromatographic conditions of the HPLC-ICPMS system were applied in the present thesis. However, some specific modifications and changes for each research study depending on the type of food analysed were performed. Thereby, in all studies presented in **Chapters 4 and 5**, arsenic species content was determined by HPLC-ICPMS after an extraction method (**Table 6**). Four methods for the extraction of arsenic species were applied throughout the thesis, i.e. method B, C, D and E. Three of them, method B, C and D, were evaluated and applied throughout the present research studies. Two were based on microwave-assisted extraction with 0.2% HNO_3/ 1% H_2O_2 and with 0.2% HNO_3 for method B and C, respectively and the other on mechanically shaking and extraction with water extraction (method D). For all them further determination of arsenic species was carried out by HPLC-ICPMS (**Table 6**).

Assessment of the extraction methods for arsenic speciation in foodstuffs

To evaluate the suitability of extraction methods for arsenic speciation analysis (B, C and D, **Table 6**) some preliminary studies were carried out in the present thesis. The competence of the extraction methods was tested for their ability to extract arsenic species. These studies were specially focused on the extraction, and accurate quantification of inorganic arsenic (iAs), the most toxic form of arsenic. Thereby, in the following paragraph, these preliminaries studies of method evaluation are shown. As an example, the competence of the extraction methods for their ability to extract arsenic species in three *L. edodes* samples is summarised and discussed below.

Methods B, C and D were tested for their ability to extract the arsenic species present in three *L. edodes* samples studied (named *L. edodes* I, II and III). Chemical extractions, either end-over-end shaker-assisted or MW-assisted, were investigated (**Table 6**). Water, nitric acid and nitric acid with hydrogen peroxide were used as solvents in chemical extractions. Sample weight and volume of extraction solvent were 0.25 g and 10 mL for all of the selected extraction procedures, respectively. Arsenic species, total arsenic and total extracted arsenic contents and the percentage of the detected species as iAs for each tested method are shown in **Table 11.** Furthermore, column recovery and extraction efficiency were calculated in order to check mass balances **(Table 11)**. Methods showed extraction efficiencies (EE) >60% for the all of the tested samples and average EE was 98%, 100% and 72% for procedures B, C and D, respectively.

Furthermore, the extracted As eluted almost entirely form the chromatographic column, average column recovery (CR) was 97%, 97% and 99% for procedures B, C and D, respectively. These mass balances indicate that method D is less effective extractant from the point of view of As. Besides, methods B and C showed high extraction yields indicating a quantitatively extraction of As present in mushrooms. Furthermore, all extracted arsenic species were quantitatively determined for each method as high CR was obtained for each method.

Table 11. Evaluation study of extraction methods. Concentration of arsenic species in the assayed mushrooms (expressed as mg As kg^{-1}, dry mass).

Samples	Extraction method [a]	Total extracted As	Arsenic species										Sum of species	EE [c] (%)	CE [d] (%)
			As (III)	DMA	MA	As (V)	iAs value	iAs (%) [b]	AB	AC	TMAO	TETRA			
Lentinula edodes-I	B	0.428 ± 0.009	<LOD	0.013 ± 0.0004	0.024 ± 0.001	0.343 ± 0.012	0.343 ± 0.012	87	<LOQ	<LOD	<LOQ	0.015 ± 0.001	0.395 ± 0.012	95	92
tAs= 0.451 ± 0.026 [a]	C	0.442 ± 0.020	0.295 ± 0.025	0.012 ± 0.0005	0.023 ± 0.002	0.055 ± 0.004	0.350 ± 0.025	88	<LOQ	<LOD	<LOQ	0.014 ± 0.002	0.399 ± 0.025	98	90
	D	0.271 ± 0.011	0.160 ± 0.012	0.009 ± 0.0005	0.018 ± 0.001	0.058 ± 0.004	0.218 ± 0.011	86	<LOD	<LOD	<LOD	0.008 ± 0.001	0.253 ± 0.013	60	93
Lentinula edodes-II	B	0.616 ± 0.029	<LOD	0.032 ± 0.004	0.016 ± 0.001	0.550 ± 0.019	0.550 ± 0.019	90	<LOQ	<LOD	<LOQ	0.014 ± 0.001	0.612 ± 0.019	103	99
tAs= 0.600 ± 0.040 [a]	C	0.600 ± 0.028	0.449 ± 0.032	0.027 ± 0.003	0.017 ± 0.001	0.111 ± 0.009	0.560 ± 0.032	91	<LOQ	<LOD	<LOQ	0.013 ± 0.001	0.617 ± 0.033	100	103
	D	0.454 ± 0.024	0.301 ± 0.024	0.026 ± 0.003	0.010 ± 0.001	0.123 ± 0.012	0.424 ± 0.02	91	<LOD	<LOD	<LOD	0.004 ± 0.0006	0.464 ± 0.027	76	102
Lentinula edodes-III	B	0.896 ± 0.054	<LOD	0.028 ± 0.003	0.017 ± 0.002	0.824 ± 0.050	0.824 ± 0.050	93	<LOQ	<LOD	<LOQ	0.015 ± 0.002	0.884 ± 0.056	98	99
tAs= 0.918 ± 0.043 [a]	C	0.928 ± 0.063	0.723 ± 0.065	0.027 ± 0.003	0.018 ± 0.002	0.123 ± 0.011	0.846 ± 0.056	94	<LOQ	<LOD	<LOQ	0.013 ± 0.002	0.904 ± 0.066	101	97
	D	0.724 ± 0.055	0.525 ± 0.045	0.016 ± 0.002	0.008 ± 0.0003	0.189 ± 0.016	0.714 ± 0.048	96	<LOD	<LOD	<LOD	0.007 ± 0.0004	0.745 ± 0.048	79	103

[a] Details of extraction methods are shown in **Table 6.** [b] Percentage of iAs with respect to sum of extracted As species. [c] EE means Extraction efficiency. [d] CR means Column recovery. LOD and LOQ are shown in **Article IV**

Several arsenic species were determined in all tested methods, i.e: As(III), DMA, MA, As(V) and TETRA (**Table 11**). Other species such as AB, AC and TMAO were below de quantification limits. As expected for the low EE, lower content for the majority of As species in the extracts of method D was found than those found in methods B and C. Similar levels were found comparing DMA, MA and TETRA species between method B and C (**Table 11**).

High differences were found between As(III) and As(V) contents in methods B, C and D. As we expected, high interconversion between As(III) and As(V) was observed for each tested procedures. **Figure 12** shows HPLC-ICPMS chromatograms obtained for the fresh *L. edodes*-III sample extracted with the three different procedures B-D. The major peaks corresponding to As(III) or As(V) appears depending on the extraction method, followed by DMA and MA as the next abundant species and TETRA as a minor compound. Similar chromatograms were obtained for the other two *L. edodes* samples (I and II). Oxidation transformation of As(III) and As(V) was complete with procedure B, as is shown in **Figure 12**, As(III) was not found in extracts of method B and all iAs was found as As(V), meanwhile both As(III) and As(V) were found in procedures C and D. Inorganic arsenic content was calculated as sum of As(III) and As(V) for the evaluated methods. In all cases, low iAs values were obtained for method D in the three samples (**Table 11**). However, no differences were found between method B and C: 0.343 ± 0.012 vs 0.350 ± 0.025, 0.550 ± 0.019 vs 0.560 ± 0.032 and 0.824 ± 0.050 vs 0.846 ± 0.056 mg As kg^{-1} for *L. edodes*-I, *L. edodes*-II and *L. edodes*-III, respectively. This indicate that the use of HNO_3/H_2O_2 (method B) produced a quantitative oxidation of As(III) into As(V) without degradation or transformation of other organoarsenicals into As(V).

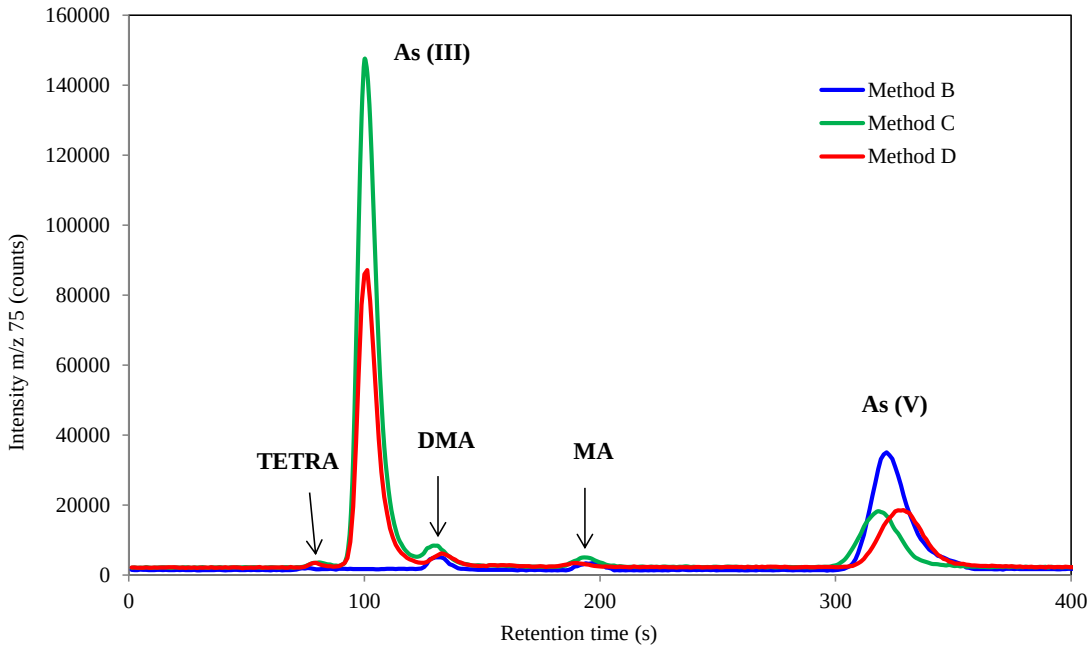


Figure 12. HPLC-ICPMS chromatograms of extracts of the same *L. edodes*-III sample extracted with the procedures B (blue line), C (green line) and D (red line).

Therefore, the two MW-assisted procedures with HNO_3, i.e: B with H_2O_2 and C without H_2O_2, were the most effective in extracting the As species from samples, which were generally satisfactorily recovered from the chromatographic column. No differences were observed in DMA, MA and TETRA contents between them and no significant transformation of these organoarsenicals were observed (**Table 11 and Figure 12**). Regarding iAs, the sum of As(III) and As(V) was in agreement between two methods highlighting that both methods could be useful for the determination of iAs in mushrooms. Method C preserves the original state of oxidation of As(III) and As(V) in the sample, and could be useful to determine As(III) and As(V) separately and to know the ratio between As(III)/As(V) in the sample. However, as a method drawback is that a satisfactory chromatographic separation of both As (III) and As(V) from other As species is needed to provide reliable iAs data. When using the present HPLC-ICPMS method with a strong anionic exchange column and a mobile phase of ammonium phosphate, As(III) elute near the void volume as can be seen in **Figure 12.** Therefore, arsenite could co-elute with other cationic species present in mushrooms, such as AB, TETRA, TMAO or AC [40, 42, 160–163] leading to an overestimation of iAs. Regarding the advantages of method B, the most notable factor is the conversion of As(III) into As(V) allowing the quantification of iAs as As(V). There is no conversion (demethylation) of organic arsenic species like DMA or MA to arsenate. This means that method B has some advantages comparing to method C and some possible problems are minimised: the co-elution of As (III) with cationic species are solved and iAs could be determined quantifying As (V) which is well separated from other As species. Furthermore, to determine iAs content is not necessary to quantify two peaks minimizing the associated errors.

On the basis of the above, it is concluded that the extraction of MW-assisted with HNO_3/H_2O_2 solvent (B) was the most suitable procedure for a quantitative extraction of all arsenic species in mushrooms and for an accurate quantification of the toxic inorganic arsenic species without degradation of other arsenocompounds. Therefore, this method was applied in other related research studies shown in **Chapter 4 and 5**. For instance, a method was developed and validated for determination of As species in rice products (**Article II**) and in cereal-based products (**Article III**). In these studies the extraction method using a HNO_3/H_2O_2 solution proved to be an effective solvent for arsenic speciation in rice and cereals. As is reported in **Article II**, the extraction method completely oxidises As(III) into As(V), without conversion of the methylated arsenic species into iAs. The method was satisfactory validated in rice and cereal-based foods and specific discussion of the validation is shown in **section 6.2.2**. Furthermore, method B was applied in other type of foods: mushrooms (**Articles IV and V)**, and fish and shellfish samples **(Article VIII).** Spiking experiments were performed in these studies to assess the quantification of iAs. Both As (III) and As(V) standards were added to solid samples before the extraction to evaluate the oxidation between species and the recovery of all iAs as As(V). The recovery of inorganic arsenic was adequate in all assayed samples (results are shown in **section 6.2.2**).

Standards and separation of As species

More than 50 different naturally occurring As-containing compounds have been identified, comprising both organic and inorganic forms [5]. Among them, the standards available in our research group of arsenic species are: As(III), DMA, MA and As(V), AB, TMAO and AC. Stock standard solutions for arsenic speciation were home-made prepared as dilution of the corresponding salts. For our internal quality control, arsenic concentration in these standards was determined by ICPMS and were standardised against two arsenate certified standard solutions (Merck and Inorganic Ventures) as well as against a primary standard, As_2O_3 solution. All stock solutions were kept at 4 ºC, and further diluted solutions for the analysis were prepared daily. Arsenic speciation was carried out in the extracts by HPLC–ICPMS. After extraction two chromatographic modes were used for separation of the arsenic species. Anionic species were analysed by anion exchange chromatography, based on the method described by Gailer et al. [164]. Cationic species were separated by cation-exchange chromatography, based on the method described by Madsen et al. [165]. Operating conditions of the HPLC-ICPMS system, both ICPMS parameters and chromatographic conditions, were described in **Articles III, IV and VI.** The performance of the separation methods is adequate for most samples and work properly with the ICP-MS. As an example of separation of standards for arsenic speciation, two HPLC-ICPMS chromatograms are shown in **Figure 13**, anionic-exchange column (a) and cationic-exchange column (b).

One important drawback in arsenic speciation is the lack of standards for all arsenic species. Unfortunately, there are no commercially available arsenosugars (derivatives of dimethylarsinoylribosides) and trimethylarsonioribosides, **Figure 1).** Therefore, an aliquot of freeze-dried extract of *Fucus serratus*, containing the four common arsenosugars, that is, phosphate (PO_4-sug), sulfate (SO_4-sug), sulfonate (SO_3-sug), and glycerol (Gly-sug), was used to identify the arsenosugar peaks in the chromatograms. This extract was obtained from the brown seaweed *Fucus serratus* prepared by Madsen et al. [165] to identify the arsenosugars present in seaweed samples and the authors identified the arsenosugars peaks in chromatograms by analysing the extract by HPLC–ICPMS and LC-ESI-MS. Therefore, *F. serratus* was used in the present thesis to assure the identification of arsenosugars in all sample extracts as well as the accuracy of its determination by comparing the obtained values with those reported by Madsen et al. [165] and by other authors in the literature. As an example, *F. serratus* chromatograms obtained from cation and anion exchange chromatography are shown in **Figure 14**. It shows the presence of phosphate, sulfonate and sulphate sugar in the anion exchange chromatogram **(Fig.14a)** and Gly-sug in the cation exchange chromatogram **(Fig.14b).**

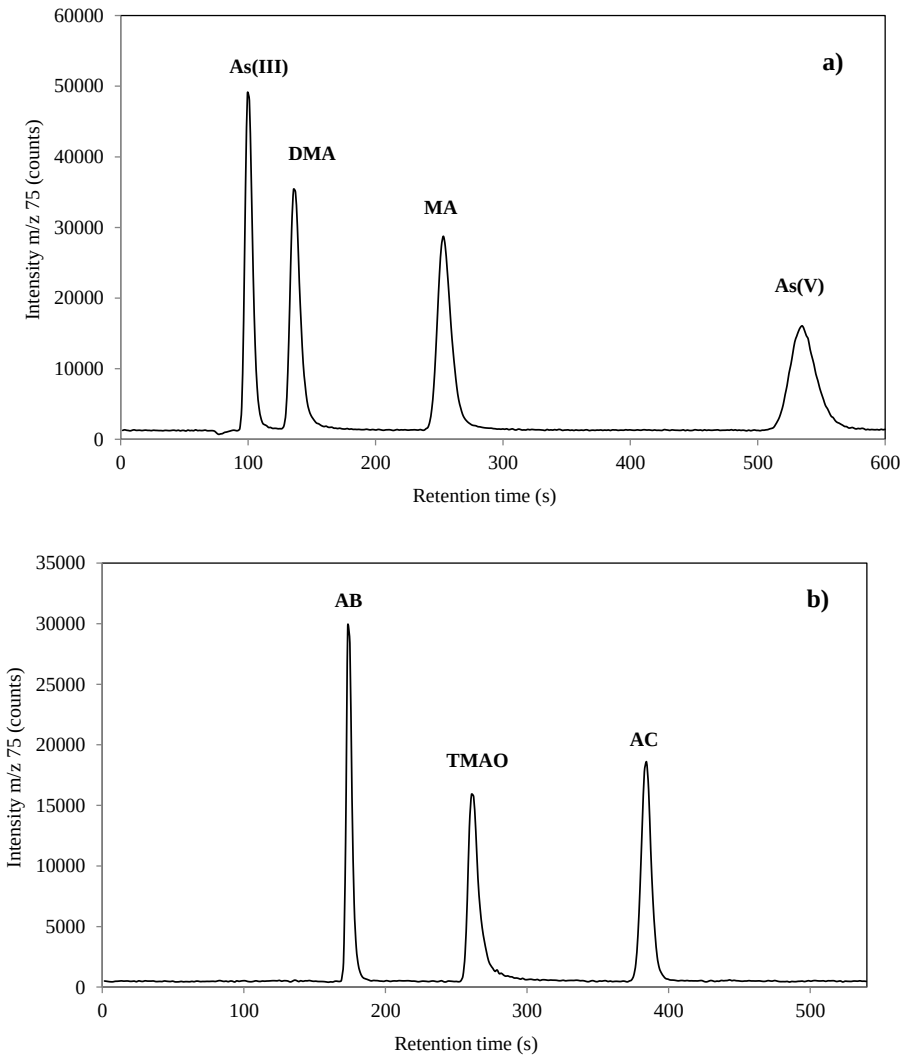


Figure 13. Standards separation of anionic (a) arsenic species by HPLC-ICPMS using PRP-X100 Hamilton anion-exchange column, 20mM $NH_4H_2PO_4$, pH 5.6, 1.5 mL min^{-1}. Standards separation of cationic (b) arsenic species by HPLC-ICPMS using ZORBAX 300-SCX cation-exchange column, 20 mM pyridine, pH 2.6, 1.5 mL min^{-1}

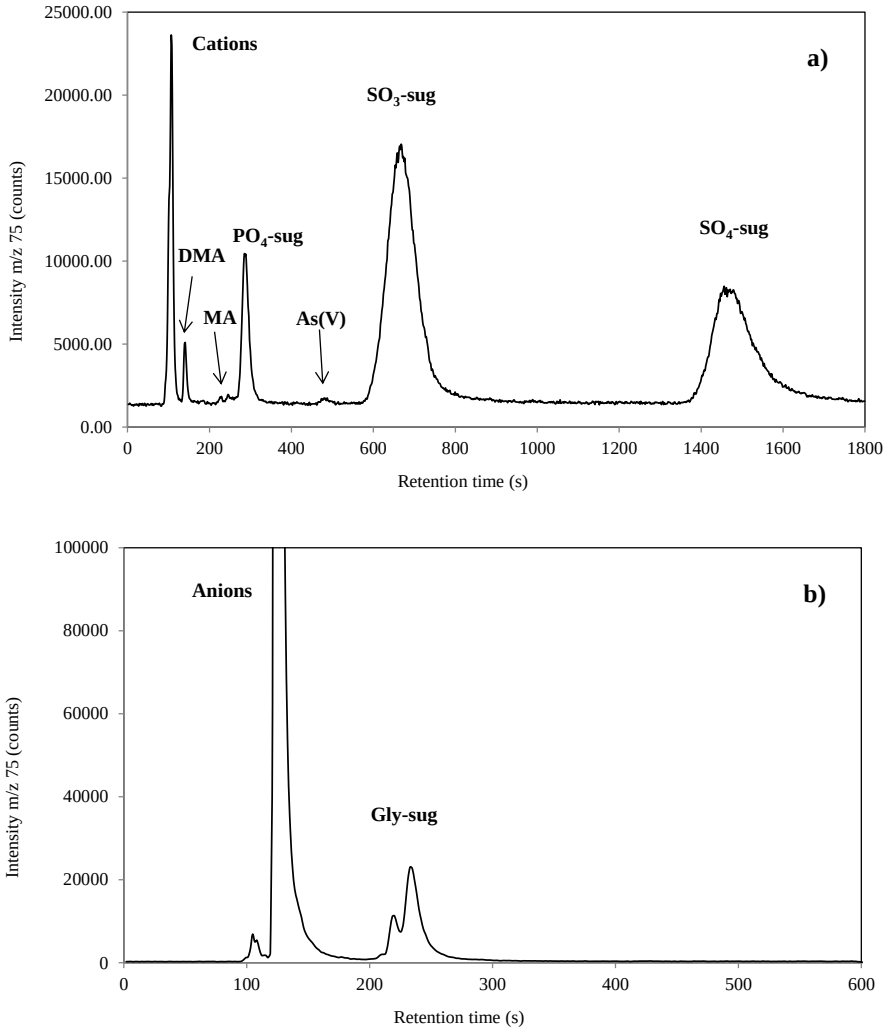


Figure 14. Chromatograms of the *F. serratus* extract from anion exchange with the PRP-X100 column (a) and cation exchange with the Zorbax SCX300 column (b).

Measurement techniques

The coupling of HPLC and ICPMS for arsenic speciation is used throughout the studies performed during the thesis. Specific HPLC-ICPMS operating conditions for measuring arsenic species content were reported in **Articles III, IV and VI.** This technique provides high specificity and sensitivity which allow determine arsenic species at trace level concentrations and applied it to routine analysis. The performance of the technique is adequate for arsenic speciation in most samples. However, some unknown arsenic compounds were found in some types of food that cannot be identified. The technique relies on an extraction step (usually from a solid sample) that can be incomplete or alter the arsenic compounds; and it provides no structural information, relying on matching sample peaks to standard peaks. Identification of peaks relies in retention time matching with standards and standard addition and therefore, the method depend on the availability of standard compounds. The lack of structural information provided by the ICPMS is an important drawback of this approach, and other techniques could be used to provide structural information for the identification of organoarsenical species.

Considering these drawbacks, an alternative method to identify and characterize the unknown arsenic compounds is the liquid chromatography with a mass spectrometry detector (MS). For instance, a chromatographic method coupled to a molecular mass spectrometer: to MS (electrospray, ESI-MS or HPLC-ESI-MS) [165] or to tandem MS (HPLC–ESI–MS/MS system) [63, 166] are reliable techniques to identify and confirm unknown arsenic species when the retention time matching and the standard addition were not enough for confirming their identity. A chromatographic method compatible with these coupling techniques have to be used. The low volatility of $H_2PO_4^-$ discouraged the use of $NH_4H_2PO_4$ as the mobile phase in the coupling, so NH_4HCO_3 could be an alternative due to its compatibility with the detector. Summarising, the information obtained from HPLC-ICPMS analysis can be greatly enhanced by the complementary use of molecular mass spectrometry (MS) to identifying co-eluting compounds and unknown compounds.

Furthermore, the use of additional analytical methods in a complementary manner introduces the ability to address these disadvantages. The use of X-ray absorption spectroscopy (XAS) with HPLC-ICPMS can be used to identify compounds not extracted and measured for HPLC-ICPMS and provide minimal processing steps for solid state analysis that may help preserve labile compounds such as those containing arsenic sulfur bonds, which can degrade under chromatographic conditions. On the other hand, HPLC-ICPMS is essential in confirming organoarsenic compounds with similar white line energies seen by using XAS, and identifying trace arsenic compounds that are too low to be detected by XAS [122].

Moreover, an alternative technique to determine arsenic compounds could be the hydride generation atomic fluorescence spectrometry (HG-AFS) coupled to a chromatographic system (HPLC-HG-AFS). However, only those species that form the hydride can be detected which makes this detector unsuitable for arsenic speciation in some seafood matrix, i.e: algae since arsenosugars cannot be detected as the hydride cannot be performed. The technique has adequate detection limits and is a low cost alternative and is a suitable technique to be applied to terrestrial foods (especially rice and cereal-based food) in which iAs and methylated species are predominant and the generation of hydride is performed (**Article I**).

6.2.2 Analytical quality assurance

A rigorous quality assurance – quality control study was conducted in all research studies performed to develop an analytical method for total arsenic. For this the following items were assessed: internal QC evaluation, validation parameters assessment and the participation in proficiency tests as external QC.

6.2.2.1 Internal Quality Control

A summary of internal QC parameters evaluated in HPLC-ICPMS analysis for arsenic species determination is shown in **Table 12.** Information of quality criteria to evaluate internal QC is shown in **Table 13**. Arsenic species were determined by HPLC-ICPMS measuring the ion intensity at m/z 75 (^{75}As). Usually, no gas was used in the collision cell to avoid loss of sensitivity in speciation analysis, ICPMS work on standard mode. Therefore, to detect possible argon chloride ($^{40}Ar^{35}Cl$) interference at m/z 75, the ion intensities at m/z ^{77} ($^{40}Ar^{37}Cl$) and m/z

35 (^{35}Cl) were monitored. The selectivity of the method regarding this interference for the As species studied was verified in **Article II** and the ($^{40}Ar^{35}Cl$) peak eluted at 7.92 min, whereas near arsenic species in the chromatogram, i.e. As(V) eluted at 6.0 min.

Arsenic species in the chromatograms were identified by comparison of the retention times with those of the standards when available. When this is not the case, as for arsenosugars, a brown seaweed *F. serratus* extract [165] is used for comparison, as a well-characterised sample extract. External calibration curves were used to quantify arsenic species against the corresponding standards. Curves ranged from 0.125 to 5 μg As L^{-1}, for low As content or from 5 to 100 μg As L^{-1} for high As content. Unknown arsenic compounds were quantified using the calibration curves of the nearest eluting standard compound. Each sample preparation was performed and analysed in triplicate to eliminate batch-specific error and to monitor repeatability. Glassware, polypropylene centrifuge tubes and PTFE microwave vessels were tested to ensure that they did not contribute significantly to background levels of As. Both water blanks and reagent blanks were also analysed by HPLC-ICPMS in each batch of samples to monitor possible cross-contamination or memory effects. In each speciation run, quality control standard solutions at two concentrations levels were measured to assess instrumental response and run quality. Furthermore, a CRM was measured every some samples and at the end of the sequence to ensure stable instrument sensitivity.

A mass balance study was performed to evaluate the arsenic speciation methods. For this, extraction efficiency (EE) and column recovery (CE) were calculated for each assayed samples and CRMs. The mass balance between total arsenic extracted and total arsenic content in the matrix provides an estimation of the extraction yield. Extraction efficiency (EE) values from 80 – 110 %, calculated in this way, can be considered acceptable. In several food groups values were >80%, for instance EE ranged from 73% to 104% in rice, mushrooms, fish and shellfish samples. On the other hand, low extraction efficiency values were found in some seaweed samples. It has to be taken into account that a suitable procedure for extraction of arsenosugars in seaweeds can yield to low extraction efficiency values for an algae specie (e.g. *Undaria pinnatifida*, EE= 29%) indicating that non-water soluble compounds are present in the raw sample. The fraction of arsenic not extracted with water could be associated with lipids fraction and might account for up to 50% of the total arsenic in algae [167]. For quality assessment, column recovery must also be established to guarantee the correctness of the chromatographic separation. To this end, the ratio of the sum of the species eluted from the chromatographic column to the total arsenic in the extract injected into the column was calculated. Depending on the combination matrix and arsenic species, CE values from 70 to 110 % could be obtained. For example, CR values were usually >80% in rice, cereal-based foods, mushrooms, fish and shellfish samples whereas low values were obtained in seaweeds, i.e. 29%, 53% and 59% for *U. pinnatifida*, *C. effusum* and *C. vermilara*, respectively. This low column recovery values could indicate the presence of several arsenic compounds in the sample that cannot be evaluated with the chromatographic separation used

Table 12. Performance of internal quality control assessment in arsenic speciation studies.

Publication	Food commodity	Analyte	Method [a]	Internal QC						
				Calibration	Internal QC	Instrumental drift	Internal STD	CRMs	Spike Recovery	Mass balance study (EE and CR)
Article II	Rice	DMA, MA and iAs	B and C	✓	✓	✓	**n.e**	✓	✓	✓
Article III	Cereal-based foods	DMA MA and iAs	B	✓	✓	✓	**n.e**	✓	✓	✓
Article IV	*L. edodes* products	DMA, MA, iAs, AB, AC, TMAO, unknowns	B	✓	✓	✓	**n.e**	✓	✓	✓
Article V	Edible mushrooms	DMA, MA, iAs, AB, AC, TMAO, unknowns	B	✓	✓	✓	**n.e**	✓	✓	✓
Article VI	Marine seaweeds	DMA, MA, iAs, AB, AC, TMAO, arsenosugars and unknowns	D	✓	✓	✓	**n.e**	✓	✓	✓
Article VII	Edible seaweeds	DMA, MA, iAs, AB, AC, TMAO, arsenosugars and unknowns	D	✓	✓	✓	**n.e**	✓	**X**	✓
Article VIII	Fish and shellfish	DMA, MA, iAs, AB, AC, TMAO and unknowns	B	✓	✓	✓	**n.e**	✓	✓	✓

[a] Methods are shown in **Table PPP.**

EE means Extraction efficiency. CR means Column Recovery

n.e means not evaluated

Table 13. Summary of criteria to evaluate internal quality control parameters in arsenic speciation analysis.

Quality control parameter	Evaluation	Frequency	Quality criteria
Calibration	External calibration curve. Home-made standards and *F. serratus* extract	Before and at the end of the each sample series	Standardisation of speciation standards with other standard or against a primary standard
Instrumental drift	QC standard solutions at two concentrations levels	Post-calibration, every five samples and at the end of the run	Values within 90 and 110% of the 90–110% of the expected value
Blank	Water and method blanks	One for each batch of samples	Blank values<LOQ
CRMs	CRM (or RMs) available with certified values for As species	One for each batch of samples	Values within 85 and 115% of the certified value
Replicates	Sample preparation was performed in triplicate.	All samples analysed in triplicates	Acceptable if RSD between replicates <15%
Spike recovery	Spiking experiments of inorganic arsenic	>3 spiked samples per experiment	Values within 85 and 115% of the theoretical spiked standard value
Mass balance study	Extraction Efficiency (EE) and Column Recovery (CR)	Estimation for each analysed sample	Values within 80 and 110% for EE and CR

Several CRMs exist for the measurement of tAs but few of them are certified for iAs (**Article I**). Given this fact and with the aim to assure that the oxidation of As(III) to As(V) was quantitative, spiking experiments were performed in several speciation studies. Thereby, to assure the accurate identification and quantification of inorganic As species, food samples were spiked by adding As(III) and As(V) standards to solid samples and then homogenised. Recoveries of iAs were calculated to assess the trueness and to verify the specificity of the method. Excellent iAs recoveries (mean% ± SD, n) were obtained: 100± 1% (n=9), 97 ± 3% (n=9), 95 ± 9% (n=3), 95 ± 8% (n=5), 102 ± 7% (n=5) in rice (**Article II**), cereal-based foods (**Article III**), *L. edodes* products (**Article IV)**, edible mushrooms (**Article V)**, fish and shellfish samples (**Article VIII)**, respectively. In addition, satisfactory values were obtained for DMA: 100 ± 2% (n=9) and 104 ± 5% (n=9) and for MA: 101 ± 1% (n=9) and 104 ± 4% (n=9) in rice (**Article II**) and cereal-based foods (**Article III**), respectively. Furthermore, ERM-BC211 and TORT-2 CRMs were also spiked with As (III) and As(V) standards in several research studies and the recovery of iAs was satisfactory in both: 102 ± 4% (n=3) and 96 ± 6% (n=3) in BC-211 rice in two research studies (**Articles IV and VIII**) and 106 ± 2 (n=3) in TORT-2 (**Article VIII**).

6.2.2.2 Establishment and evaluation of validation parameters

Several validation parameters were evaluated in speciation studies presented above in **Chapter 4**. The following validation parameters were established as specified elsewhere [146]: detection (LOD) and quantification limits (LOQ), Linearity, Repeatability, Intermediate Precision, Trueness, Accuracy, Selectivity and Uncertainty. A summary of parameters evaluated in the developed methods for arsenic speciation analysis is shown in **Table 14**. The criteria acceptance for each validation parameter is shown in **Table 15.**

In general, to evaluate the precision and accuracy of the methods applied in this thesis CRMs were analysed during sample analysis run. Although numerous CRMs exist for the measurement of tAs, few of them are certified for arsenic species analysis. One of the most commonly used practices within the scientific community to evaluate accuracy without a certified value is to perform arsenic speciation analysis on CRMs in which the tAs content or other arsenic species are certified. For validation purposes, the data obtained is compared with data reported in the literature by different researchers (**Article I**).

Therefore, in this thesis obtained results were compared with the few certified arsenic species available or in some cases with several published results on non-certified arsenic species. Thus, the following CRMs with certified values for arsenic species have been analysed in our studies: ERM-BC211 rice where tAs, DMA and iAs are certified; tuna fish tissue BCR-627 (tAs, AB and DMA are certified); CRM 7503-a rice (tAs, DMA and iAs are certified); CRM 7405-a Hijiki (tAs and iAs are certified). Average measured values obtained in several studies and certified values are shown in **Figure 15.** As can be noted, measured values (orange bars) were in agreement with the certified values (green bars) performing an excellent accuracy assessment of arsenic species.

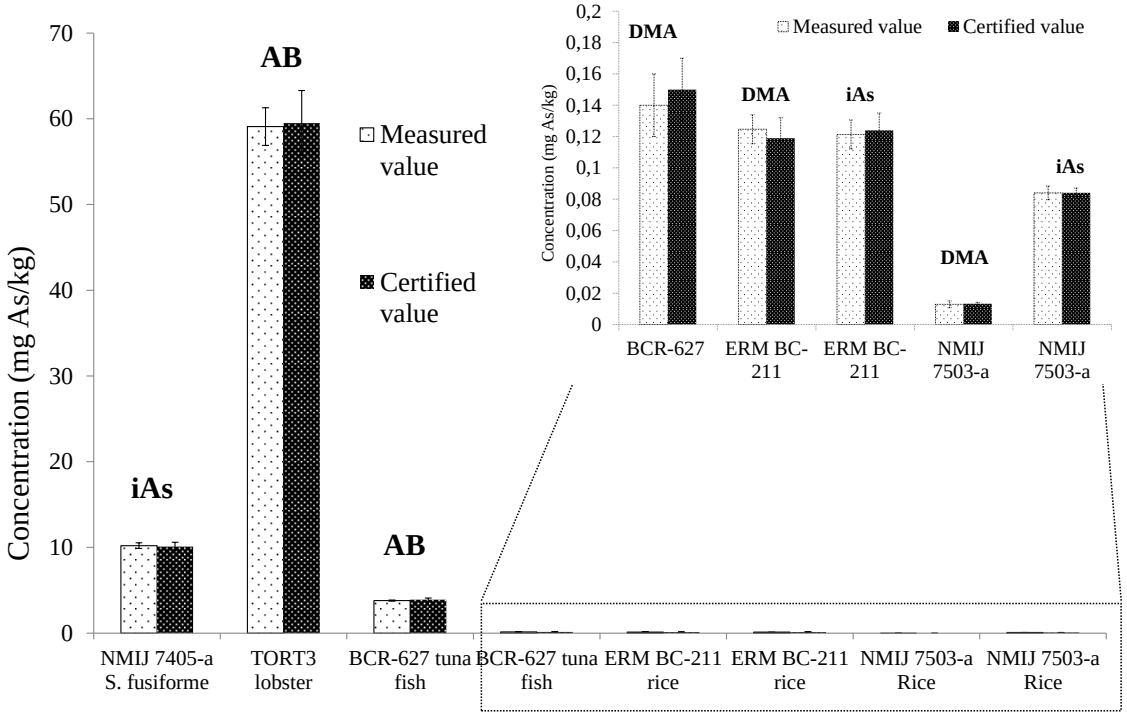


Figure 15. Evaluation of accuracy of the arsenic speciation methods by analysis of CRMs. Average concentrations are expressed as mg As kg^{-1} (mean ± U). Error bars denote combined standard uncertainty.

Table 14. Summary of validation parameters evaluated in the developed methods for arsenic species determination.

Publication	Food commodity	Analyte	Method [a]	Validation parameters									
				LOD	LOQ	Linearity	Repeatability	Intermediate Precision	Trueness	Accuracy	Selectivity	Uncertainty	External QC
Article II	Rice	DMA, MA and iAs	B and C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Article III	Cereal-based foods	DMA MA and iAs	B	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Article IV	*L. edodes* products	DMA, MA, iAs, AB, AC, TMAO, unknowns	B	✓	✓	**n.e**	**n.e**	**n.e**	✓	✓	**n.e**	**n.e**	✓
Article V	Edible mushrooms	DMA, MA, iAs, AB, AC, TMAO, unknowns	B	✓	✓	**n.e**	**n.e**	**n.e**	✓	✓	**n.e**	**n.e**	**n.e**
Article VI	Marine seaweeds	DMA, MA, iAs, AB, AC, TMAO, arsenosugars and unknowns	D	✓	✓	**n.e**	**n.e**	**n.e**	**n.e**	✓	**n.e**	**n.e**	**n.e**
Article VII	Edible seaweeds	DMA, MA, iAs, AB, AC, TMAO, arsenosugars and unknowns	D	✓	✓	**n.e**	**n.e**	**n.e**	**n.e**	✓	**n.e**	**n.e**	**n.e**
Article VIII	Fish and shellfish	DMA, MA, iAs, AB, AC, TMAO and unknowns	B	✓	✓	**n.e**	**n.e**	**n.e**	✓	✓	✓	**n.e**	**n.e**

[a] Methods are shown in **Table 6.** n.e means not evaluated

Table 15. Acceptance criteria of the method parameters evaluated in the research studies

Parameter	Evaluation	Acceptance criteria
LOD	Blanks or Standard solutions	Suitable for detect As species at the levels found in the assayed food samples
LOQ	Comparison with method quantification limit (MQL) (lowest level validated)	1/10 maximum level (ML) a of Legislation (if not ML, preliminary analysis of >10 samples to select the minimum LOQ needed)
Linearity	5 calibration points	$R^2 \geq 0.9990$. Residual error of <15% for the lowest calibration level and <10% for the others [a]
Repeatability	%RSD, same day, same analyst and same analytical run	%RSD (repeatability) ≤ 2/3 * %RSD (intermediate precision)
Intermediate Precision	%RSD, 3 different analysis days over 3 weeks, different analysts and different standard preparation	For each validation level, % RSD ≤ 2/3 Horwitz-Thomson function [b]
Trueness	%Recovery of spiking experiments	Rec= 85%-115% [c]
Accuracy	CRMs or RMs	Values within 85% and 115% of the certified value
Selectivity	Argon chloride ($^{40}Ar^{35}Cl$) interference at m/z 75	Free of interferences and good chromatographic resolution within As species
Expanded Uncertainty	Combination of trueness and intermediate precision parameters	U_{max} (k=2) ≤ 2 * %RSD Horwitz function [d]
External QC	Proficiency tests and certification studies	Z score or comparison with certified value

[a] As recommended by Thompson [146] .

[b] Acceptance criterion [168] . In %RSD: 14.7% for values ≤ 100 μg kg^{-1}, 13.6% for 200 μg kg^{-1} and 12.2% for 400 μg kg^{-1} [169, 170].

[c] Acceptance criterion set by CODEX is 60%-115% for 10 μg kg^{-1} and 80%-110% for 0.1-10 mg kg^{-1} [171].

[d] Horwitz, 1982 [168]; Thompson et al.., [146]

Fully validated methods for the determination of arsenic species in rice and cereal-based foods

Among the speciation studies performed, two of them were specially focused on validate a method (method B, **Table 6)** for the determination of arsenic species in rice and rice products (**Article II**) and in cereal-based food (**Article III**). In the following paragraph, routes towards the establishment and validation of these research studies are discussed.

As presented in **Article II**, a full validation of an analytical method for the determination of iAs, MA, and DMA in rice and rice products was performed in two laboratories: (A) the Department of Analytical Chemistry of the University of Barcelona and (B) the Public Health Agency of Barcelona under different instrumental and operating conditions. Furthermore, the applicability was also assessed by applying the validated method to 29 samples of rice and rice-based baby cereals. Although method validation performance was

satisfactory, several infant cereal samples were below the validated LOQs (established as the lowest limit validated) and were not determined. Because cereals and infant cereals deserve special attention with respect to iAs exposure in European population [5], we aimed to optimize our method in rice samples (**Article II)** to improve the LODs to analyse these kinds of food. Therefore, the main objective of **Article III** was to optimize our method (**Article II**), then evaluate and validate a new method in cereal-based products that could substitute the above-mentioned method and could be used in routine analysis for food control purposes. First, instrumental conditions of the HPLC-ICPMS technique were optimised to improve the LODs. Second, the validation parameters for the determination of iAs, MA and DMA were evaluated and validated. Finally, several cereal-based foods were analysed to expand the method applicability and provide iAs occurrence data on these type of foods.

First of all, several HPLC-ICPMS parameters were modified and optimized from our previous validated method in rice samples to improve the LODs. Regarding the ICPMS tuning parameters, a deep study was carried out to investigate possible factors that could significantly affect the sensitivity of detecting arsenic. For this, the reaction cell mode, make up and carrier gas flows, difference between the octopole and quadrupole voltage, cell exit, ion lens, omega bias, sample depth and the addition of organic solution were tested to achieve the best signal-to-noise ratio for arsenic at m/z 75. Given that the range of signal intensities could depend on the degree of wearing of the sampler and skimmer cones, regularly-cleaned Ni cones were used throughout the work. To adjust and test all the tuning parameters, a standard solution containing 5 μg As L^{-1} as As(V) in the mobile phase was applied as a tuning solution. A peristaltic pump was used to introduce samples of the analyte solution at 1 mL min^{-1}. A blank solution was measured before and after the tuning solution to ensure the background level.

Among the evaluated parameters, increase of injection volume, non-use of gas in the reaction cell, and the addition of a 10% IPA solution after the column were those most increased arsenic sensitivity (**Article III**). Furthermore, other parameters were evaluated without obtaining significant effects on arsenic signal enhancement. For instance, the flow of both carrier gas and make-up gas were tested. Carrier gas controls both the nebulization efficiency and the uptake rate of the sample, while the make-up gas makes up Ar gas in the spray chamber and is mixed with the carrier gas. From the different combinations of the gas flows tested with a concentric micromist nebulizer, the optimum total flow (carrier + make up) was 1.15 L min^{-1} (0.95 and 0.15 L min^{-1}, respectively). Regarding the ion lens parameters, the energy difference between the octopole exit (OctP bias) and quadrupole entrance (QP Bias) were also evaluated. For this, differences between 1 and 4 V OctP and QP biases were tested (1V increments). An increase in As signal selecting a difference value of 2 V was observed. As the OctP bias voltage was changed, cell exit value was also evaluated and simultaneously changed by the same degree. Thus, cell exit voltage was adjusted within the common range (-30 to -70V, using 10-V increments) to find an optimum value near the OctP bias. High cell exit voltages provided high arsenic sensitivities and therefore a -70V value was selected. Furthermore, other parameters such as RF power, ion lens, omega bias voltages and sample depth were assessed, with no observed significant effects on As signal enhancement. Finally, the optimized tuning parameters (presented in **Article III**) were tested in the coupled HPLC-ICPMS system, analysing mixed

standard solutions (As(III), DMA, MA and As(V)) from 0.05 to 5 μg As L^{-1}. The base-line corrected heights of the chromatographic peaks were evaluated visually and calibrated against standard arsenic mixtures. The lowest calibration level (0.05 μg As L^{-1} for each species) was satisfactorily quantifiable and distinguishable from background noise.

Thereby, the LODs were estimated applying the optimized conditions and were lower than those obtained into the rice method (**Table 16**) illustrating that the optimization was satisfactory. The optimization of the rice method (**Article II**) allowed us to validate a method at a lower concentration level suitable for cereal-based foods and infant cereals (**Article III**): 4 vs 10 μg As kg^{-1} for DMA and MA and 4 vs 20 μg As kg^{-1} for iAs. All parameters evaluated in the two fully validated methods are summarised in **Table 16**. To establish and evaluate intermediate precision, trueness and expanded uncertainty in these two methods, spiking experiments were performed. For this, different type of samples at three concentration levels (in triplicate) were fortified with As(III), As(V), DMA, and MA standards. Similar performance of intermediate precision, trueness and expanded uncertainty were obtained between them (**Table 16**) and in both cases these parameters were satisfactory in terms of acceptance criteria (**Table 15**). Furthermore, in both cases, acceptable accuracy was obtained by analysing some CRMs (NIST SRM 1568a, NMIJ CRM 7503-a and NCS ZC73008 Rice). For selectivity, no interferences of the ArCl interference at m/z 75 were observed in both methods. Besides, the separation of As species was adequate in all assayed samples and no co-elution of species was observed. Furthermore, as external quality control, methods were tested in proficiency tests (IMEPs, FAPAS) and in certification studies of CRMs obtaining acceptable results in all cases (**Table 16**). Finally, the applicability was demonstrated analysing several samples. Rice samples including several types of rice, rice products, and infant rice products were determined in **Article II**, meanwhile cereal-based food samples, i.e. bread, biscuits, breakfast cereals, wheat flour, corn snacks, pasta and infant cereal were analysed in **Article III**.

Since maximum limits of iAs in rice have been recently established by European Union [107], food control laboratories have to be ready to determine iAs in food, especially rice. Furthermore, they should be ready to expand the applicability to other foodstuffs since the European Commission has recently published a recommendation on the monitoring of arsenic in food by Member states during the years 2016, 2017 and 2018 [109]. The monitoring should include a wide variety of foodstuffs and Member States should carry out the analysis of arsenic, preferably by determining the content of iAs and tAs and, if possible, other relevant arsenic species. Our proposed speciation method is successfully validated according to ISO/IEC 17025:2005 standard [172] and is sensible and selective for iAs and could be a valuable tool for the determination of iAs in rice and cereal-based foods currently a subject of high interest in food control analysis [5, 103, 104, 107]

Table 16. Summary of the parameters evaluated in the two fully validated methods.

Foodstuffs	Analyte	Method parameters										Reference
		LOD ($\mu g\ L^{-1}$)	LOQ ($\mu g\ L^{-1}$)	MQL [a] ($\mu g\ kg^{-1}$)	Linearity ($\mu g\ L^{-1}$)	Repeatability %(RSD)	Int. Precision %(RSD)	Trueness %(Recovery)	Accuracy [b] % (of CRM)	Selectivity	Uncertainty %(U, k=2)	Ext. QC	
Rice													
	DMA	0.03	0.12	10	0.25–10.0	2.0	5.7	101.8	88.0		11.6	n.e	
	MA	0.04	0.14	10	0.25–10.0	2.4	4.0	102.3	n.e	Control the ArCl interference at m/z 75	9.3	n.e	**Article II**
	iAs	0.06	0.20	20	0.50–10.0	1.8	3.8	99.3	101.0		7.8	IMEP-107	
Cereal-based foods													
	DMA	0.008	0.027	4	0.05–5.0	2.5 and 3.7	6.2	104.2	101.5		16.2	Certification of ERM-BC211	
	MA	0.007	0.023	4	0.05–5.0	3.6	5.3	103.7	n.e	Control the ArCl interference at m/z 75	14.1	n.e	**Article III**
	iAs	0.009	0.030	4	0.05–5.0	2.7 and 1.9	4.1	97.2	99.5		11.5	IMEP-112, FAPAS 07151 and Certification of ERM-BC211	

[a] Method quantification limit is the lowest limit validated by means of spiking experiments. [b] Accuracy was evaluated by analysis of CRMs and results were expressed as % of recovery compared with the certified value. n.e means not evaluated**.**

6.2.2.4 External Quality Control

For external QC, the developed methods for determination of arsenic species were tested in several proficiency tests (PTs), in a method validation study and in various certification studies of CRMs. Specific information for each one are summarized in **Table 17** and arsenic speciation results are shown in **Table 18.**

Proficiency tests

Our research group was selected by IRMM as expert laboratory in arsenic speciation field and we were invited to participate in various PTs organised by IMEP from IRMM of the JRC. This collaboration project allowed us to corroborate the suitability of our developed methods for the determination arsenic species in foodstuffs. Our main objective was to analyse tAs and iAs content in the test samples with the aim to assign a reference values for these measurands. Therefore, we participated in various PTs and the general aim is to: "judge the state of the art of analytical capability for the determination of tAs and iAs in several foodstuffs with a view to future discussions on the need for possible regulatory measures and future discussions on risk management and the possibility of introducing maximum levels for iAs in the European Union". As the test materials were not certified for iAs, some expert laboratories were requested by IRMM to analyse iAs content in the test samples. Thus, homogeneity, stability and certification studies of iAs content in the test samples were performed by expert laboratories in the field. Several types of food samples were analysed in these PTs covering a wide range of foodstuffs: rice, fish, wheat, vegetable, algae, mushroom, and canned food (peas in brine). Information and specific details of each IMEP can be found in the corresponding IRMM report (**Table 17**). A summary of these PTs is discussed and reported in **Article I** and information related to objectives, target analytes, assigned values, and results of participants (z-score), comments and main conclusions of each IMEP can be found (**Article I)**.

In general, we were requested to analyse the test materials using methods of our choice and no further requirements were imposed regarding methodology. Test items were storage following the conditions supplied by IRMM until analysis. For the determination of water content the procedures supplied by IRMM were strictly followed in each IMEP. We performed an accurate quality assessment to assure the reliability of our results obtained. This includes internal QC assessment, analysis of some CRMs to evaluate precision and accuracy and spiking experiments of iAs to evaluate the trueness. Our inorganic arsenic results obtained in several IMEPs are shown in **Table 18.** In the following paragraphs, further details of our participation in these IMEPs are presented and discussed.

Table 17 . Summary of our participation in proficiency tests for the determination of total and inorganic arsenic.

Proficiency test	Method [a]	Description	Target analyte	Analyte evaluated	Sample	Sample Origin	References
IMEP-107	B and C	*Proficiency test*	tAs and iAs	tAs and iAs	Rice	Provided by Aberdeen University and produced by IRMM)	[147, 148]
IMEP-30/109	E	*Proficiency test*	Cd, Pb, tAs, iAs Hg and MeHg	iAs	Fish	NRC CRM DOLT-4	Article X; [149, 150]
IMEP-112	B	*Proficiency test*	tAs and iAs	tAs and iAs	Wheat	Provided by Istituto di Sanita and produced by IRMM	
	B	*Proficiency test*	tAs and iAs	tAs and iAs	Vegetable	NIST SRM 1570a spinach leaves	Article XI; [151]
	B	*Proficiency test*	tAs and iAs	tAs and iAs	Algae	Produced by IRMM	
IMEP-116/39	B	*Proficiency test*	Cd, Pb, tAs, Hg and iAs	iAs	Mushroom	Provided by University of Barcelona and produced by IRMM	Article XII; [152, 153]
IMEP-118	B	*Proficiency test*	As, Cd, Pb, Hg, Sn and iAs	iAs	Canned food	Produced by IRMM.	[154]
FAPAS 07151	B	*Proficiency test*	tAs and iAs	tAs and iAs	Rice	Produced by FAPAS	[155]
IMEP-41	B	*Method validation*	iAs	iAs	Foodstuffs	Samples and CRMs	Article XIII; [173]

[a] Methods are summarised in **Table 6**.

Table 18. External QC assessment for arsenic speciation methods: participation in PTs and method validation study. Concentrations are expressed as mg As kg^{-1}, mean value ± U for measured and for assigned/certified values.

External QC	Sample	As speciation method [a]	Inorganic arsenic		References
			Measured value	Assigned/ Certified value	
Proficiency test					
IMEP-107	Rice	B and C	0.105 ± 0.005 and 0.106 ± 0.004	0.107 ± 0.014	[147, 148]
IMEP-30/109	Fish	E	Not detected [b]	not assigned [c]	Article X; [149, 150]
IMEP-112					
	Wheat	B	0.154 ± 0.003	0.169 ± 0.25	
	Vegetable	B	0.060 ± 0.002	0.054 ± 0.012	Article XI; [151]
	Algae	B	0.190 ±0 .010	0.188 ± 0.025	
IMEP-116/39	Mushroom (*L.edodes*)	B	0.348 ± 0.026	0.321 ± 0.026	Article XII; [152, 153]
IMEP-118					
	Peas in brine. Drained product	B	0.106 ± 0.008	0.098 ± 0.020	[154]
	Peas in brine. Solid/liquid composite:	B	0.086 ± 0.006	0.082 ± 0.008	
FAPAS 07151	Rice	B	0.424 ± 0.005	0.390 ± 0.072	[155]
Method Validation					
IMEP-41					
	Rice	B	0.109 ± 0.008	0.108 ± 0.011	
	Wheat	B	0.173 ± 0.013	0.165 ± 0.021	
	Mussels	B	0.084 ± 0.006	0.0863 ± 0.008	
	Cabbage	B	0.080 ± 0.006	0.091 ± 0.016	Article XIII; [173]
	Mushroom	B	0.348 ± 0.026	0.321 ± 0.026	
	Seaweed	B	9.74 ± 0.72	10.10 ± 0.50	
	Fish	B	0.228 ± 0.017	0.271 ± 0.061	

[a] Methods are summarised in **Table 6.** [b] The LOQ of the method used is 0.031 mg kg^{-1} for As(III) and 0.084 mg kg^{-1} for As(V).

IMEP-107: Determination of total and inorganic As in rice

This PT was organized in 2009 and focused on the determination of total As and iAs in rice (IMEP-107) [147, 148]. Two bottles were received and analysed on two different days (one bottle/day/ performing three independent replicates per bottle (six replicates /measurand). Inorganic arsenic content in the rice test sample was determined from the speciation carried out after application of methods B and C (**Table 6**). As commented before, Method B oxidises As(III) to As(V) and iAs content was determinate quantifying As(V) peak in the extracts. Moreover, method C preserves the original state of species and both As(III) and As(V) were found in the extracts and iAs was quantified as sum of As(III) and As(V). Comparing the two methods, similar inorganic arsenic results were found: 0.105 ± 0.005 and 0.107 ± 0.005 mg As kg^{-1} for method B and C, respectively. As an example, a chromatogram obtained applying method B for the extractable species in the rice test material is shown in **Figure 16.**

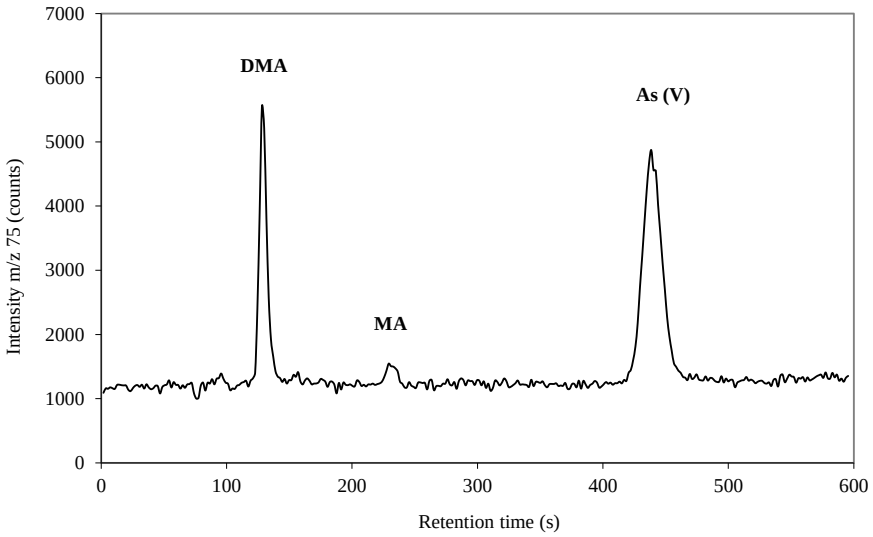


Figure 16. Chromatogram obtained by HPLC-ICPMS of the extractable arsenic species in the rice test material (IMEP-107).

Furthermore, similar results were obtained between all expert laboratories and consequently an iAs value was satisfactory assigned: 0.107 ± 0.014 mg As kg^{-1} (**Table 18 and Figure 17**). Our laboratory is presented as Cert.ID "1" and as can be observed in **Figure 17**, our results for tAs and iAs content (dark circles) were in agreement with the assigned values (continuous lines). As our internal QC, the SRM 1568a from NIST with a certified concentration of tAs= 0.29 ± 0.03 mg As kg^{-1} was used to evaluate the accuracy of our results. The NIST SRM 1568a was run together with the samples (three replicates each day) and arsenic species results were in agreement with those reported in the literature [174, 175] proving the accuracy of the speciation results.

The organizers commented that the main conclusion is that the concentration of iAs determined in rice does not depend on the analytical method applied, as was proved by the results submitted by six expert laboratories and the participants in IMEP-107. Finally, they

conclude that there is no reason not to consider the option of introducing possible maximum levels for iAs in rice in further discussions on risk management and it should not be postponed due to analytical concerns [147, 148].

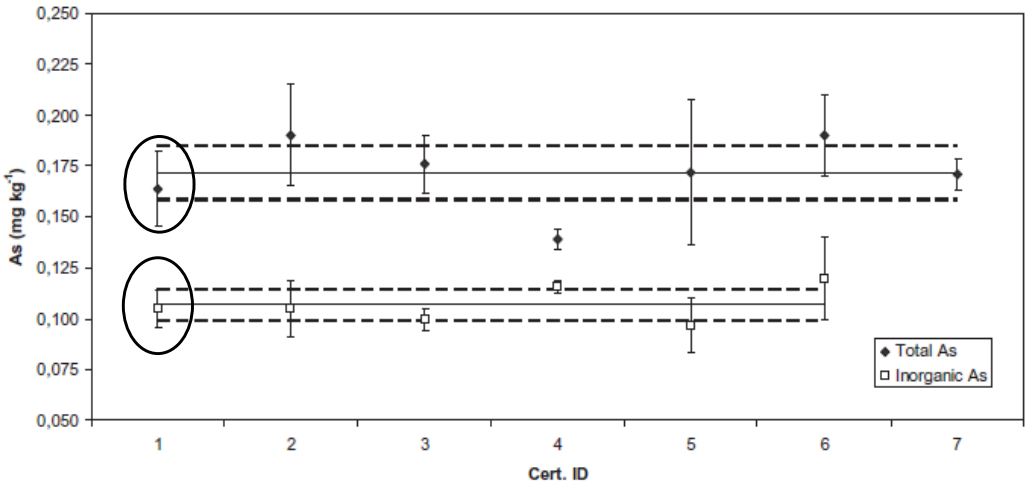


Figure 17. Results reported by the expert laboratories for tAs and iAs in the test material of IMEP-107 (adapted from de la [147]. Our results of tAs and iAs content are marked with dark circles (Cert.ID "1"). The continuous black lines correspond to Xref for total As and inorganic As, respectively, the dotted lines mark the expanded standard uncertainties of the characterization by the expert laboratories, $2U_{car}$

IMEP-109/30: Analysis of total Cd, Pb, As and Hg as well as MeHg and inorganic As in seafood

Two inter-laboratory comparisons, IMEP-109 and IMEP-30, were performed in 2010 focused on the measurement of trace elements as well as MeHg and iAs, in seafood (**Article X**). Only the EU NRLs took part in IMEP-109 [150] , while IMEP-30 was open to all laboratories [149]. The commercially available CRM DOLT-4 dogfish liver from the National Research Council of Canada (NRC) was used as the test material for these PTs.

Our group has successfully established a method for the determination of arsenic species in oyster tissue [176]. The method is based on a MW-assisted extraction with MeOH/H_2O (1:1) solution and then clean-up step with a C18 cartridge. One bottle was received and was analysed under repeatability conditions. Therefore, we decided to apply this method (method E in **Table 6)** to perform arsenic speciation analysis in the test sample of IMEP-30/109. One bottle was received and was analysed under repeatability conditions. AB was the predominant species and iAs was below the LOD (**Table 18**). As an example, chromatograms obtained applying method E for the extractable species in the seafood test material are shown in **Figure 18.** DMA and traces of an unknown anion species (UK-A) were determined by anion exchange column (**Figure 18a**) and AB and unknown cation species (UK-C) (**Figure 18a**). To assess the accuracy of our results, two seafood CRMs, BCR-627 and ERM-CE278, were analysed during our participation in the PTs. BCR-627 is certified for DMA and AB and values are: 0.15 ± 0.02 and 3.9 ± 0.2 mg As kg^{-1} for DMA and AB, respectively. Our results for DMA

and AB contents were in agreement with the certified values: 0.152 ± 0.004 and 3.69 ± 0.03 for DMA and AB mg As kg^{-1}, respectively.

As commented in **Article X**, strong discrepancies among the iAs results and the expert laboratories were not able to agree on a value for the iAs within a reasonable degree of uncertainty (our laboratory is presented as "Certifier 2" in Table 1 of **Article X**). For this reason, it was not possible to establish an assigned value for iAs and therefore the results from the laboratories for iAs could not be scored (**Article X**). Inorganic arsenic results were spread over a wide range, but the majority of laboratories agreed that the iAs content of the test material did not exceed 0.25 mg kg^{-1}. According to the results, the determination of iAs in seafood presented serious analytical problems and iAs is clearly more difficult to analyze in this seafood matrices than in rice (IMEP-107). Further information and possible causes for the dispersion of the results are widely discussed (**Article X;** [149, 150].

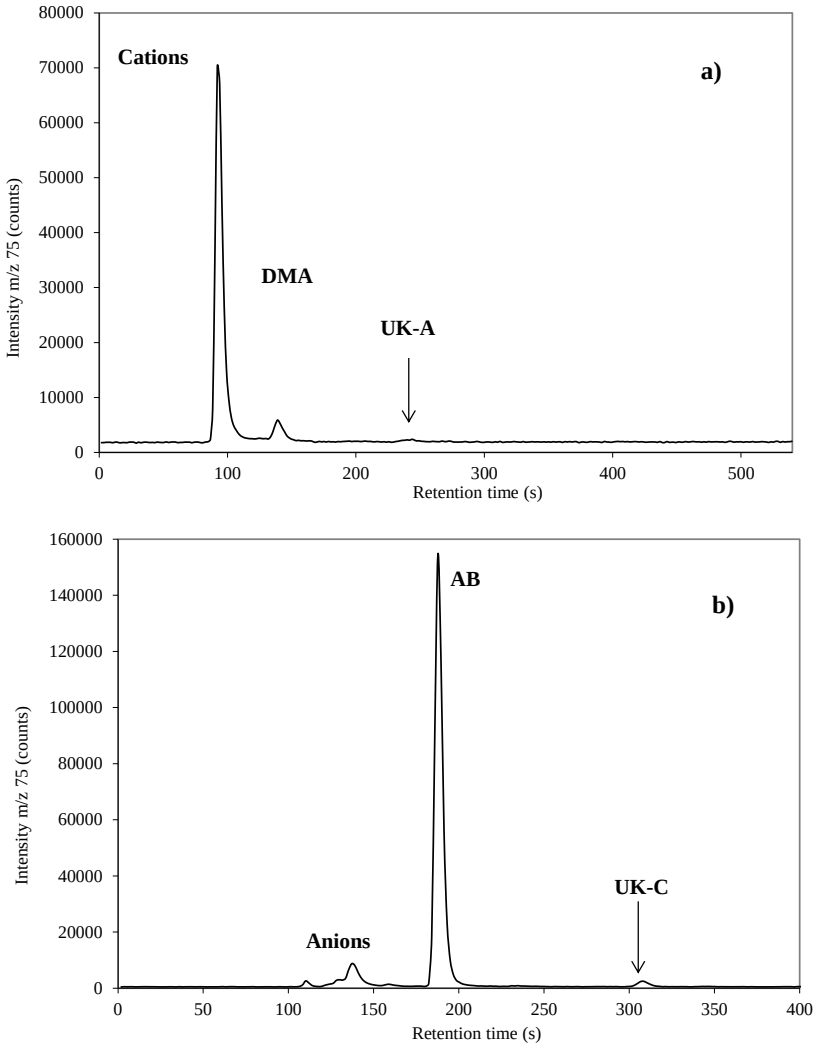


Figure 18. Chromatograms obtained by HPLC-ICPMS for seafood test sample of IMEP-109/30 from anion exchange column (a) and cation exchange column (b)

From our results, iAs was below the LOD in the extracts of the test sample. Regarding our performance, we are able to say that possible causes for the discrepancies could be attributed to the extraction and/or detection steps. Thereby, the use of MeOH/water (1:1) (method E), as extracting reagents might not have provided quantitative extraction and recovery of iAs. Most naturally occurring As species in marine tissues are polar and soluble in water, thus the use of MeOH:H_2O mixtures provides a good compromise between As solubility and ease of solvent removal. However, methanol is a poor extractant of iAs species [177] and in several cases, low As extraction efficiencies were obtained with methanol-water extraction in some type of food samples. Moreover, acids and bases have been used with varying success to improve extraction efficiencies [10]. Other cause could be related to co-elution of As(III) with other species. Using method E, we expected to found both As(III) and As(V) species in the extracts as the method preserves the original state of species but as can be observed in **Figure 18**, As (III) and As(V) were not detected. Applying this chromatographic conditions, As(III) elute at the void volume and could be co-eluted with cationic species (specially AB in fish and shellfish samples) performing a possible underestimation of iAs content. As is presented in **Article VIII**, a possible solution could be the using of an acid solvent with and an oxidant such as H_2O_2 to oxidise As(III) to As(V) and then determine total inorganic arsenic as a As(V) which is usually well-separated from other species. As an example, two chromatograms are shown in Figure 1 and 2 of **Article VIII** illustrating this approach to oxidise As(III) to As(V) and quantify total inorganic arsenic as As(V).

Other alternatives for a selective determination of iAs in seafood have been reported in the literature (**Article I**). For example, the introduction of an extra step of HG between the HPLC and the ICPMS which would allow the selective determination of iAs. This could be an alternative since arsenobetaine, arsenocholine or arsenosugars cannot be generate the hydride and therefore cannot be detected and only iAs and methylated species can be detected. Other alternative without a chromatographic system could be the use of a strong anion exchange solid-phase extraction (SPE) cartridge to separate iAs from AB and other organoarsenicals and further detection by HG-AFS or AAS [178, 179]. In addition, a selective extraction of iAs using specific extracting media can separate iAs from organic compounds that remain in the sample matrix could be a suitable approach. For instance, the extraction method proposed by [180, 181], based on the solubilisation of the protein matrix with a high concentration of HCl, which denaturates the proteins and allows the release into the solution of all the arsenic species, and the subsequent extraction with chloroform of the iAs present in the acid medium.

The conclusion of IMEP-109/30 was that more research is needed in the future to find appropriate and effective extraction procedures, as well as chromatographic conditions for reliable separation and quantification of iAs [149, 150] (**Article X)**. Based on IMEP-30/109 conclusions and from our PT performance, we think that further work on the comparison of different extraction methods for iAs determination should be performed and would be a valued contribution to the topic, and a matter of high interest. Furthermore, the production of seafood CRM with an iAs certified value would help in the validation of iAs methods and in providing reliable iAs data which could be useful in PTs since as is commented, these IMEPs have shown unsatisfactory performance.

IMEP-112: Determination of total and inorganic in wheat, vegetable food and algae

IMEP-112 focused on the determination of tAs and iAs in wheat, vegetable food and algae (**Article XI;** [151]). Two bottles for each three test materials were received and were analysed on two different days (one bottle/day) performing three independent replicates per bottle (six replicates/measurand). Our group participated by applying method B (**Table 6)** was applied to determine iAs content in the test samples. Results provided by expert laboratories were in agreement and assigned values were satisfactorily established (**Table 2, Article XI**). Our laboratory is presented as Certifier "4" (**Table 2, Article XI**) and for the three test samples, our iAs results were acceptable compared to the assigned values (**Table 18**). Inorganic arsenic was the major compound in wheat and vegetable test items meanwhile arsenosugars were predominant in algae sample. Chromatograms for the extractable species in the wheat and algae test materials are shown in Figure 5 of **Article XI**. As our internal QC, the NIST SRM 1568a rice and CRM BCR-279 *Ulva lactuca* were used to evaluate the accuracy of our results. As both materials are certified for tAs, our iAs results were compared with those reported in the literature, for SRM 1568a [174, 175] and for BCR-279 [182] proving the accuracy of the obtained results.

The organizers of IMEP-112 concluded that the concentration of iAs determined in any of the matrices does not depend on the analytical method applied, as proven by the results submitted by the seven expert laboratories and by the participants. Furthermore, the participating laboratories performed, in general, satisfactorily for the determination of iAs in wheat and vegetable food; however, only a few laboratories obtained a satisfactory score for iAs in algae. Finally, it was also highlighted that, purely from the analytical point of view, there is no reason not to consider the option of introducing maximum levels for iAs in wheat, vegetable food and algae in further discussions of risk management (**Article XI**; de la Calle et al., 2011a).

IMEP-116/39: Total Cd, Pb, As, Hg and inorganic As in mushrooms

Our research group was selected to perform a screening of total Cd, Pb, As and Hg and inorganic arsenic mass fractions in commercially available mushrooms with the aim to select a candidate test item for the proficiency test IMEP-116/39. For this, we selected thirteen fresh mushroom species and four dehydrated mushrooms for the initial screening. Samples were purchased from local markets and shops in Barcelona. Fresh mushrooms were hand cleaned for soil and moss as is described in **Article XII**. Method A was applied to determine total content of Cd, Pb, As and Hg in the seventeen mushrooms. Results ranged from: 0.053 to 2.83, 0.089 to 8.33, 0.025 to 1.71 and 0.075 to 10.72 mg As kg^{-1} dry mass for As, Pb, Cd and Hg, respectively. Two CRMs, NIST 1570a spinach leaves and BCR-679 white cabbage, were used throughout the study to assess the accuracy of the analytical results. To determine arsenic species as well as iAs content, method B was used and results ranged from 0.016 to 1.02 mg As kg^{-1} for iAs. From all assayed mushrooms, we selected *Lentinula edodes* since is one of the most cultivated mushrooms worldwide and the concentrations of the target analytes were suitable for accurate determination by National Reference Laboratories (NRLs), official control laboratories (OCLs) and other laboratories using AAS, ICPOES or ICPMS based methods. Therefore, we purchased

6 kg of *L. edodes* from a local market in Barcelona. We randomly selected a subsample of 1 kg as representative for our analysis in UB and we sent the remaining 5 kg to IRMM under refrigerated conditions. The subsample (1 kg) was pre-treated as described in **Article XII**. The homogenized material was randomly distributed into 7 plastic bottles and one replicate of each bottle was analysed. Concentrations of As, iAs, Pb, Cd and Hg in this mushroom were: 0.610 ± 0.017, 0.331 ± 0.015, 0.111 ± 0.011, 4.47 ± 0.19 and 0.073 ± 0.004 mg As kg^{-1} dry mass, respectively. Inorganic arsenic was the predominant arsenic species (accounting for 54.3% of tAs) and other As species such as DMA and MA were also found in minor proportions. Furthermore, good reproducibility (expressed as RSD% precision) between the bottles for the test material was obtained as follows: 4.0%, 12.8%, 2.7%, 5.4% and 4.4% for Cd, Pb, As, Hg and iAs, respectively. Since these concentrations were suitable for accurate quantification by laboratories participating in a proficiency test, we proposed *L. edodes* as a candidate test item for a further PT.

After this initial screening of several commercial mushrooms, IRMM selected *L. edodes* as a test item. Thus, two PTs were organized by IRMM in 2013 using the same test item (shiitake mushroom) (**Article XII**): IMEP-116 (for NRLs) [152] and IMEP-39 (for OCLs and other laboratories) [153]. Two bottles were received and analysed on two different days (one bottle/day/ performing three independent replicates per bottle (six replicates /measurand). Our group participated applying method B to determine iAs content in the mushroom test sample. Results provided by expert laboratories were in agreement between them and therefore and iAs assigned value was established (Table 2, **Article XII**). Our iAs result was acceptable compared to the assigned value (**Table 18**). A chromatogram obtained in the mushroom test sample is represented in upper part of Figure 7 of **Article XII**. As can be seen, iAs was the major compound in the mushroom test item and also DMA, MA and an unknown species were found in minor proportions. As there is no certified reference material available for iAs content in mushrooms, three rice CRMs (NIST SRM 1568a, NMIJ CRM 7503a and ERM BC-211) were used throughout the study to assess the accuracy and the reliability of iAs results. Additionally, to assess the trueness of the results, spiking experiments were carried out by adding As(III) and As(V) standards to solid and homogenized samples. The mixtures were then left to stand for 30 min before extraction. Excellent recoveries of iAs were found in the test sample: 98 ± 3% (mean value ± RSD, n = 4).

The organizers concluded that, the performance of the network of NRLs for all the investigated measurands can be considered satisfactory. Furthermore, a high percentage of satisfactory results were obtained for iAs in IMEP-116 (NRLs). Additionally, it is also pointed out that in IMEP-39; several laboratories obtained a satisfactory z-score for iAs using AAS-based techniques, showing that sound determinations of iAs can be made without the need for expensive sophisticated instrumentation. Specific comments of other measurands can be found (**Article XII;** [152, 153]).

IMEP-118 Determination of total As, Cd, Pb, Hg, Sn and inorganic As in canned food

In 2014, a PT program was produced focused on the determination of total As, Cd, Pb, Hg, Sn and iAs in canned food (peas in brine) (IMEP-118) [154]. The PT was mandatory for all NRLs having experience in this kind of analysis and was open for food control laboratories and other interested laboratories in the determination of heavy metals in canned food. The raw material was originally spiked with such elements during sample preparation. Four bottles containing each 100g of test material (glass jars containing peas in brine) were received and were stored at 4ºC until the analyses were carried out. We are asked to analyse iAs in the canned vegetables, in both the drained product and the solid/liquid composite following the procedure established in the AOAC official methods 968.30 ([183]) and 945.68 [184] for canned vegetables. Briefly, the procedures applied are:

· **Drained product** (according to AOAC Official Method 968.30): weigh full can, open and pour entire contents on No. 8 sieve (2.38mm particle size and 20 cm of diameter). Without shifting product, incline sieve at ca 17-20º angle to facilitate drainage. Drain 2min, directly weigh either drained solids or free liquid, and weigh dry empty can. From weights obtained, determine % liquid and % drained solid contents.

· **Solid/liquid composite** (according to AOAC Official Method 945.68): thoroughly grind entire contents of can in food processor. In all cases, mix portion used and store balance in glass-stoppered container. Unless analysis is to be completed in reasonably short time, determine water in portion of test sample prepared as above. To prevent decomposition, dry reminder, grind, mix thoroughly, and store in a glass-stoppered container (second water determination is required in this method). Run analysis immediately after the sample preparation to avoid decomposition and dessication.

From the four bottles of the test item, two were used for the certification of the mass fraction of iAs in the "drained product" and the other two for the certification of iAs in the "solid/liquid composite". Our group participated using method B to determine iAs content in the test samples. Results provided by expert laboratories were in agreement between them and assigned values were established for iAs in both "drained product" and "solid/liquid composite". These results are shown in Tables 1 and 2 and also in Figure 1 of IMEP-118 Report [154] and our laboratory is presented as "Expert 1". As can be observed in **Table 18**, our iAs results were satisfactory compared to the assigned values. Only iAs species were found in the extracts of test samples. As an example, two chromatograms of the extractable species in the "solid/liquid composite" and "drained product" are shown in **Figure 19**.

As our internal QC, the following CRMs: NIST SRM 1570a spinach leaves, NMIJ CRM 7503a rice and ERM BC-211 rice were used to evaluate the accuracy. Our results were in agreement with certified values, iAs content was: 0.126 ± 0.002 vs 0.124 ± 0.0011 and 0.082 ± 0.002 vs 0.084 ± 0.003 for BC-211 and NMIJ 7503a, respectively.

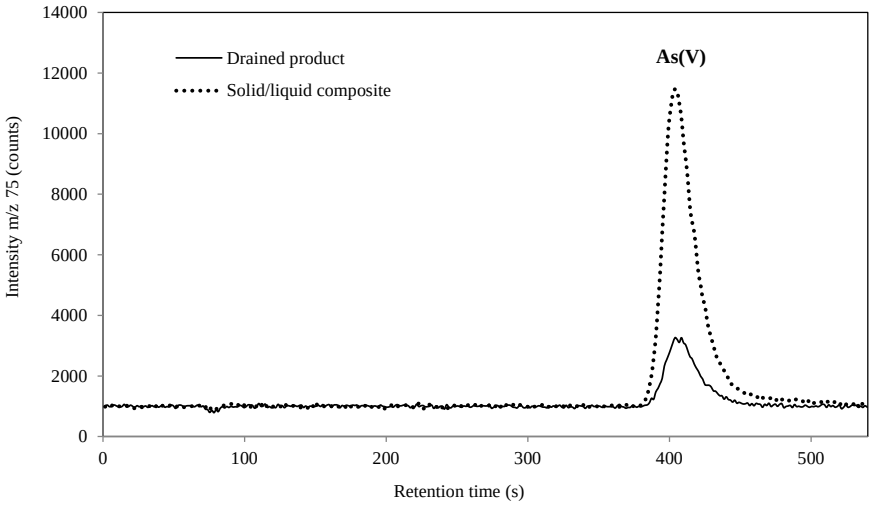


Figure 19. Chromatograms of IMEP-118 test samples, dotted line: "solid/liquid composite" and continuous line: "drained product" obtained by anion exchange HPLC-ICPMS

From the PT results, organizers concluded that the performance of the participating laboratories at determining iAs was satisfactory for both sample preparation approaches. However, few laboratories carried out analysis for iAs determination (only 33% reported values). More information, specific comments and conclusions related to other target analytes are widely discussed in the IRMM report [154].

IMEP-41: Determination of inorganic arsenic in food

An interlaboratory comparison (IMEP-41) was performed to assess the performance characteristics of a method by means of a collaborative trial for the determination of iAs in food products by FI-HG-AAS (**Article XIII**,[173]) and was organised in support to Commission Regulation 1881/2006 [108]. The method under evaluation and validation was previously developed and in-house validated [181]. The organizers clearly stated that the standard operating procedure (SOP) was to be strictly followed by participating laboratories and any deviation from the method should be reported. The seven test items used in this exercise were all reference materials (either certified reference materials or test items of former IMEP proficiency tests) covering a broad range of matrices and concentrations. The mass fraction of iAs was not known for all the test items used. For this reason, five laboratories with recognised experience in the analysis of iAs were asked to analyse the test items using a method of their choice, different from the one being validated, in order to compare with the FI-HG-AAS method.

Thereby as an expert laboratory, we are requested to analyse iAs content in the seven test samples and we decided to apply method B to determine iAs content. Results provided by expert laboratories were generally in agreement between them and after rejection of outliers (**Table 4, Article XIII**) assigned values were established for iAs in all test materials. Our results are shown in **Table 18** and are summarised in **Figure 1** of **Article XIII** where our laboratory is

presented as "C5" and as can be observed, our iAs results were in agreement with the assigned values in all assayed test items. Our chromatograms are presented in **Figure 20** and as can be observed, inorganic arsenic was the major compound in rice (a), wheat (b), vegetable (d) samples meanwhile it was minor a compound in mussels (c) and fish matrices (f). Furthermore, as expected high iAs content was found in seaweed sample (e) (Hijiki, approx. 10 mg As kg^{-1}) (**Article XIII).** Additionally to our participation and to assess the trueness of our results, spiking experiments of iAs were performed by adding As(III) and As(V) standards to solid samples as described above. Satisfactory recoveries are obtained and values are shown in **Table 19.** Furthermore, some CRMs (or RMs) were analysed throughout the study to assess the accuracy and reliability of iAs results (**Table 20**).

Table 19. Inorganic arsenic recoveries in the test materials of IMEP-41

Samples	iAs Recovery (%)
Rice	102.0 ± 2.0
Wheat	101.2 ± 1.2
Mussels	97.6 ± 4.2
Cabbage	100.2 ± 1.2
Seaweed	101.4 ± 1.7
Fish	98.9 ± 1.0

Table 20. Inorganic arsenic content in CRM or RM. Concentrations are expressed as mg As kg^{-1} dry mass (mean ± U, n = 3).

Materials	Matrix	Inorganic arsenic	
		Measured value	Assigned/Certified values
ERM-BC211	Rice	0.121 ± 0.007	0.124 ± 0.011
NMIJ CRM 7503a	Rice	0.084 ± 0.001	0.084 ± 0.003 [a]
FAPAS-07129	Seaweed (Hijiki)	107.0 ± 1.7	102 ± 12.5 [b]
FAPAS-0792	Seaweed (Hijiki)	66.5 ± 1.6	67.2 ± 5.33 [c]

[a] NMIJ 7503a rice is certified in As species: As(III) = 71.1 μg As kg^{-1} and As(V) = 13.0 μg As kg^{-1} so the value for iAs is 84.10 μg As kg^{-1}. [b] Assigned value on FAPAS report 07129. [c] Assigned value on FAPAS report 0792

From the results of IMEP-41, the organizers concluded that 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. The proposed method can be used to monitor iAs in food and help providing more data on the fraction of As with the highest toxicity in the human diet **(Article XIII**, [173]). Specific comments and problems of the laboratories participating in the IMEP-41 PT are reported in the IRMM report [173].

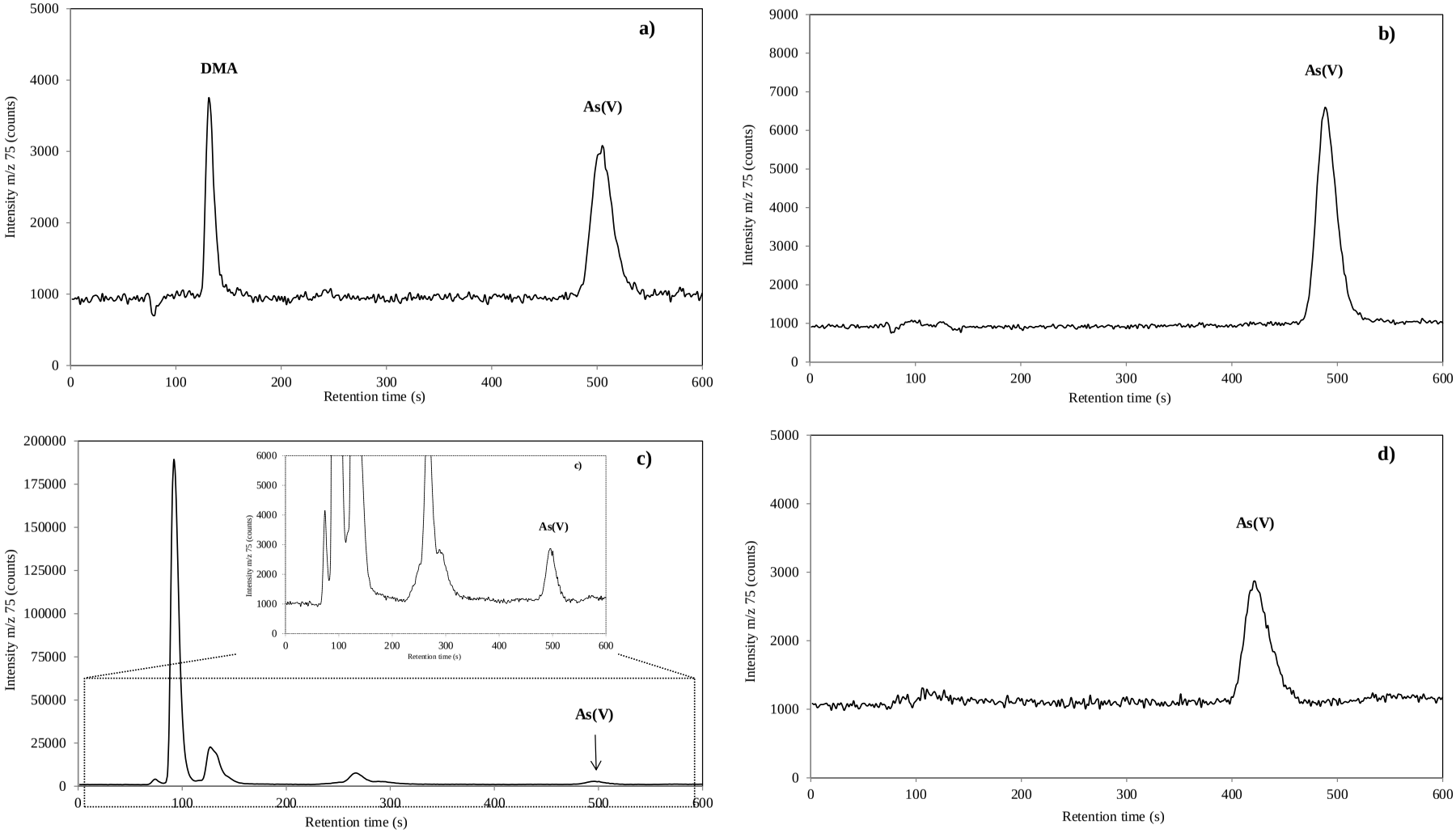


Figure 20. Anion exchange HPLC-ICPMS chromatograms obtained from the analysis of the test sample materials of IMEP-41: rice (a), wheat (b), mussels (c) and cabbage (d).

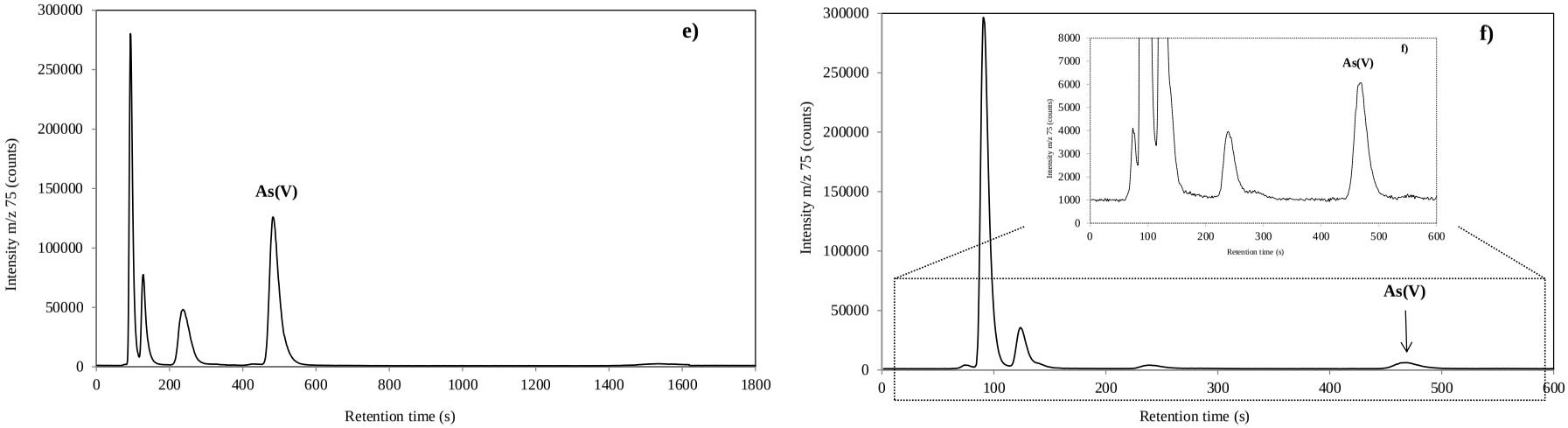


Figure 20 (continued). Anion exchange HPLC-ICPMS chromatograms obtained from the analysis of the test sample materials of IMEP-41: seaweed (e) and fish (f).

FAPAS round 07151

Furthermore, our method was tested participating in a PT organised by Central Science Laboratory-Food Analysis Performance Assessment Scheme (CSL-FAPAS) to determine tAs and iAs levels in rice [155]. The measured value for iAs content was satisfactory (**Table 18)** and % of recovery compared with an assigned reference value was acceptable: within ± 15%.

Certification studies of CRMs

Our research group participated in various certification studies organised by IRMM to certified mass fraction of arsenic species in CRMs. This collaboration project allowed us to corroborate the suitability of our developed methods for the determination arsenic species. We were requested to analyse the CRMs using methods of our choice and no further requirements were imposed regarding methodology. Test materials were storage following the conditions supplied by IRMM until analysis. For the determination of water content the procedures supplied by IRMM were strictly followed in each study. An accurate quality assessment was performed to assure the reliability of our results. In the following paragraphs, further details of our participation in these studies are presented and discussed. Specific information for each one are summarized in **Table 21** and arsenic speciation results are summarised in **Table 22.**

ERM-BC211 rice

We were requested to participate in the certification study of inorganic arsenic (as sum of arsenite and arsenate) and DMA mass fractions in ERM-BC211 rice material [156]. The certification was organised by IRMM and the starting material was purchased and supplied by the University of Aberdeen. The rice was milled, sieved, dried, homogenised, filled in vials by IRMM and sterilised. Homogeneity and stability studies were satisfactory performed prior the characterization study.

We participated in the characterization study of iAs and DMA contents using our method validated for determination of arsenic species in rice and cereal-based foods. Two bottles were received and analysed on two different days (one bottle/day/ performing three independent replicates per bottle (six replicates /measurand). As described above, an accurate QC assessment was performed during this study, i.e. calibration, instrumental drift, interferences, blanks, replicates and mass balances were evaluated. Furthermore, NIST SRM 1568a rice and NMIJ CRM 7503a rice were analysed to check the accuracy. Our tAs results are shown above in **Table 10** and DMA and iAs results for the characterization study are presented in **Table 22**.

Table 21. Participation in certification studies and stability tests of arsenic species in CRMs. Method B was applied to determine arsenic species content. All studies were organized by IRMM.

Certification of CRMs	**Description**	**Matrix**	**Sample**	**Target analyte**	**Analyte evaluated**	**Technical specifications**	**References**
ERM-BC211	*Certification studies*	Rice	2 bottles and 1 QC sample	tAs, iAs and DMA	iAs and DMA	Three independent measurements for each bottle on two different days (one bottle/day) (6 determinations/measurand).	
ERM-BC211	*Stability testing 2013*	Rice	4 bottles	tAs, iAs and DMA	tAs, iAs and DMA	Three independent measurements for each bottle under repeatability conditions (one analytical run) (12 determinations/measurand)	[156]
ERM-BC211	*Stability testing 2014*	Rice	4 bottles	tAs, iAs and DMA	tAs, iAs and DMA	Three independent measurements for each bottle under repeatability conditions (one analytical run) (12 determinations/measurand)	
BCR-627	*Stability testing 2014*	Tuna fish tissue	4 bottles	tAs, DMA and AB	tAs, DMA and AB	Three independent measurements for each bottle under repeatability conditions (one analytical run) (12 determinations/measurand)	[157, 158]
ERM-AC626	*Confirmation measurements*	Arsenobetaine solution	3 ampoules of AB and 1 ampoule of QC sample	tAs and AB	tAs and AB	Two independent measurements per ampoule divided into 3 days, one ampoule per day (6 determinations/measurand)	Unpublished results

Table 22. Summary of participation in certification studies of CRMs as external QC assessment for arsenic speciation analysis. Concentrations are expressed as mg As kg^{-1}, mean value ± U. Method B was applied to determine arsenic species content.

Certification of CRMs	**Sample**	**Inorganic arsenic**		**DMA**		**AB**		
		Measured value	**Assigned/ Certified value**	**Measured value**	**Assigned/ Certified value**	**Measured value**	**Assigned/Certified value**	**References**
ERM-BC211	Rice							
Characterization study		0.106 ± 0.008	0.124 ± 0.011	0.120 ± 0.009	0.119 ± 0.013	n.e [a]	n.e [a]	
Stability testing		0.123 ± 0.009	0.124 ± 0.011	0.121 ± 0.009	0.119 ± 0.013	n.e [a]	n.e [a]	[156]
Stability testing		0.123 ± 0.009	0.124 ± 0.011	0.124 ± 0.009	0.119 ± 0.013	n.e [a]	n.e [a]	
BCR-627	Tuna fish tissue							
Stability testing		n.e [a]	n.e [a]	0.153 ± 0.004	0.15 ± 0.02	3.8 ± 0.1	3.9 ± 0.2	[157, 158]
ERM-AC626	Arsenobetaine solution							
Confirmation measurements		n.e [a]	n.e [a]	n.e [a]	n.e [a]	100.4 ± 1.5	UR [b]	Unpublished results

[a] n.e. means not evaluated.

[b] Unpublished results

As an example a chromatogram of a rice test material is shown in **Figure 21**, and as can be observed, DMA and iAs species were predominant in rice test sample of ERM-BC211 and MA was found in minor proportions. Our results were considered acceptable compared with the certified values of ERM-BC211 rice.

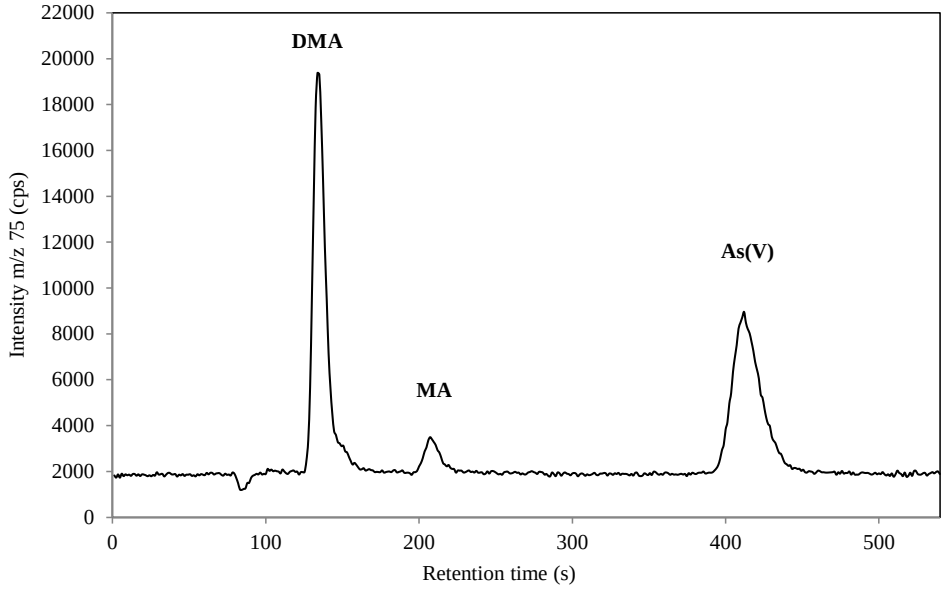


Figure 21. Chromatogram of rice test sample of ERM-BC211 obtained by anion exchange HPLC-ICPMS

Following we present a summary of the results reported by the participants in the characterisation study of the mass fraction of DMA and iAs in rice test material [156]. Eighteen laboratories were participated in the characterization study. After rejection of some results, the characterisation campaign resulted in 13 datasets for total arsenic, 13 datasets for arsenite/arsenate and 8 datasets for DMA. Regarding methods used for the characterization of iAs and DMA a variety of extraction procedures with different extractants was applied: HNO_3/H_2O_2, TFA, HNO_3, HCl, HCl/H_2O_2 and enzymatic digestion. Thereafter, detection and quantification was carried out *via* different analytical techniques (HPLC-ICPQMS, HPLC-AFS, HG-AAS). In the case of HG-AAS two different reduction protocols (HBr/hydrazine sulphate and KI/ascorbic acid) were applied.

Accepted results of the participants in the certification of DMA and iAs are shown in **Figure 22.** Average results with uncertainty bars as submitted by each individual lab are reported. Our laboratory is presented as "L13" and our results (dark circles) are presented in **Figure 22a** and **22b,** for DMA and iAs, respectively. Our data were accepted for the assignment of the certified values of DMA and iAs. The performance of the certification study was satisfactory and ERM-BC211 was certified for mass fractions of total arsenic, the sum of arsenite and arsenate DMA using an inter-laboratory comparison approach (EUR 25366 EN – 2012). Furthermore, we were selected to participate in further stability studies of ERM-BC211 material (2013, 2014) and the results were in agreement with the certified values (**Table 22)**.

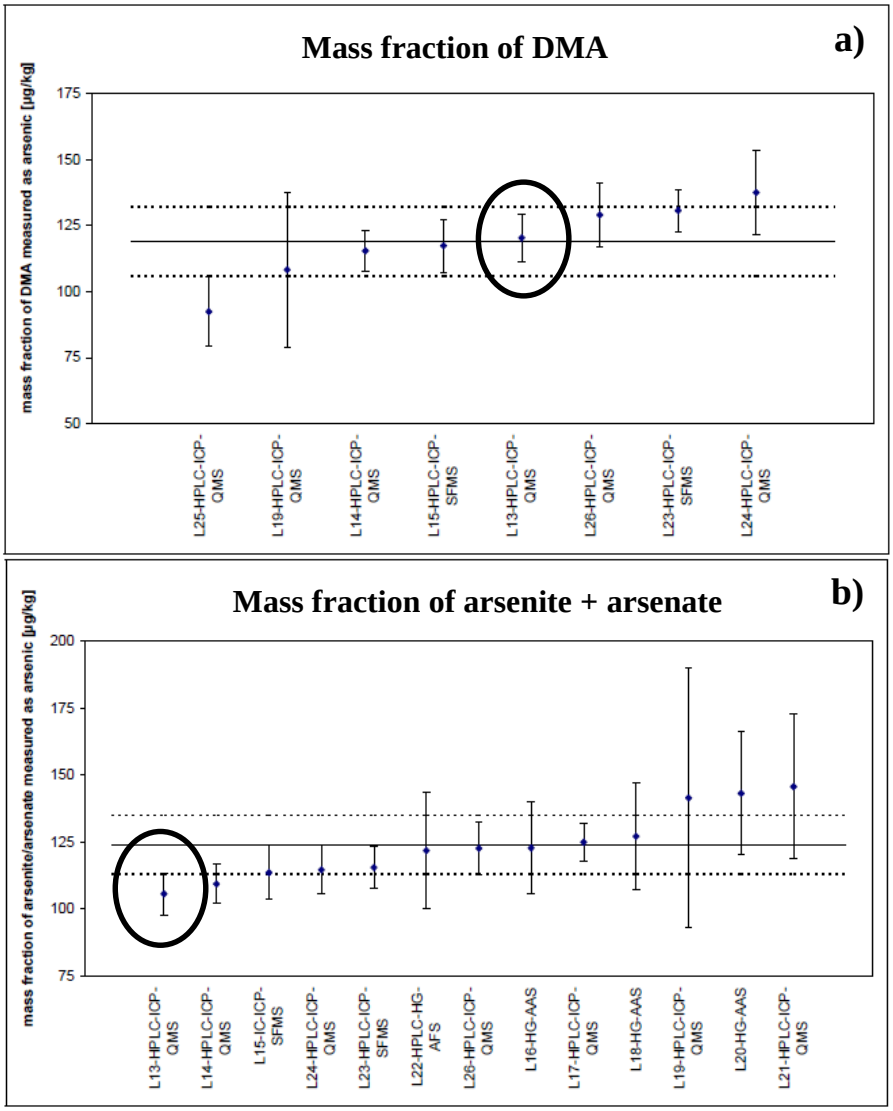


Figure 22. Results of the characterisation study for the mass fraction of DMA (a) and iAs (b) in rice test material (ERM-BC211) (adapted from [156]). Our laboratory is presented as "L13" (dark circles). Continuous line: certified value; dashed line: expanded uncertainty with k=2; results with uncertainty bars as submitted by each individual lab.

Stability testing of BCR-627 (tuna fish tissue)

We were selected to participate in the stability study of CRM BCR-627 tuna fish performed in 2014. The material was one of the first materials certified for As species and it was produced by IRMM in 1997 [157, 158] and was certified for tAs, DMA and AB contents.

We received four bottles of the CRM BCR-627 to participate in the stability testing of DMA, AB and tAs contents. Following the instructions of IRMM, determinations were performed under repeatability conditions, i.e. in one analytical run. These determinations have equally distributed over 4 bottles, i.e. three independent replicates per bottle, performing 12 determinations for measurand. Our developed speciation method (B) was applied to determine AB and DMA concentrations in the BCR-627 material. As described above, an accurate QC

assessment was performed during this study, i.e. calibration; instrumental drift, interferences, blanks, replicates and mass balances were evaluated. Furthermore, DORM-4 Fish protein and DOLT-4 dogfish liver were analysed throughout the study for analytical quality control purposes. Furthermore, to evaluate the accuracy of AB measurements, TORT-3 lobster hepatopancreas which is certified for arsenobetaine content was analysed throughout the stability study of BCR-627. Measured value of arsenobetaine in TORT-3 was in agreement with the certified value: 53.2 ± 1.0 vs 54.9 ± 2.5 mg As kg^{-1} on dry mass (mean ± SD). Total arsenic results are shown above in **Table 10** and measured AB and DMA results for the stability testing are shown in **Table 22** and were considered acceptable compared with the certified values of BCR-627 demonstrating the validity and reliability of the applied method. As an example, two chromatograms of the test material are shown in **Figure 23**. As can be observed **in Figure 23a** DMA was found in minor proportions accounting of 3% of the tAs and AB was predominant (**Figure 23b)** (accounted of 80% of the tAs) and also other minor cationic species were found such as TMAO, AC and two unknown species (UC-A and UC-B).

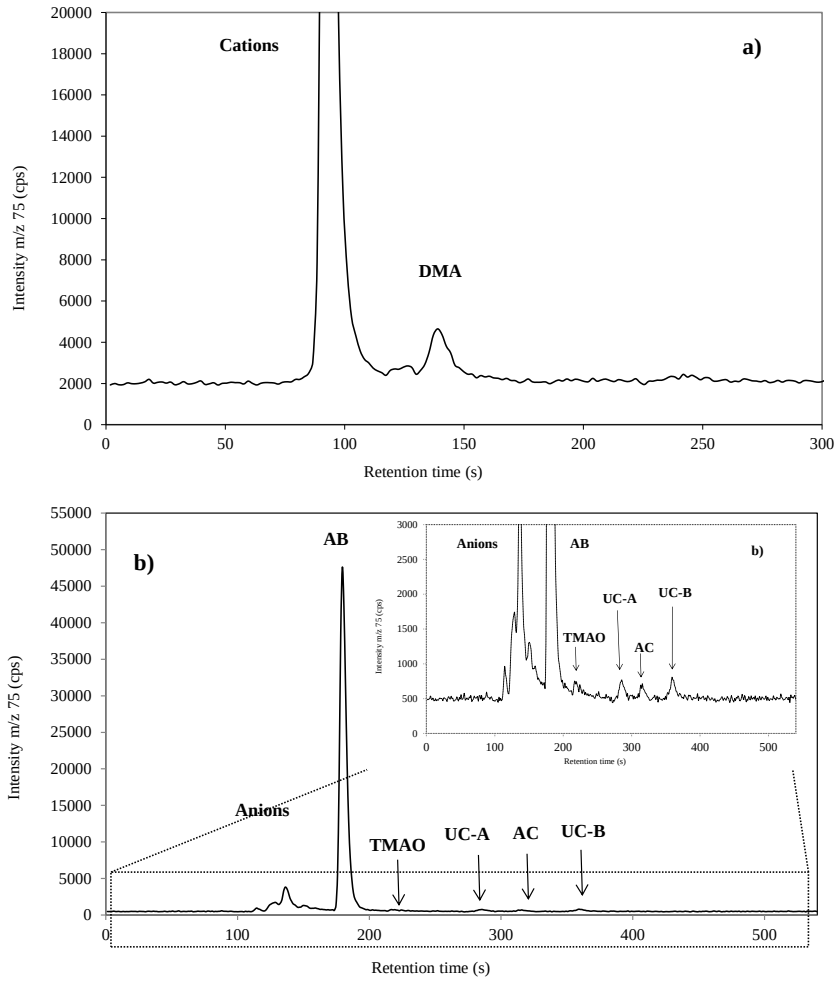


Figure 23. Chromatograms of BCR-627 test sample from anion exchange column (a) and cation exchange column (b) by HPLC-ICPMS

Confirmation measurements of ERM-AC626 arsenobetaine solution

The IRMM is developing a certified reference material ERM-AC626 "Arsenobetaine in water" which will replace the former BCR-626 (currently not available). This material will support the analysis of the mass fraction of arsenobetaine allowing harmonization and quality assurance of measurements in this field. The starting material used was synthesized by an external collaborator and the material was prepared by water dissolution of solid arsenobetaine which purity was carefully determined. Thereafter, the obtained raw material underwent two independent purity determinations, the first approach was carried out by quantitative nuclear magnetic resonance (qNMR) spectroscopy. The second approach was based on impurity measurements, with special emphasis on the impurities likely to occur based on the route of synthesis. By doing this, analytical techniques such as gas chromatography-flame ionization detection (GC-FID), gas chromatography-mass spectrometry (GC-MS), HPLC-ICPMS, headspace-gas chromatography-mass spectrometry (HS-GC-MS), ion chromatography (IC), Karl Fischer titration (KFT), ICP-QMS and ICP-SFMS were applied. Currently, the production of this material is in its final phase and will be available from IRMM in the near future.

In 2014, the IRMM selected some experienced laboratories that can perform the analyses for one or more of the studies shown above. Therefore, we were selected to perform the confirmation measurements of ERM-AC626 arsenobetaine solution. Following the instructions of IRMM, this confirmation analysis was done by different methods with the aim to determine: arsenobetaine; all other arsenic species and total arsenic. Three amber glass ampoules containing 1 mL water solution of arsenobetaine (ERM-AC626) together with 1 ampoule of quality control sample (QCS) were received. Following the IRMM guidelines, we strictly applied the technical specifications for confirmation measurements of ERM-AC626. Briefly, the samples should be stored at 18 °C. Just before analysis, they should be kept at room temperature to equilibrate. An ampoule, before being opened, should be shaken by turning upside down for 30 s to ensure homogenisation of the material. Two independent analyses per ampoule (two replicates per each ampoule, also for the QCS) of the mass fraction of AB, other arsenic species and tAs should be performed. Each replicate should include the entire procedure of sample preparation and detection. The measurements should be divided into 3 days-one ampoule per day. Furthermore, the use of BCR-626 as a calibrant or quality control sample was not allowed. Finally, no particular quantification method is prescribed, but the measurement method must be validated and more than one method can be applied.

An accurate QC assessment was performed during this confirmation study following our internal QC protocol described above. So, blanks, QC standard solutions and calibrants were included in our HPLC-ICPMS run. Furthermore, all stock solutions and test samples and further diluted solutions were prepared daily and were performed by weight. The arsenobetaine solution, CRM 7901-a from NMIJ was used as a calibrant. An AB solution (Fluka) prepared from $(CH_3)_3As^+CH_2COO^-$ dissolved in water, was as used as internal QC in AB measurements. Furthermore, repeatability, reproducibility and trueness were evaluated throughout the study. No sample treatment was required prior to the measurement of the measurands since test material was a water solution of AB. Therefore, tAs concentration was directly determined by ICPMS analysis (tAs results are presented above in **Table 10**) and AB and arsenic species were

determined by both cation and anion exchange HPLC-ICPMS analysis and applying our arsenic speciation method (HPLC-ICPMS conditions are reported in **Article IV and VI).** AB content is shown in **Table 22** and as an example a chromatogram of the test material is presented in **Figure 24.** As can be seen only AB was found in the ERM-AC626 sample and other arsenic species were not detected.

According to IRMM, the arsenobetaine synthesized is pure enough for the production of a CRM. Since the material is still developing by IRMM, results of the certification study of ERM-AC626 cannot be presented here. The ERM-AC626, certified for the mass fraction of arsenobetaine in water, is expected to be available in the near future (Boertz, 2015, poster Winter).

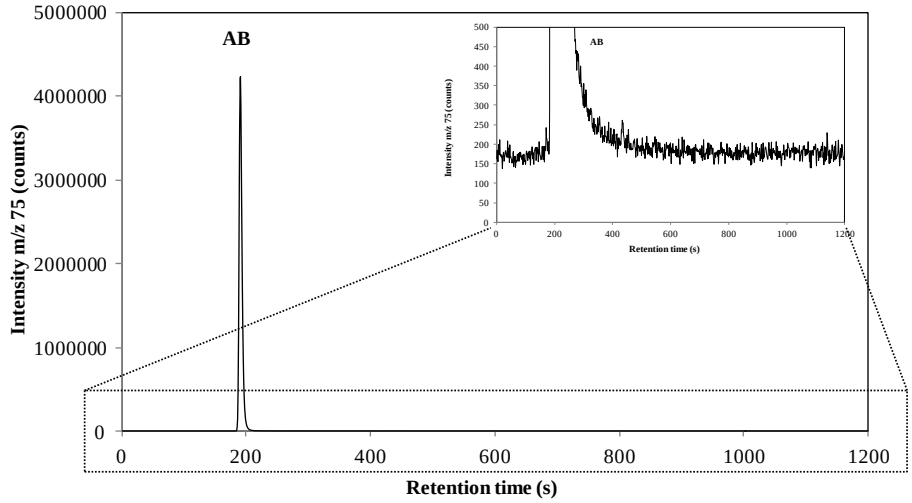


Figure 24. Chromatogram of the ERM-AC626 test sample from and cation exchange column by HPLC-ICPMS

6.3 Collaborative trial on the determination of inorganic arsenic in foodstuffs of marine and plant origin by HPLC-ICPMS (CEN TC275/WG10)

6.3.1 Project background

In 2010 a tender for a project with the aim to develop a European standard (EN) method for the determination of inorganic arsenic in foodstuffs of marine and plant origin was set up by DIN on behalf of CEN TC275. Several proposed methods were discussed within the CEN TC 275/WG10 working group, that finally selected the proposal from Technical University of Denmark (DTU) and Dr. Jens J. Sloth was assigned as project leader. The selected proposal was based on a method for the determination of iAs in foodstuffs of marine and plant origin by HPLC-ICPMS technique. The method was developed and validated at DTU Food during the period 2012-2013. A collaborative trial was conducted in 2013 with participants from 15 different laboratories from 10 different countries to evaluate the performance characteristics of the method for the determination of iAs in foodstuffs of marine and plant origin [185]. Our

research group was invited to participate in this international collaborative study. The outcome of this collaborative trial are presented and discussed in this section.

6.3.2 Participation in the collaborative trial

We participated in this collaborative study following the instructions of the method procedure which was supplied by organization. The method was strictly followed and any deviation from the instruction or method protocol was reported to the organizers. Two bottles of six different sample materials were received: white rice, wholemeal rice, leek powder, mussel powder, fish muscle and seaweed (Table 2, [185]). The samples included several RMs or CRMs and proficiency test materials, for which suitable homogeneity already has been verified by the supplier. For each one of these materials a certified value for iAs has been established and this value was used to evaluate the accuracy of the methodology evaluated in this collaborative trial. Furthermore, one bottle with a standard solution containing a mixture of 3 arsenic species: AB; MA and As(V) in a hydrochloric medium was received. Samples were stored in a dark and cold place (at maximum 4 ºC) until analysis.

Method description

The method principles are based on waterbath extraction followed by selective determination of iAs by HPLC-ICPMS [185]. Briefly, a representative test portion of the sample is treated with an extraction solution of dilute nitric acid and hydrogen peroxide in a waterbath at 90˚C for 60 min. Hereby the analytes of interest are extracted into solution and As(III) is oxidised to As(V). The inorganic arsenic is subsequently determined as As(V) by a method based on anion-exchange HPLC coupled ICPMS (HPLC-ICPMS). External calibration with solvent matrix-matched standards is used for quantification of the amount of inorganic arsenic.

Following the instructions of organisers, sample materials were analysed in duplicate on two separate days following the method procedure. To minimise the contamination, all apparatus and equipment that come into direct contact with the sample and the solutions were carefully pre-treated. The use of glassware was avoided, since this may cause contamination with arsenate. A specific test portion size was used for each test material. Sample was wetted sufficiently prior to putting it in the waterbath: tubes were thoroughly shaken and sample and extractant solution were in contact for an extended time period, i.e. overnight prior to the waterbath extraction step. Following the waterbath extraction step the tubes were centrifuged. The supernatant was then transferred to clean containers. All sample extracts were filtered and transferred to HPLC vials prior to analysis.

According to method procedure, a mobile phase of ammonium carbonate in 3% methanol at pH 10.3 must be used but the optimal concentration of ammonium carbonate in the mobile phase depend on the analytical column used (e.g. brand, particle size and dimensions). The concentration of ammonia carbonate may be optimised and should be decided by the participant without compromising the criteria for sufficient resolution of the arsenate peak. Thus, the nearest peak in the chromatogram should be separated from the analyte peak by at least one full peak width at 10% of the analyte peak height. As a guideline the minimum acceptable retention time for the analyte is twice the retention time corresponding to the void

volume of the column. The method procedure recommended the use of 50mM ammonium carbonate in 3% methanol at pH 10.3 to obtain a satisfactory resolution between MA and As(V) as well as between As(V) and Cl^-. If resolution is inadequate, chromatographic conditions should be optimised, e.g. by changing the mobile phase concentration or the mobile phase flow rate.

A strong anion exchange column (SAX), suitable for selective separation of As(V) from other arsenic compounds present in the sample extracts was used. Sample and reagent blank solutions were analysed by anion exchange HPLC-ICPMS. Arsenic is evaluated at a mass/charge ratio (m/z) of 75 and m/z 35 was also monitored to check chloride interference. The retention time of As(V) was identified from the analysis of the calibration standard solutions and the concentration of iAs in the test solutions was calculated using the calibration curve. Finally, dry matter content was determined as specified: oven drying of two portions of minimum 0.2 g at 103 ± 2°C until constant mass, and results were corrected in order to report them in mg As kg^{-1} dry matter as iAs with at least 3 significant figures.

Optimization HPLC-ICPMS of conditions

Taking all this into account and prior to analysis, chromatographic conditions for HPLC-ICPMS analysis were optimized. Using an anion exchange column (Hamilton PRP-X100, 250 mm x 4.1 mm, 10 µm), we tested several concentrations of ammonium carbonate (20, 30, 40 and 50 mM) at various flow rates (0.7, 1.0 and 1.5) to optimize the chromatographic separation of arsenic species and Cl^- interference. For this, the standard solution (CEN –sample 7) was used since contained a mixture of three arsenic species: AB; MA and As(V) and HCl. This mixture was diluted 10 fold in the extraction solvent and was analysed to obtain a satisfactory resolution between MA and As(V) as well as between As(V) and Cl^-. After several assays, the optimal conditions without compromising the criteria for sufficient resolution of the arsenate peak were: 20mM ammonium carbonate in 3% methanol at pH 10.3, flow rate at 1.5 mL/min and injection volume of 50 µL. Applying the optimised mobile phase conditions and as example, a chromatogram of the CEN standard solution is shown in **Figure 25.** As observed, AB eluted at void volume of the chromatographic system and a satisfactory resolution between MA and As(V) as well as between As(V) and Cl^- was achieved using the optimized conditions (**Figure 25)**.

No further requirements were imposed regarding the HPLC and ICPMS operating conditions. Therefore, we applied similar ICPMS conditions than those used in the developed speciation methods in the present thesis and which are reported in **Articles IV and VI**. Briefly, ICPMS operated in standard mode without using gas in the collision cell, and the ion intensities at m/75 and m/35 were monitored. Furthermore, due to the use of an organic solvent in the mobile phase (3% MeOH), a low spray chamber temperature (2ºC) was applied to maintain suitable plasma conditions. Prior to analysis, HPLC was equilibrated by turning on the mobile phase flow in advance and ICPMS was tuned to maximise ion signals and to ensure sufficiently low levels of oxides, doubly-charged ions.

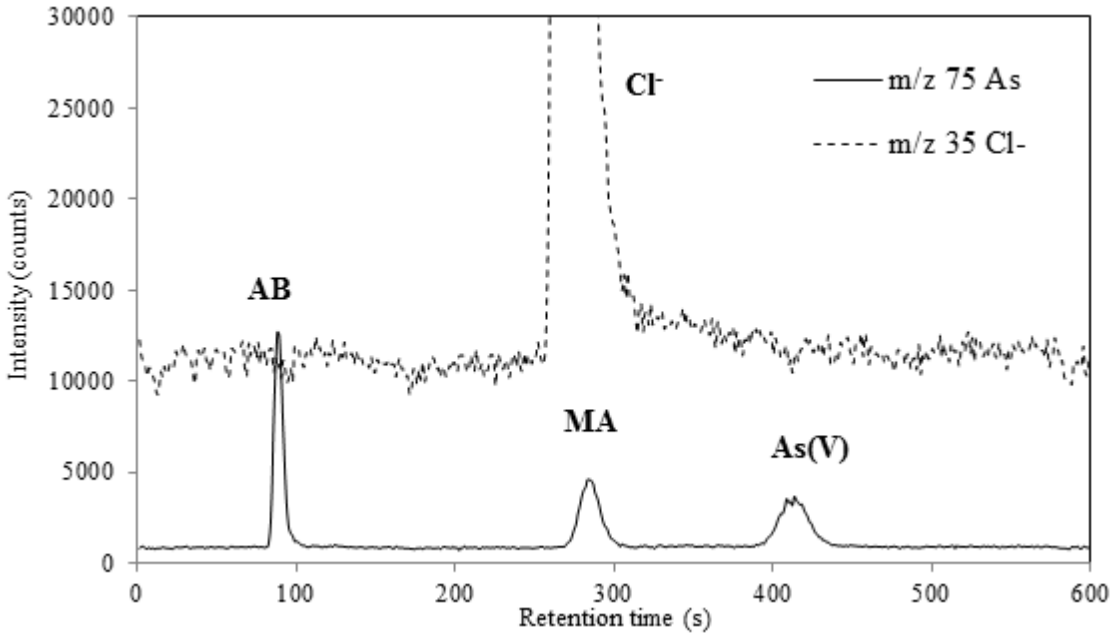


Figure 25. Chromatogram of standard solution (CEN sample 7) containing AB, MA, As(V) and Cl^- from anion exchange by HPLC-ICPMS. Continuous line shows m/z 75As and dotted line m/z $35Cl^-$

Results

Once the HPLC-ICPMS conditions were optimised, test materials were analysed following the method procedure [185]. We determined inorganic arsenic as As(V) and quantified by matrix-matched external calibration. We prepared an arsenate calibration curve ranged from 0 to 10 µg As L^{-1} (five calibration points) and extracts were diluted with the extraction solution when needed. No interferences were identified throughout the application of the method. For our internal QC, three rice CRMs (SRM 1568a Rice Flour, NMIJ CRM 7503a White Rice Flour and ERM-BC211 Rice) were analysed throughout the study to assess the accuracy and the reliability of iAs results. To control the stability of the instrument sensitivity, the standards of the calibration curve were run before and after each sample series. Moreover, sample solutions were analysed in batches including internal quality control, such as a standard solution every six samples and also at the end of the sequence, to monitor instrument drift. Furthermore, limit of detection for arsenate was estimated as 0.057 µg As L^{-1}.

Our results of the collaboration study are shown in **Table 23** and were in agreement compared with the mean values obtained from all the laboratories participants. Chromatograms obtained from the analysis of the six test materials analysed in the collaborative trial are shown in **Figure 26**: white rice (a), wholemeal rice (b), leek (c), blue mussel (d), fish muscle (e) and seaweed (f). Inorganic arsenic is eluting as As (V) (arsenate) at a retention time of approximately 400s. The other peaks in the chromatograms represent organoarsenic compounds (**Figure 26)**.

Table 23. Measured results (expressed in mg As kg^{-1}) of inorganic arsenic content and dry matter in the sample materials of the collaborative trial.

CEN iAs Food Sample	Bottle no	Dry matter (%)	Inorganic arsenic		
			Measured value (replicate 1)	Measured value (replicate 2)	Assigned value [a]
White rice	67	11.6	0.067	0.065	0.073 ± 0.008
Wholemeal rice	19	9.2	0.431	0.443	0.47 ± 0.043
Leek	10	10.6	0.087	0.077	0.086 ± 0.012
Blue mussel	18	6.6	0.317	0.313	0.33 ± 0.049
Fish muscle	35	10.4	0.288	0.280	0.27 ± 0.038
Seaweed	7	8.9	9.315	10.158	10.3 ± 1.2
Standard solution	13	-	16.029 (µg As L^{-1})	17.117 (µg AsL^{-1})	
Procedural blank	-	-	<LOD	<LOD	

[a] Mean value of all reported results ± Reproducibility standard deviation (mg As kg^{-1})

6.3.3 Overall evaluation of the collaborative trial

A summary of the results reported by the participants in the collaborative trial on the determination of iAs in foodstuffs of marine and plant origin by HPLC-ICPMS is presented below. The method was tested in a collaborative trial with 16 participating laboratories from 10 different countries (Denmark, Sweden, Norway, Belgium, Germany, France, Spain, Switzerland, UK and USA). The reported results from the participating laboratories can be found in Annex 8 of the Report [185]. An overview of the ICPMS instrumentation used as well as details regarding the chromatography (column type and dimensions, injection volume, mobile phase concentration and flow rate) is reported [185]. Various types of ICPMS instruments and three different anion-exchange columns have been used. A wide range of different injection volumes are reported from 5 -100 µL as well as variation in mobile phase concentration (20–180 mM) and flow rates 0.8 – 1.5 ml min^{-1}.

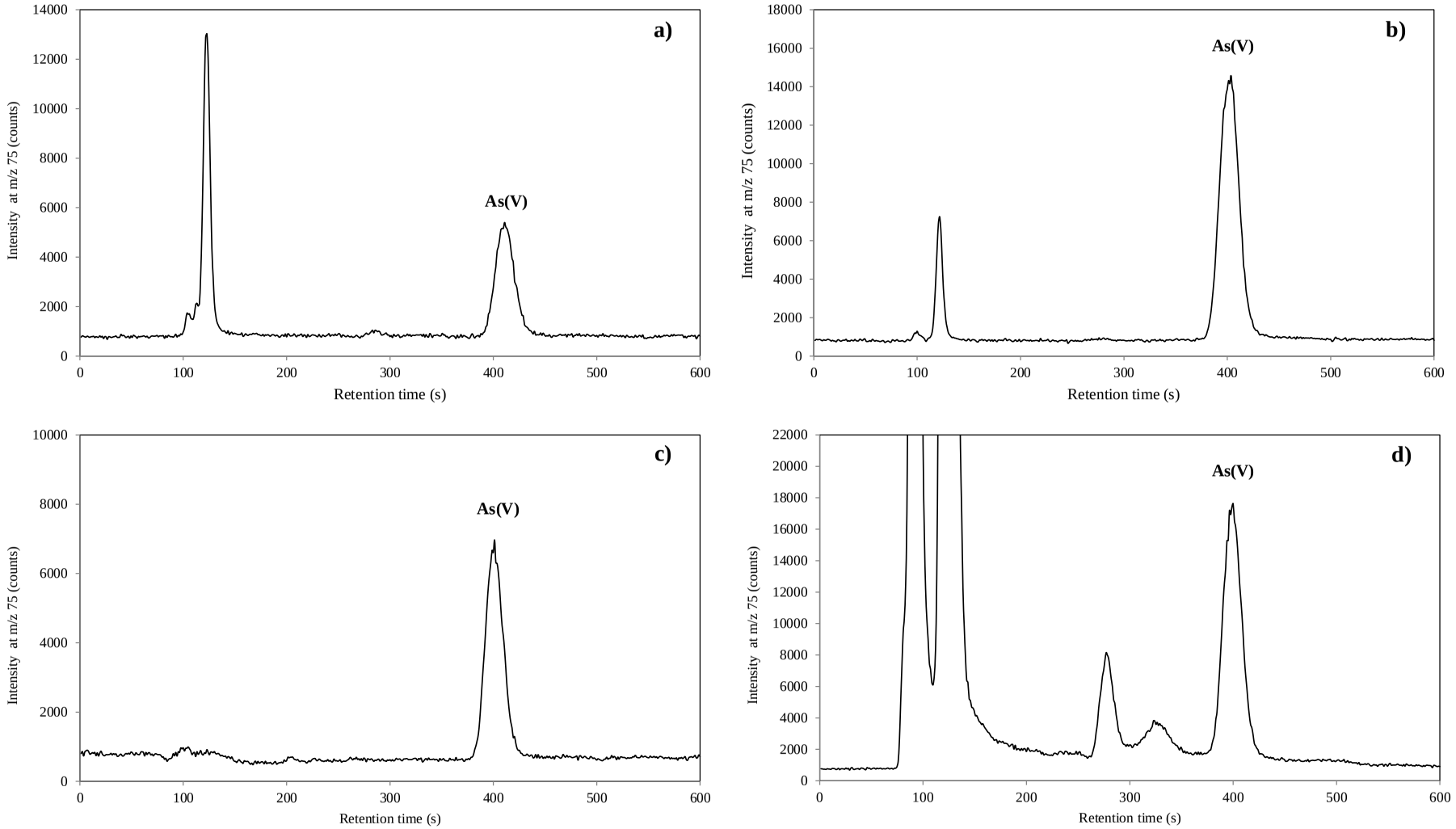


Figure 26. Chromatograms obtained from the analysis of the six sample materials analysed in the collaborative trial: white rice (a), wholemeal rice (b), leek (c) and blue mussel (d) by HPLC-ICPMS. Inorganic arsenic is eluting as As (V) (arsenate) at a retention time of approximately 400s. The other peaks in the chromatograms represent other arsenic compounds.

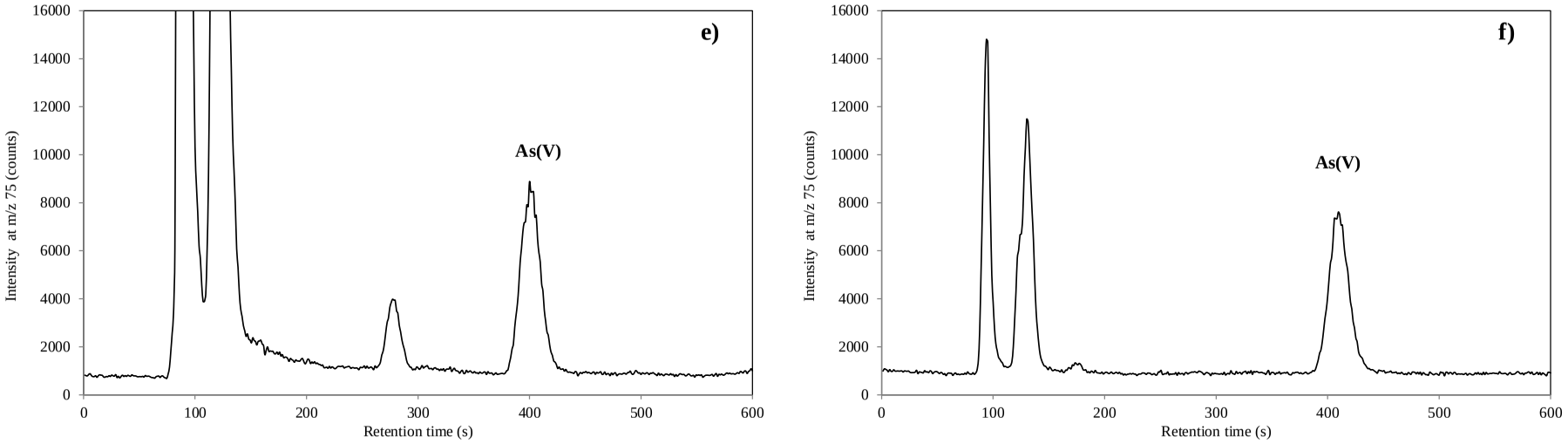


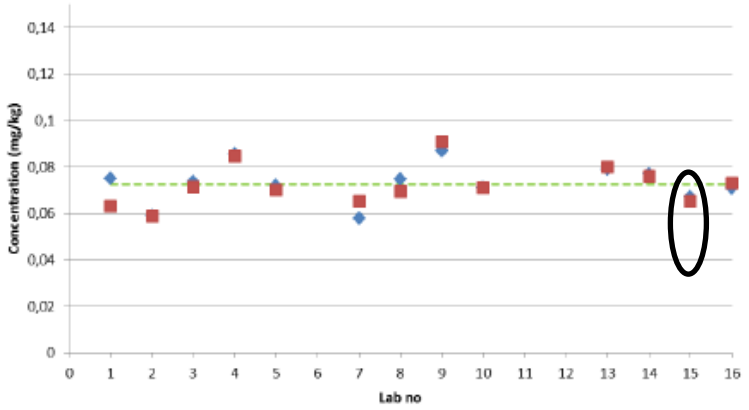
Figure 26 (continued). Chromatograms obtained from the analysis of the six sample materials analysed in the collaborative trial: fish muscle (e) and seaweed (dilution 1/50) (f) by HPLC-ICPMS. Inorganic arsenic is eluting as As (V) (arsenate) at a retention time of approximately 400s. The other peaks in the chromatograms represent organoarsenic compounds.

Sixteen laboratories signed up to participate in the collaborative trial. One laboratory did not report any results and two laboratories reported deviations from the method procedure (use a different extraction solution and different mobile phase than stated in the method procedure) and were judged as non-compliant [185]. Their results were excluded from the statistical analysis of the data from the collaborative trial. Results from the remaining 13 laboratories were subjected to statistical analysis. First step was to identify outliers and stragglers by the Cochran and Grubbs tests. Following exclusion of outlying results the remaining measurements were used to evaluate relevant performance characteristics related to trueness and precision of the method under validation and an overview of the method performance characteristics is presented (Table 5 [185].

Plots of results from participant laboratories are shown in **Figure 27.** Results are shown as were submitted by each individual lab, two replicates for test sample: represented by rhombus and squares (**Figure 27**). Our laboratory is represented as "L15" and our results (dark circles) were in agreement with the assigned values for each one of the test materials. The organizers concluded that a method for the determination of iAs in foodstuffs of marine and plant origin was developed. The method performance characteristics were assessed in a collaborative trial on six different food samples within the concentration range of 0.073 – 10.3 mg kg^{-1}. It was also concluded that the proposed method was suitable for the quantitative analysis of iAs in foodstuffs of marine and plant origin.

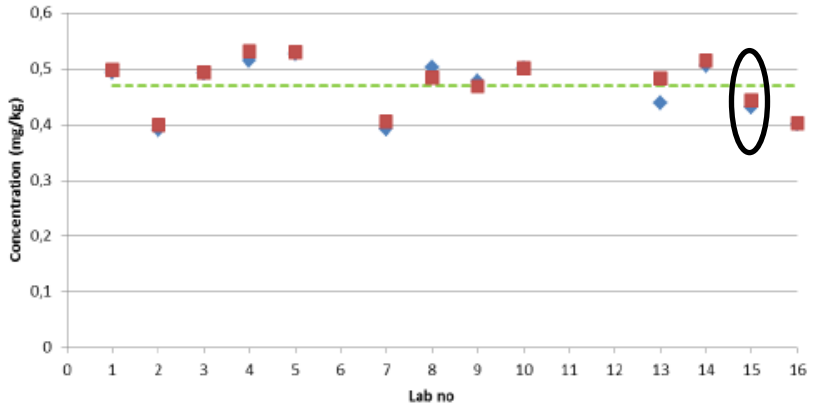
The results from the collaborative trial were presented and discussed by the project leader at the CEN TC275/WG10 meeting in Paris (October 2013) and comments were received from the expert group members of WG10. The report of the collaborative trial [185] was prepared in January-February 2014 and is recently published in August 2015. To date, the method is in the current status of "Technical Review" and the Standard: "BS EN C16802/WG10 Foodstuffs" is developing.

Sample 1 White rice (mean value +/- u_{obs} = 0.073 + /- 0.006 mg/kg)



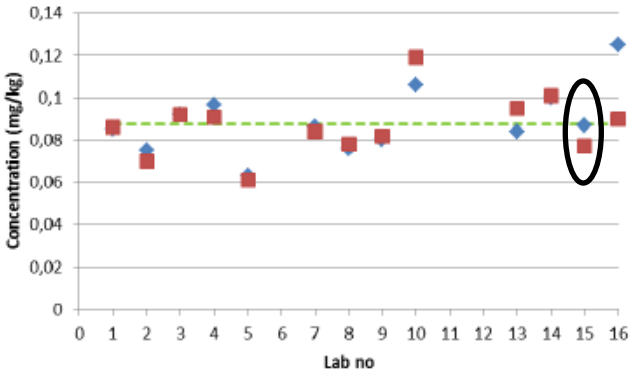
L01 is a Cochran straggler

Sample 2 Wholemeal rice (mean value +/- u_{obs} = 0.47 +/- 0.05 mg/kg)



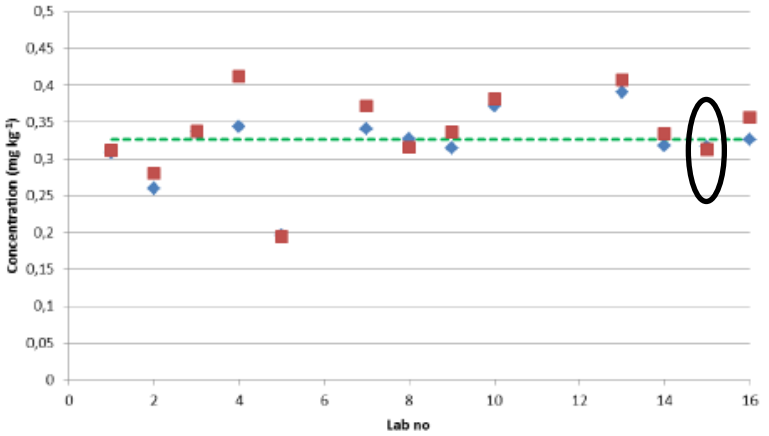
L13 is a Cochran outlier

Sample 3 Leek (mean value +/- u_{obs} = 0.086 +/- 0.013 mg/kg)



L16 is a Cochran outlier

Sample 4 Blue mussel (mean value +/- u_{obs} = 0.33 +/- 0.05 mg/kg)



L05 is a Grubbs straggler

Figure 27. Plots of results from participant laboratories (adapted from Repor [185]). Our laboratory is represented as "L15" (dark circles)

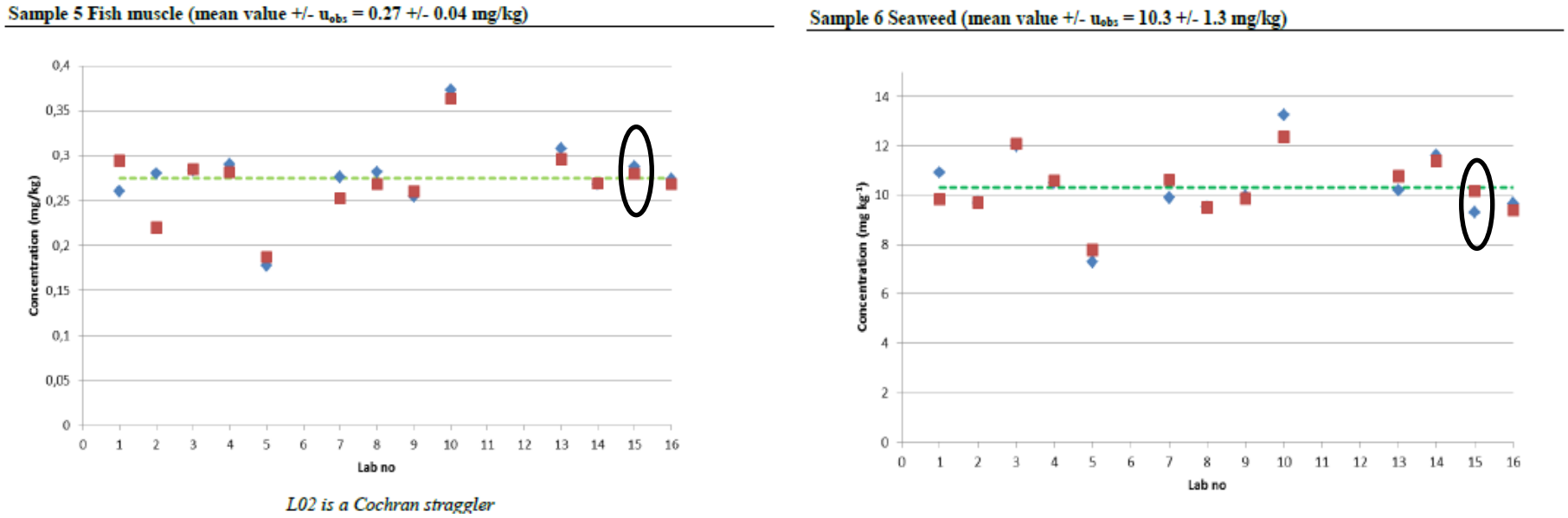


Figure 27 (continued). Plots of results from participant laboratories (adapted from Report [185]). Our laboratory is represented as "L15" (dark circles)

6.4 Arsenic bioaccessibility methods

A discussion of the selection and development of the applied method to determine bioaccessible arsenic (**Table 6**, method F**)** is presented in this section. Furthermore, as a method quality assurance, the main QC parameters are discussed and evaluated in the following paragraphs.

6.4.1 PBET method

As commented in the Introduction section, several *in vitro* studies have been conducted to estimate arsenic bioaccessibility (BA) in food items involving the conditions similar to those found in the human body during digestion [125–127]. Our research group has developed an *in vitro* physiologically based extraction test (PBET) method for selenium bioaccessibility [186] based on the previously described [187]. The method was satisfactory applied in cabbage samples showing the reliability of the approach [186]. Thus, in the present thesis, this PBET method was selected to estimate arsenic bioaccessibility. We aimed to apply this *in vitro* PBET method to estimate the bioaccessible fraction of arsenic in raw and cooked mushrooms as a previous step of bioavailability, as indirect estimation of arsenic bioavailability. The suitability of this method was evaluated in **Article IX** and the scheme of this *in vitro* PBET method is shown in **Figure 28.**

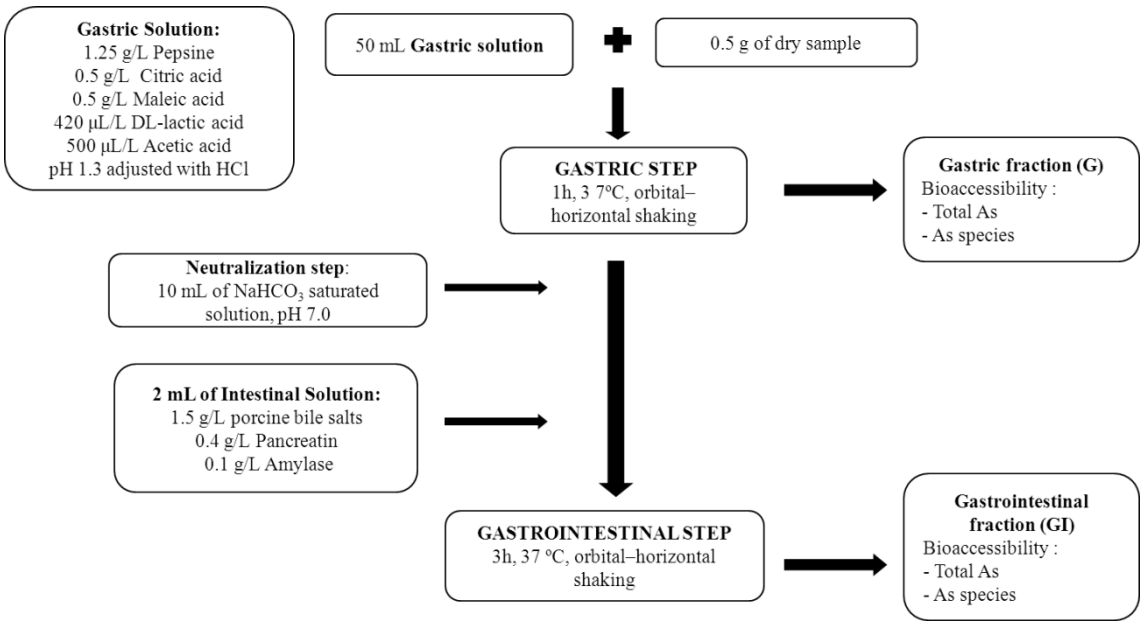


Figure 28. Scheme of the applied *in vitro* PBET method

Ruby et al. [187] proposed that the PBET method process takes place in two or three steps: saliva, stomach and intestine. The PBET method applied in this thesis was carried out in two stages; gastric (G) and gastro+intestinal (GI) simulating stomach and intestine conditions, respectively. Few modifications were made in relation to the previous developed in our research group [186]. The method was carried out in two stages. Gastric digestion (G) was simulated by

adding pepsin, citric acid, maleic acid, DL-lactic acid and acetic acid at pH 1.3 and 37 ºC for 60 minutes. Intestinal digestion was performed by adding the intestinal enzymes to the gastric digest (pancreatin, amylase and bile salts) at pH 7 and 37 ºC for 3 hours (**Figure 28**). Solution aliquots were separated at each stage for analysis, yielding two solutions per sample; the G and GI solutions. Arsenic bioaccessible content in both G and GI fractions was determined by ICPMS.

6.4.2 Analytical quality assurance

A rigorous quality assurance programme was conducted to evaluate suitability of the *in vitro* PBET method.

Internal Quality Control

Several quality control parameters were evaluated in the *in vitro* PBET method: type of calibration, use of internal QC; use of internal standard; check instrumental drift; analysis of blanks; analysis of CRMs; check possible interferences; sample replicates and spike recovery. The main internal QC parameters evaluated in the present thesis for bioaccessible arsenic content are summarised in **Table 24**.

Arsenic bioaccessible content in both G and GI fractions was determined by ICPMS measuring mass at m/z 75 and specific ICPMS conditions were presented in **Article IX.** As indicated before, the interference of $[^{40}Ar^{35}Cl]^+$ could interfere with arsenic determination at m/z ^{75}As in samples with significant amounts of chlorine. Therefore, helium was used as a collision gas to remove this interference in the octopole reaction system (ORS). Commercially available standards were prepared daily by dilution of a standard stock solution traceable to NIST with a certified concentration of 1001 ± 5 mg As L^{-1}. To minimise matrix effects, bioaccessible arsenic content in the gastric (G) and gastrointestinal (GI) fractions was quantified by means of a standard addition curve. Standard addition curves ranged from 0.10 to 5.0 μg As L^{-1} prepared in 2% HNO_3 for the commercially available standards. A solution of 20 μg As L^{-1} of ^{9}Be, ^{103}Rh and ^{205}Tl was used as an internal standard to monitor instrumental drift and matrix effects. In case of arsenic measurements, ^{103}Rh was used. Each sample preparation was performed and analysed in triplicate to eliminate batch-specific error and to monitor repeatability. To verify the lack of contamination in the reagents of the PBET method or during the preparation of samples, blanks were analysed together with samples. To assess instrumental response and run quality, QC standard solutions from different source of standards were measured after every some samples. Furthermore, blank spiked solutions of G and GI fractions were also analysed.

Table 24. Summary of internal QC parameters evaluated for PBET method.

Quality control parameter	Information	Frequency	Quality criteria
Calibration	NIST traceable standards. Standard addition method	Before and at the end of the each sample series	Quantification of arsenic calibration standards with other standard or against a primary standard
Instrumental drift	QC standard solutions at two concentrations levels	Post-calibration, every ten samples and at the end of the run	Values within 90 and 110% of the expected value
Blank	Reagents blanks, G and GI fractions	One for each batch of samples	Blank values<LOQ
Internal standard	^{103}Rh in standard mode	Added on-line to sample and standard solutions	Values within 80 and 120% of the target value
CRMs	ERM-BC211 rice and WEPAL-IPE-120 *A .bisporus* [a]	One for each batch of samples	Values within 85 and 115% of the established value
Spike recovery	Blank spiked solutions (G and GI fractions)	One for each batch of samples	Values within 85 and 115% of the theoretical spiked standard value
Replicates	Samples preparation was performed in triplicate	All samples analysed in triplicates	Acceptable if RSD between replicates <10%

[a] No CRMS are available for arsenic bioaccessible content. ERM-BC211 rice is certified for tAs, DMA and iAs mass fractions and tAs is reported as assigned value in WEPAL-IPE-120 *A .bisporus.*

Evaluation of validation parameters

Detection and quantification limits were calculated as three times the standard deviation and ten times the standard deviation signal of ten digestion blanks for the PBET method, G and GI fractions. Blank results in the bioaccessibility fractions analyses indicated more uncertainty in the G and GI extracts than in microwave digested samples for tAs determination, probably attributable to matrix components (e.g., chloride and enzymes). However, LOD and LOQ for G and GI fractions were suitable to determine arsenic bioaccessible in the assayed samples.

Although numerous CRMs exist for tAs content, no CRMs are commercially available for bioaccessible arsenic content. Therefore, to evaluate the PBET method we selected two of the RMs available for tAs: WEPAL IPE-120 *A. bisporus* and ERM-BC211 rice. These materials were extracted with the PBET method to control the bioaccessibility fractions throughout the study. Repeatability (%) of the PBET method was assessed analysing this materials and was expressed as relative standard deviation (RSD) of six replicates (n = 6) obtained in one day and by the same analyst. Acceptable values (%RSD) were obtained (**Article IX**) compared to other

bioaccessibility studies [135, 188]. Furthermore an as our internal QC, control limits for bioaccessible arsenic in these materials were established and the results for real samples were only accepted when RM values were 85–115% of the expected established value (**Article IX**). Since no CRMs are commercially available for bioaccessible arsenic content, a common practice within the scientific community to evaluate the accuracy without a certified bioaccessible As content is to analyse a CRM and compare the data obtained with data reported in the literature by different researchers. In this way and for comparison purposes, several CRMs have been analysed in studies focused on arsenic bioaccessibility [126, 189]. To date, no As bioaccessibility results RMs have been found in the literature on the analysed and therefore the present results cannot be compared.

A satisfactory performance of QC/QA assurance was obtained for the evaluated PBET method concluding that the method is suitable for the estimation of arsenic bioaccessibility in mushrooms. Some validation parameters were established and acceptable results were obtained according to acceptance criteria and also comparing with other bioaccessibility studies reported in the literature. Furthermore, other validation parameters such as intermediate precision, trueness and expanded uncertainty should be assessed to perform a full validation of the method. In addition, the participation of a specific proficiency tests to determine arsenic bioaccessibility in foodstuffs should be performed to test the reliability of the proposed PBET method. A priori, the method could be applied to assess arsenic bioaccessibility in other food samples; however further investigation on this applicability should be carried out. To improve the risk assessment process, the suitability of the proposed method to extract the bioaccessible arsenic species, especially iAs, in food samples should be conducted. Finally, the production of a CRM with a bioaccessible arsenic content should also be available for validation and quality control purposes

Chapter 7

Occurrence and distribution of arsenic species in foodstuffs

Food and drinking water remain the greatest source of exposure to arsenic in the general population. Human intake of arsenic species occurs mainly via food ingestion, as a consequence of bioaccumulation and biotransformation processes in biota. To assess a dietary risk in humans, assessment of the chemical forms of arsenic is more important than knowledge of their total content due to the different toxicity exhibited between organoarsenicals and inorganic arsenic species, being iAs the most toxic species.

Therefore, the main goal of the **Chapter 7** is to summarize and discuss the results to give an overview of arsenic occurrence in the analysed foodstuffs with the focus on the arsenic speciation, especially iAs, in such matrices.

Firstly, specific discussion of the results for each food group is presented as well as arsenic species distribution and the relationship between iAs and tAs. Then, a global discussion including all studied food groups is shown.

Finally, the dietary exposure to iAs to assess the potential health risks involved with the consumption of the assayed foods in the present thesis is estimated. The present daily exposure to iAs is compared to EFSA and JECFA BMDL ranges to estimate the risk assessment of the potential consumers this food commodities.

Different types of foodstuffs were analysed throughout the present thesis. Food samples were classified in the following groups: rice and rice products, cereal-based products, infant foods (including rice-based and infant cereals), mushrooms, fish and shellfish, and seaweeds and a specific discussion of .arsenic and arsenic species results is presented as follows.

7.1 Arsenic levels in rice, cereal-based foods and infant foods

To evaluate the applicability of the methods validated for the determination of arsenic species in rice and in cereal-based food, several samples were analysed: rice and rice products, cereal-based food and also infant foods (rice-based and multicereal-based formulas). First, a global discussion of the results obtained in all these studied samples is presented in this subchapter. Second, for a clear discussion of the results, samples are sorted into three groups: "rice", "cereal-based foods" and "infant foods" and the discussion is presented in the following paragraphs.

7.1.1 Total arsenic and occurrence of arsenic species in rice, cereal-based foods products and infant foods

Low levels of arsenic were found in rice, cereal-based products and infant foods samples, the average content was 0.08 mg As kg^{-1} (n=56) and ranged from 0.004 to 0.32 mg As kg^{-1} . As mentioned above, tAs levels were 1 or 2 orders of magnitude lower than those found in marine samples seafood. Median of tAs content in all samples of these food groups was 0.04 and the lowest tAs levels were found in cereal-based products and infant foods (non rice-based). Furthermore, in some samples these values were below the LOQ (6.0 µg As kg^{-1} for tAs) and were estimated for mass balance estimation.

Arsenic species distribution (%) for rice, cereal products and infant foods is shown in **Figure 29.** As can be clearly observed, inorganic arsenic (red bars) was the predominant arsenic species in these food groups and accounted for 84% of the sum of arsenic species, meanwhile DMA (blue bars) for 16% and MA (grey bars) was found at trace levels (0.04%). The concentration of iAs increases in the following order: cereal-based products infant food< rice and rice products. This fact is illustrated in **Figure 30**, which shows the relationship between iAs and tAs for these three food groups. Three groups of samples could be clearly differentiated and quite separated between them (with the exception of 3 infant food samples which are rice-based and had high proportion of rice) (**Figure 30)**. The average content of iAs was 0.112, 0.044 and 0.007 mg As kg^{-1} for rice, infant foods and cereal-based products, respectively. In general, the concentration of iAs is higher in rice (blue rhombus) than in infant foods (green triangles) and over 10 times higher than that found in other cereal-based products (red squares).

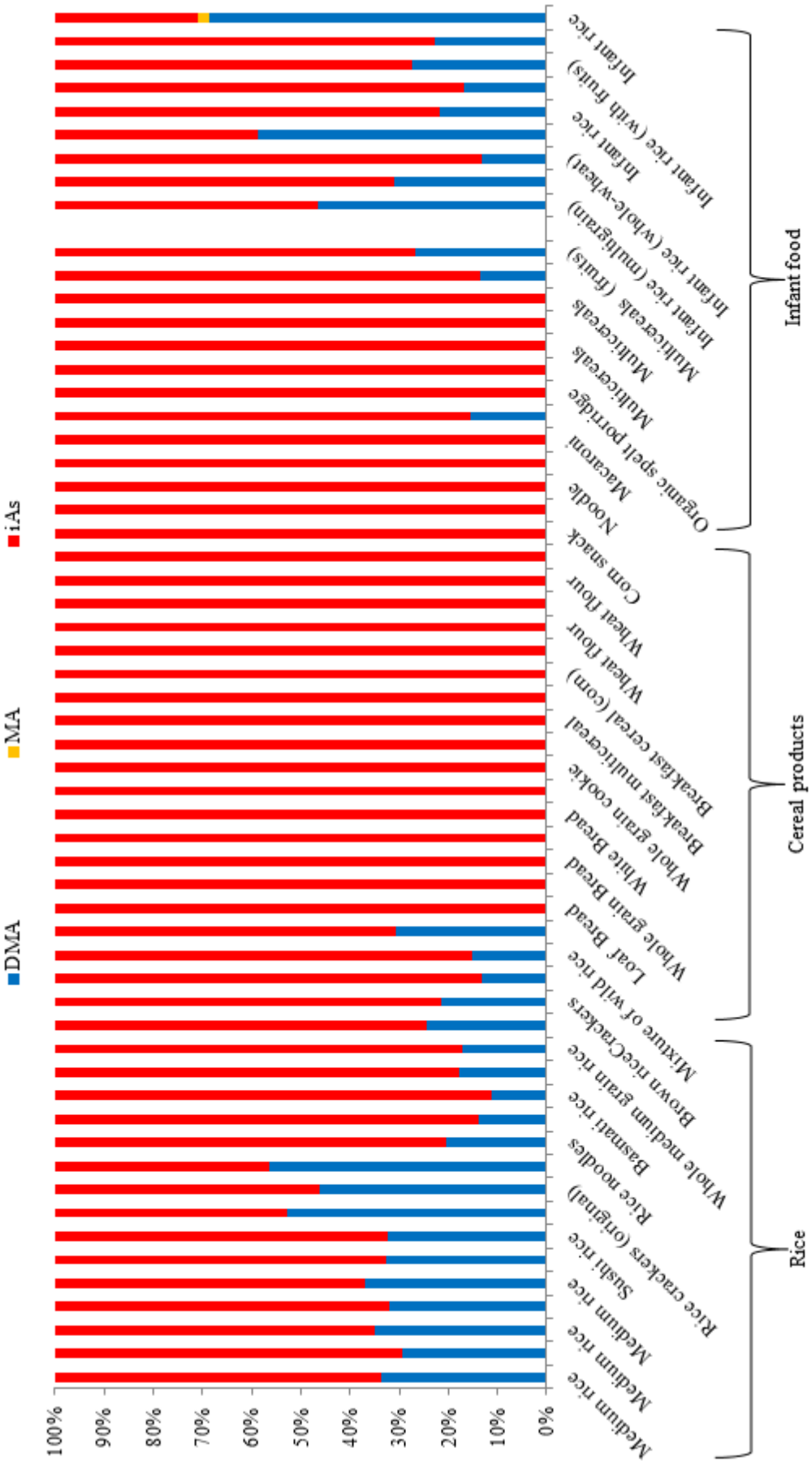


Figure 29. Proportions of arsenic species in rice, cereal products and infant food extracts with respect to the sum of arsenic species.

Considering all samples analysed in these three food groups: rice, infant foods and cereal-based food, a positive correlation between iAs and tAs is observed (**Figure 30)**. The concentration of iAs in these food groups (n=59) was positively correlated with tAs: y = 0.6097x + 0.0036, R^2 = 0.8872) highlighting that iAs content is dependent of tAs in the assayed samples. Given the number of samples analysed in the present thesis, the present relationship between iAs and tAs content cannot be extrapolated to all rice, infant foods and cereal-based food samples. Nevertheless, which seems clear is that iAs is the predominant arsenic compound in this type of samples **Figure 29** and **Figure 30**.

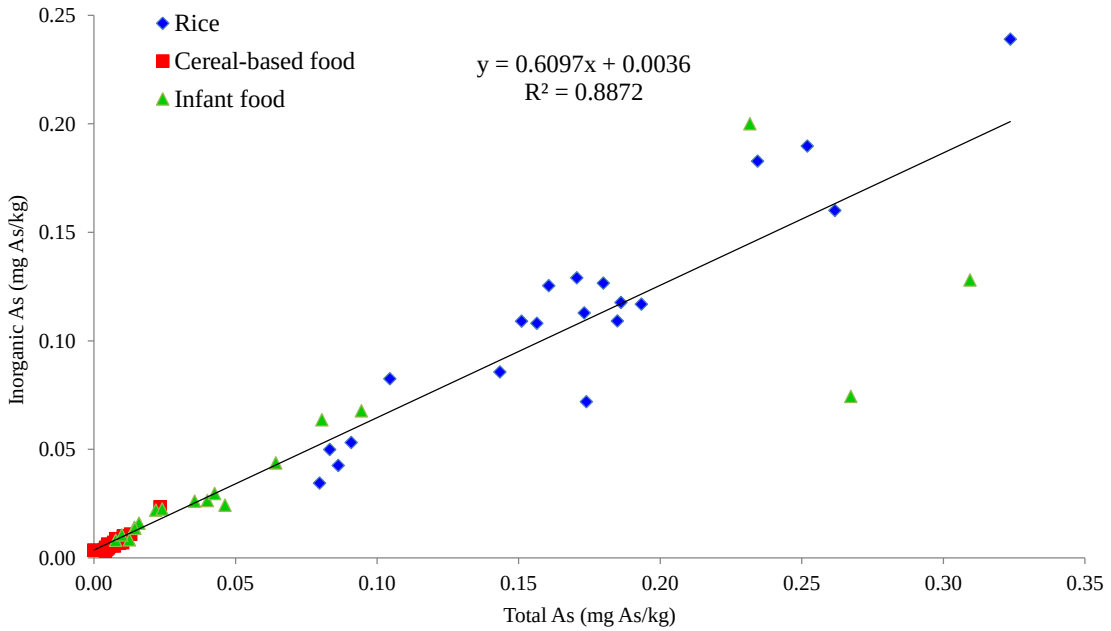


Figure 30. Relationship between inorganic and total arsenic for rice (blue rhombus), infant foods (green triangles) and cereal-based food samples (red squares)

7.1.2 Rice products

Total arsenic in the studied rice and rice products ranged from 0.08 to 0.32 mg As kg^{-1} (n=20) and the average content was 0.170 mg As kg^{-1}. Toxic inorganic arsenic ranged from 0.034 to 0.239 mg As kg^{-1} and the average content was 0.112 mg As kg^{-1} dm (median=0.111). The concentration of arsenic species in rice samples is shown in **Figure 31**. Inorganic arsenic was the predominant species in all rice samples, excluding Bomba rice and Rice crackers (sesame) samples where DMA was the major compound. Inorganic arsenic accounted for 71% of the sum of arsenic species, and DMA accounted for 29% meanwhile other arsenic species were not detected.

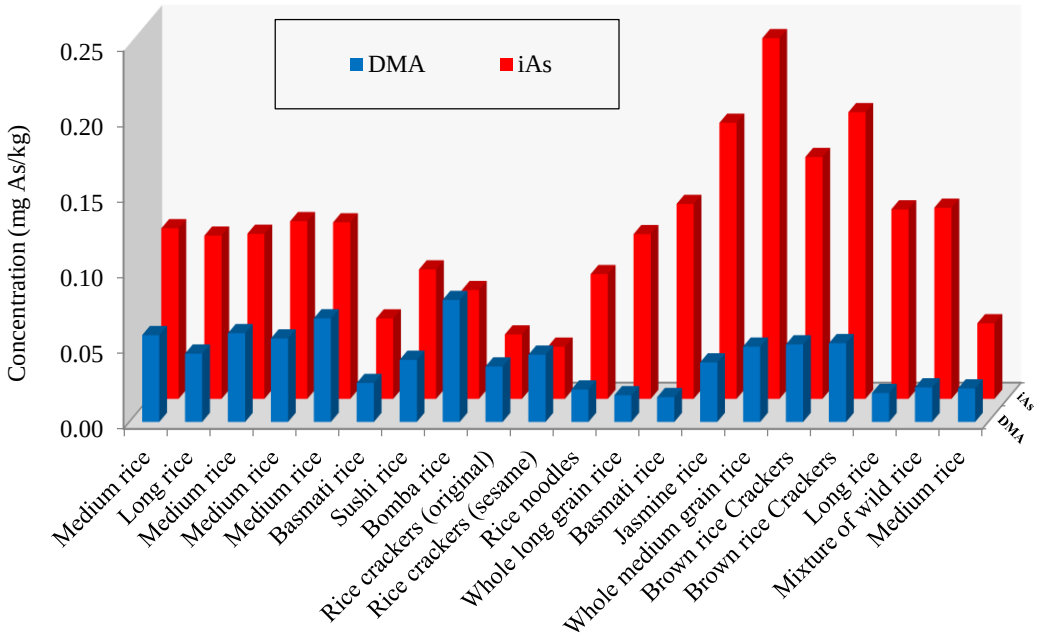


Figure 31. Concentration of arsenic species in rice and rice products.

Maximum levels of inorganic arsenic in rice

As is commented, specific maximum levels of inorganic arsenic in rice and rice products have been established by China [190], CODEX [191, 192] and European Commission [107] in the last years. Maximum levels of inorganic arsenic in rice are summarised in **Table 25.**

Table 25. Maximum levels of inorganic arsenic in rice established by European Union, CODEX and China.

	Maximum levels of inorganic arsenic in rice [a] (mg As kg^{-1})		
	EU	**CODEX**	**China**
Non-parboiled milled rice (polished or white rice)	0.20	0.20	0.20
Parbolied rice and husked rice	0.25	0.40	0.20
Rice waffles, rice wafers, rice crackers and rice cakes	0.30	n.e	n.e
Rice destined for the production of food for infants and young children	0.10	n.e	n.e

[a] Inorganic arsenic as sum of As(III) and As(V). n.e. means not established

Focusing on the recently MLs established by EU [107], inorganic arsenic content in the assayed rice samples were below these limits. This fact is illustrated in **Figure 32** in which MLs

of iAs for each specific type of rice are shown. For a better comparison between measured iAs contents and the different MLs, samples are sorted in three groups according the classification made by EU legislation **(Figure 32)**. Thus, nine samples are included in the group of 'Non-parboiled milled rice (polished or white rice)' (red rhombus), seven in 'Parbolied rice and husked rice' (green triangles), and four in 'Rice waffles, rice wafers, rice crackers and rice cakes' (blue circles).

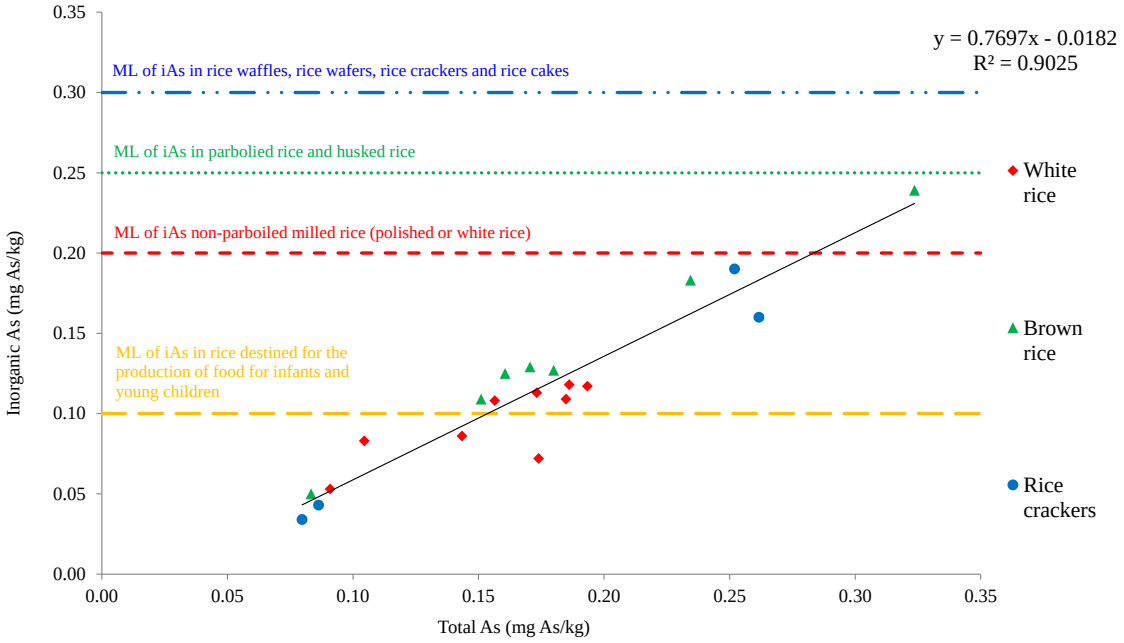


Figure 32. Relationship between inorganic and total arsenic for rice and rice products and maximum levels of iAs established by European Union [107].

Comparing our results with CODEX and China legislation, concentrations of iAs in the assayed rice samples (n=20) were below the MLs established. Only one brown rice (whole medium grain rice, iAs= 0.239 mg As kg^{-1}) exceeded the ML of 0.2 mg As kg^{-1} established by China. Excluding the mentioned sample, none of the rice samples (n=19) exceeded the specific MLs for iAs established by CODEX, China and EU. Therefore, the assayed rice samples (except for one sample) seems to have reasonable low iAs content and are safe to be marketed in the EU and China.

Relationship between As species and total arsenic in rice samples

A relationship between iAs and tAs and DMA and tAs in the analysed rice samples was found (**Article II)**. DMA and also iAs concentrations increase with tAs, being steeper the slope corresponding to iAs. This positive correlation between iAs and tAs is shown in **Figure 32** and the regression analysis confirmed this fact, highlighting that iAs does not depend on the type of rice product. Zavala et al. [193, 194] categorized rice into DMA and inorganic arsenic types. Rice from the U.S. was predominantly the DMA type, as were single samples from Australia and China, whereas rice from Asia and Europe was the iAs type. Besides, Meharg and

colleagues [14] found that the relationship between iAs content versus tAs content significantly differed among countries, with Bangladesh and India having the steepest slope in linear regression, and the U.S. having the shallowest slope. From our results, specific information about the origin of the rice grain is not always available on the product label. Therefore, taking this into account and the limited number of samples generalisations about this fact cannot be made. However, from the present results seems clear that rice contains iAs, DMA and MA at trace levels which is in agreement with other studies which reported results only for these species by the majority of the methods employed in the literature reviewed.

7.1.3 Cereal-based products

Total arsenic cereal-based products ranged from 0.004 to 0.023 mg As kg^{-1} (n= 21) and the average content was 0.007 mg As kg^{-1}. The contents of arsenic species cereal-based products are shown in **Figure 33** and as can be seen only inorganic arsenic (represented by red bars) was determined in the assayed samples, iAs ranged from 0.003 to 0.023 mg As kg^{-1} dm and the average content was 0.007 mg As kg^{-1} dm (median= 0.006). Typically, cereals and cereal products (excluding rice) had iAs content below 0.050 mg As kg^{-1} [195] which is in agreement with our results. Unlike arsenic speciation in rice, DMA species was not detected in cereal-based products.

Relationship between inorganic arsenic and total arsenic in cereal-based food

The relationship between iAs and tAs in the analysed cereal-based food is shown in **Figure 34.** Although several types of samples were analysed such as, i.e. bread (n=5), biscuits (n=3), breakfast cereals (n=4), flours (n=3), snacks (n=3) and pasta (n=3), a positive correlation between iAs and tAs was found and the regression analysis confirmed this fact.

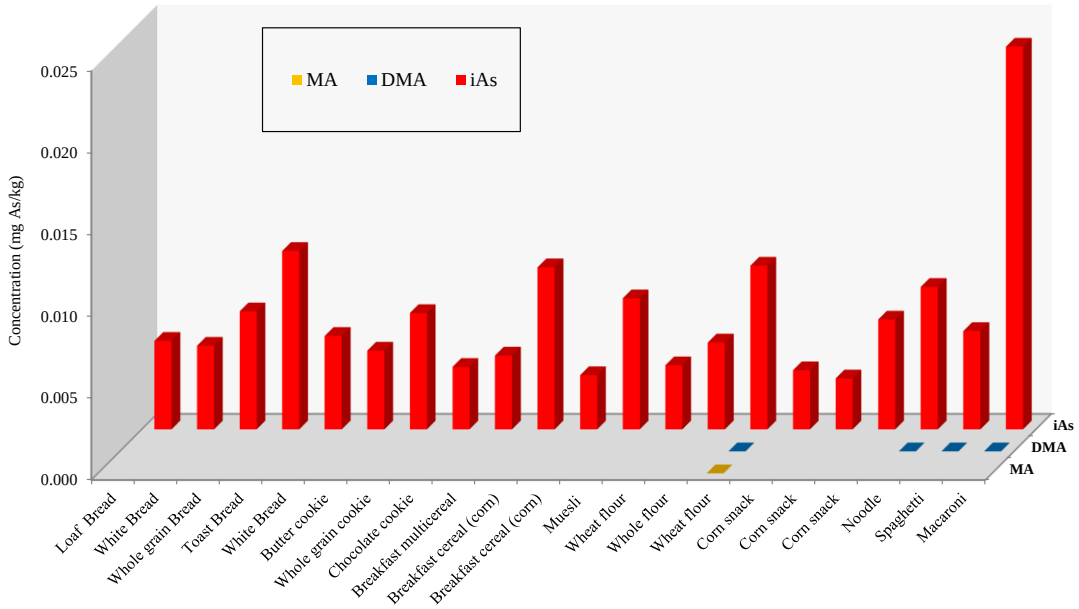


Figure 33. Concentration of arsenic species in cereal-based food.

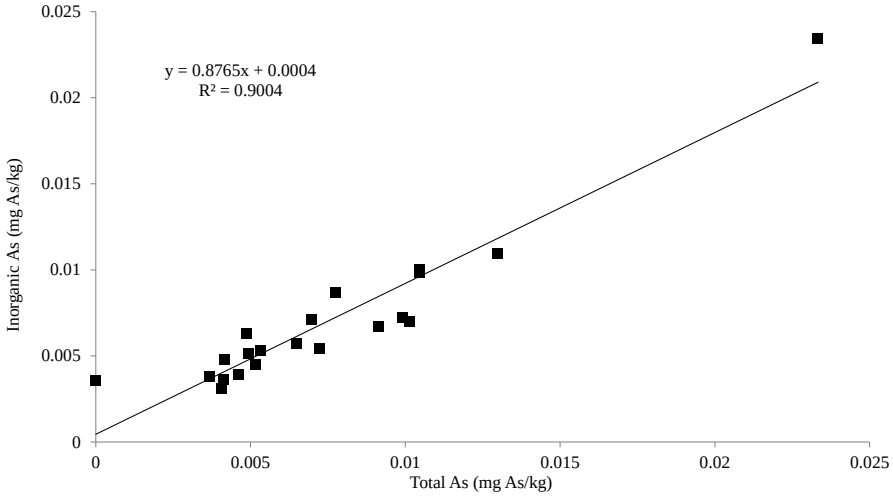


Figure 34. Relationship between inorganic and total arsenic for cereal-based food.

Maximum levels of arsenic in infant food cereal-based food

Regarding legislation, to date the content of arsenic in cereals has not regulated in the European Union. On the other hand, China established a ML in grains and their products and this maximum threshold is 0.5 mg As kg^{-1} for "grains (excluding paddy rice)" and for "processed milled grain products (excluding brown rice, white rice)" [190]. Australia and New Zealand established a ML for total arsenic content of 1 mg As kg^{-1} for "cereals" [196]. None of the assayed samples (n=21) exceeded the specific MLs for tAs established by these specific legislations and the average total arsenic content is much lower than these MLs.

7.1.4 Infant food

Total arsenic in the assayed infant food ranged from 0.008 to 0.310 mg As kg^{-1} (n=18) and the average content was 0.073 mg As kg^{-1}. Samples included in the group of 'infant foods' are mainly cereal-based complementary foods for infants and young children. Some of them are rice-based infant foods and others are made with a mixture of cereals (wheat, barley, oat, corn, rye, sorghum, millet) combined with fruit or honey. As expected, high tAs was found in rice-based than in multi-cereals samples: average content of 0.131 vs 0.018 mg As kg^{-1}, respectively. In case of rice-based formula, high variability of tAs was found depending on the proportion of rice in the infant food, values ranged from 0.040 to 0.310 mg As kg^{-1}.

The contents of arsenic species in infant foods are shown in **Figure 35.** As is shown, inorganic arsenic (red bars) was the predominant species in all samples, accounted for 78.6% of the sum of species, the average content was 0.044 mg As kg^{-1} (median= 0.025, n=18) and ranged from 0.000 to 0.200 mg As kg^{-1}. Generally, methylated species were determined as minor species, i.e: DMA (blue bars) accounted for 21.3% meanwhile MA (yellow bars) was detected 0.1%. Only two exceptions were found where DMA was the major compound and accounted for 58.8% and 68.5% of the sum of species for infant food (100% rice) and infant food (rice-based) samples, respectively (**Figure 35)**.

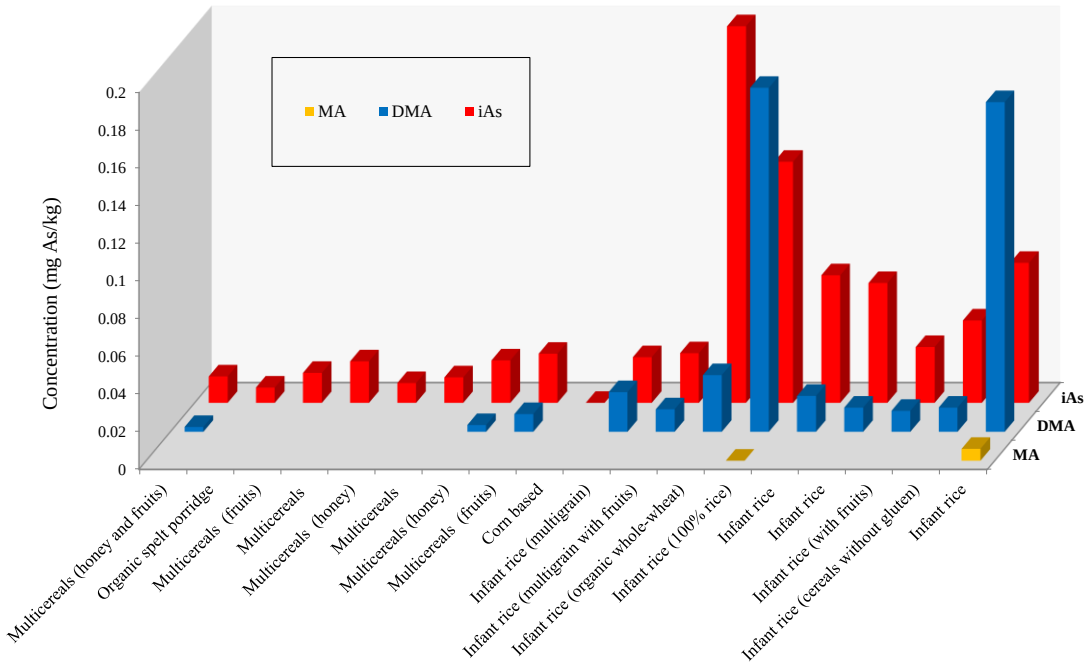


Figure 35. Concentration of arsenic species in infant foods.

Regulation of maximum levels of inorganic arsenic in infant food

China established different MLs for inorganic arsenic in "Supplementary food for infants and young children (excluding products with added algae)" and the maximum tolerated level for iAs is 0.2 mg As kg^{-1} [190]. As can be observed in **Figure 36**, almost all the samples (n=17) were below this ML for iAs highlighting that the assayed infant foods comply with Chinese legislation. As an exception, the iAs content is equal to the ML in one infant food sample (organic whole-wheat rice).

Relationship between As species and total arsenic in infant food

The correlation between iAs and tAs for the analysed infant foods is shown in **Figure 36.** As illustrated, a soft correlation was found between iAs and tAs. Sorting samples by the type of cereals: rice-based (squares) and multicereal-based (circles), it can be observed that higher iAs levels were found in rice-based (dark squares) than in multi-cereals (circles) (**Figure 36**).

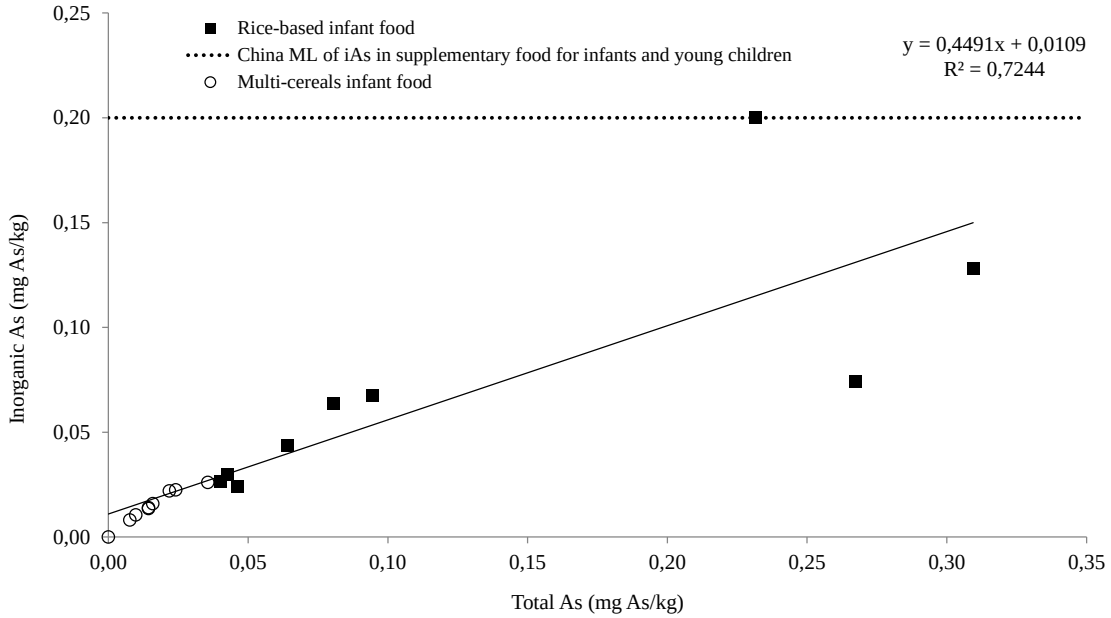


Figure 36. Relationship between inorganic and total arsenic for infant foods and maximum levels of iAs established by China [190]. Rice-based infant foods are represented by squares and multi-cereals infant food by circles

7.2 Arsenic levels in edible mushrooms

At the request of the IRMM, we performed a screening of arsenic and arsenic species mass fractions in commercially available mushrooms with the aim to select a candidate test item for the proficiency test IMEP-116/39. For this, we selected thirteen fresh mushroom species and four dehydrated mushrooms for the initial screening. From all assayed mushrooms, we selected *Lentinula edodes* as a candidate test item for a PT since is one of the most cultivated mushrooms worldwide and the high proportion of iAs in the mushroom extract. Given this fact, we performed specific study in several *L. edodes* products: fresh samples, canned, dehydrated and *L. edodes* supplements. Furthermore, a preliminary study home-cultivated *L. edodes* grown on a commercial substrate in a small-scale mushroom facility was performed.

First, a global discussion of the results obtained in all studied mushroom species is presented. Second, for a better comparison of the results, mushroom samples are divided into two groups: "*Lentinula edodes* products" and "Other mushroom species" and discussion is presented in the following paragraphs.

7.2.1 Total arsenic and occurrence of arsenic species in mushrooms

Several types of mushroom were analysed: wild species, cultivated species, mushroom supplements, canned mushrooms and dehydrated mushrooms. The average tAs content in all assayed mushrooms was 0.59 mg As kg^{-1} (median=0.28, n=32) and ranged from 0.05 to 2.8 mg As kg^{-1}. As commented, the highest tAs contents in studied terrestrial foods were found in mushroom samples, for instance 1.42 and 1.44 mg As kg^{-1} for two fresh *Lentinula edodes* and 2.37, 2.56, 2.83 mg As kg^{-1} for *Marasmius oreades, Lactarius deliciosus* and *Macrolepiota*

procera, respectively. Total arsenic levels in the assayed mushrooms and mushrooms products are in agreement to those reported in previous studies on the same wild and cultivated species growing on uncontaminated soils or substrates and with no evidence of significant variations in mushrooms from unpolluted areas [197–200].

Arsenic species distribution (%) for all mushroom samples is shown in **Figure 37.** Unlike other terrestrial foods, i.e. rice, infant food and cereal samples where iAs and methylated species were found, nine different arsenic species were determined in the mushroom extracts. There was much variability in their proportions, depending on the species of mushroom**.** As can be clearly observed in **Figure 37**, inorganic arsenic (red bars) was the predominant arsenic species in the majority of mushroom and in all *L.edodes* products and considering all the assayed mushrooms accounted for 69% of the sum of arsenic species. Meanwhile DMA (blue bars) and AB (purple bars) were found for 12.8% and 9.2% respectively. Besides, other arsenic species were found at minor proportions (below 6%), i.e: MA, TMAO, AC, TMAP and TETRA.

Mushrooms are sorted by type of mushroom product in **Figure 38**, i.e. wild species (n=14), cultivated species (n=5), mushroom supplements (n=6), canned mushrooms (n=3) and dehydrated mushrooms (n=4). As can be seen, higher tAs (blue bars) levels were found in wild species (ranged from 0.05 to 2.83 mg As kg^{-1}, median=0.61) than in the other type of mushrooms where tAs contents were similar between them. Furthermore, similar iAs levels (red bars) were found between the type of mushrooms and no differences are apparent.

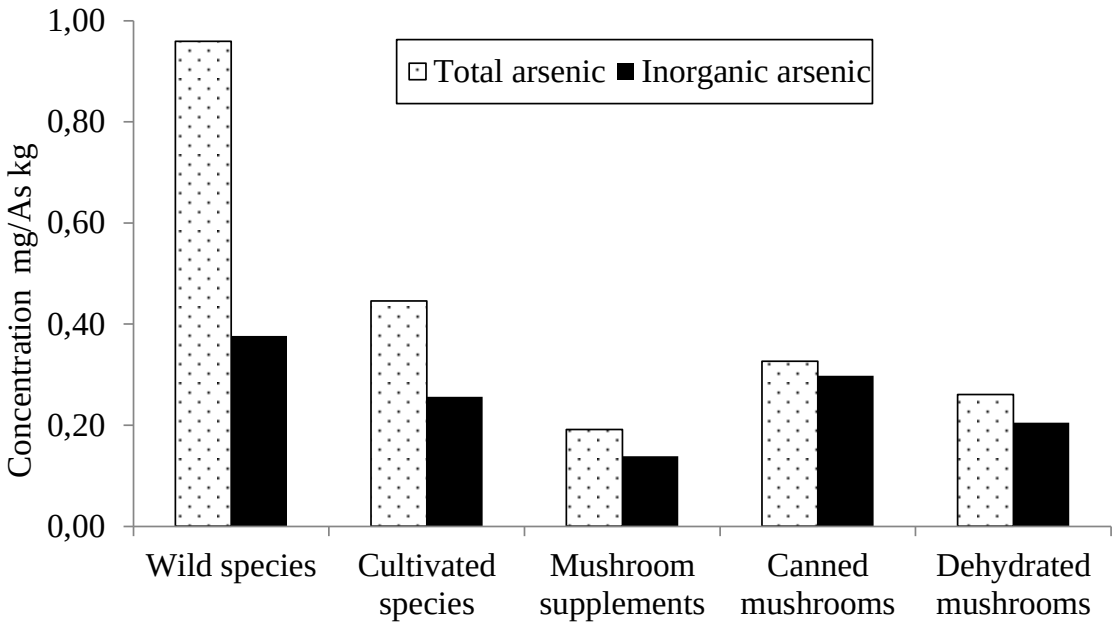


Figure 38. Total arsenic (blue bars) and inorganic arsenic (red bars) average concentrations in mushrooms (wild, cultivated, canned dehydrated species) and mushroom supplements

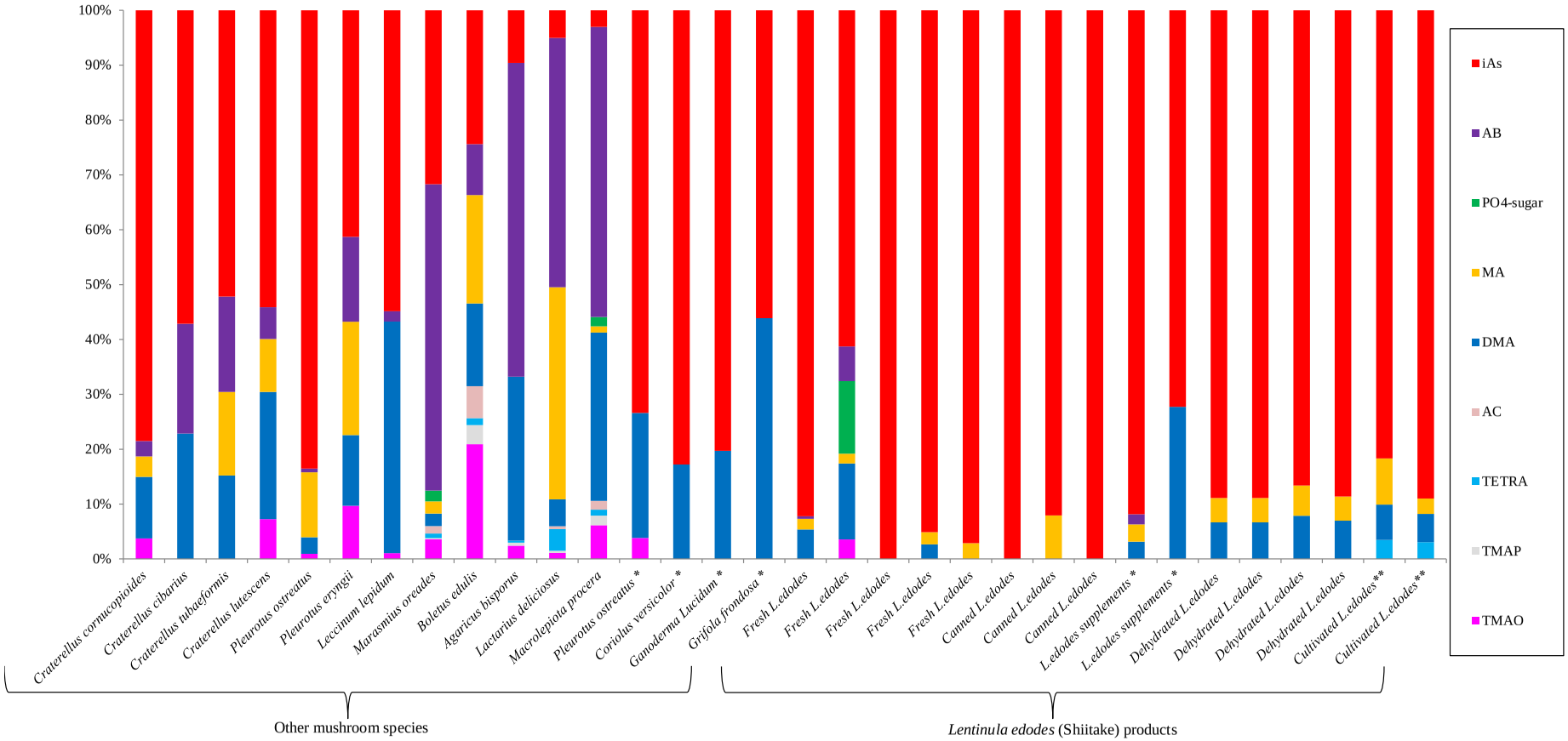


Figure 37. Proportions of arsenic species in mushroom extracts with respect to the sum of arsenic species. * Indicates mushroom supplements. ** Home-cultivated *Lentinula edodes* in a small-scale mushroom facility belonging to the University of Barcelona.

7.2.2 *Lentinula edodes* products

Average total arsenic content in *L. edodes* products was 0.49 mg As kg^{-1} and ranged from 0.11 to 1.44 mg As kg^{-1} (median=0.37, n=16). Some types of *L. edodes* products were analysed and several arsenic species were found in Shiitake products but iAs was the predominant As compound and ranged from 0.09 to 1.38 mg As kg^{-1}, with a mean value of 0.42 mg As kg^{-1} (median=0.30). Arsenic speciation pattern in *L. edodes* products is shown in **Figure 39** and as can be seen, inorganic arsenic (red bars) was the predominant species, whereas DMA, MA, AB, and TMAO were found in minor proportions. To date and to our knowledge, few studies on arsenic speciation in Shiitake are present in the literature and our results are difficult to compare. Thus, we are not able to postulate that the high proportions of iAs (84% of the tAs) are those levels commonly found in Shiitake mushrooms. Further studies analysing a considerable number of *L. edodes* products from different origins should be performed to clarify this issue.

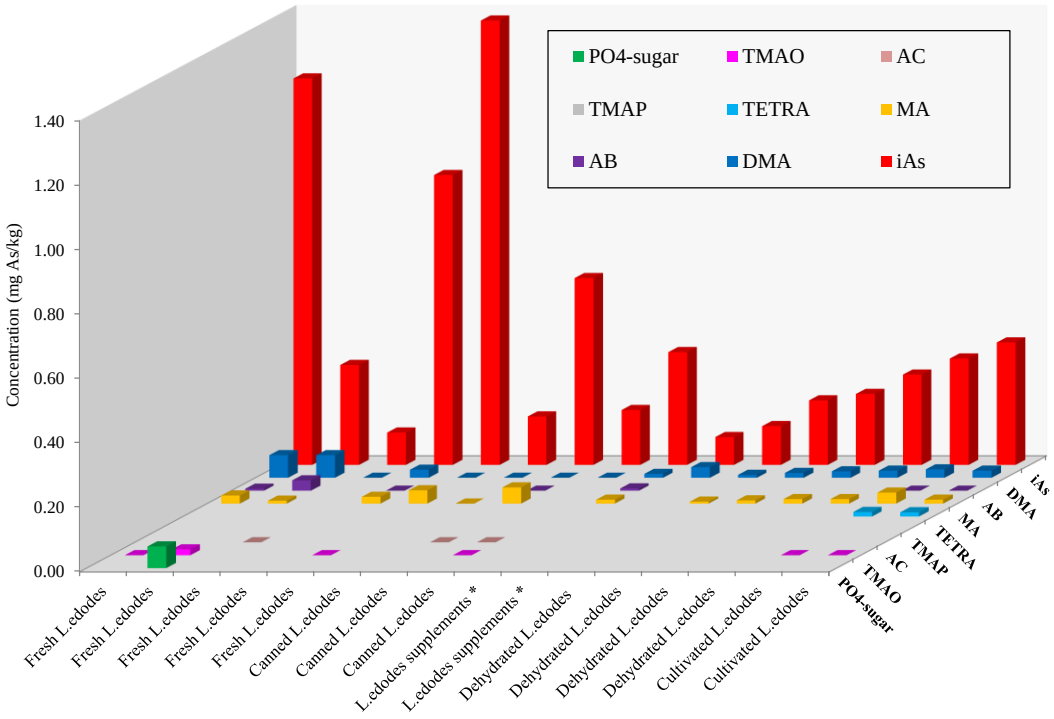


Figure 39. Contents of arsenic species in *Lentinula edodes* (Shiitake) products.

7.2.3 Other mushroom species

Average content of tAs in the group of "other mushroom species" was 0.68 mg As kg^{-1} and ranged from 0.053 to 2.83 mg As kg^{-1} (median=0.17, n=16). High variability in the proportions of As species in the other edible mushroom, depending on the species of mushroom (**Figure 40).** Inorganic arsenic was the predominant arsenic compounds (>50%) in all the studied mushrooms and mushroom supplements except in some samples: in *M. oreades*, *A. bisporus*, *L. deliciosus* and *M. procera* AB was found as the major arsenic species while in *B.*

edulis, and *P. eryngii* where some arsenic species were found in similar proportions: DMA, MA, iAs, AB and TMAO. Inorganic arsenic ranged from 0.021 to 0.613 mg As kg^{-1}, with a mean value of 0.147 mg As kg^{-1} (median=0.09, n=16) and accounted for 44.9% of the tAs. AB accounted for 0.7 to 52.8% of the tAs with a mean percentage of 19.4% and DMA accounted for 1.9 to 41.7% of the tAs, with a mean value of 18.4% of the tAs. Other arsenic compounds such as MA, AC and TMAO accounted for a few percent of the tAs.

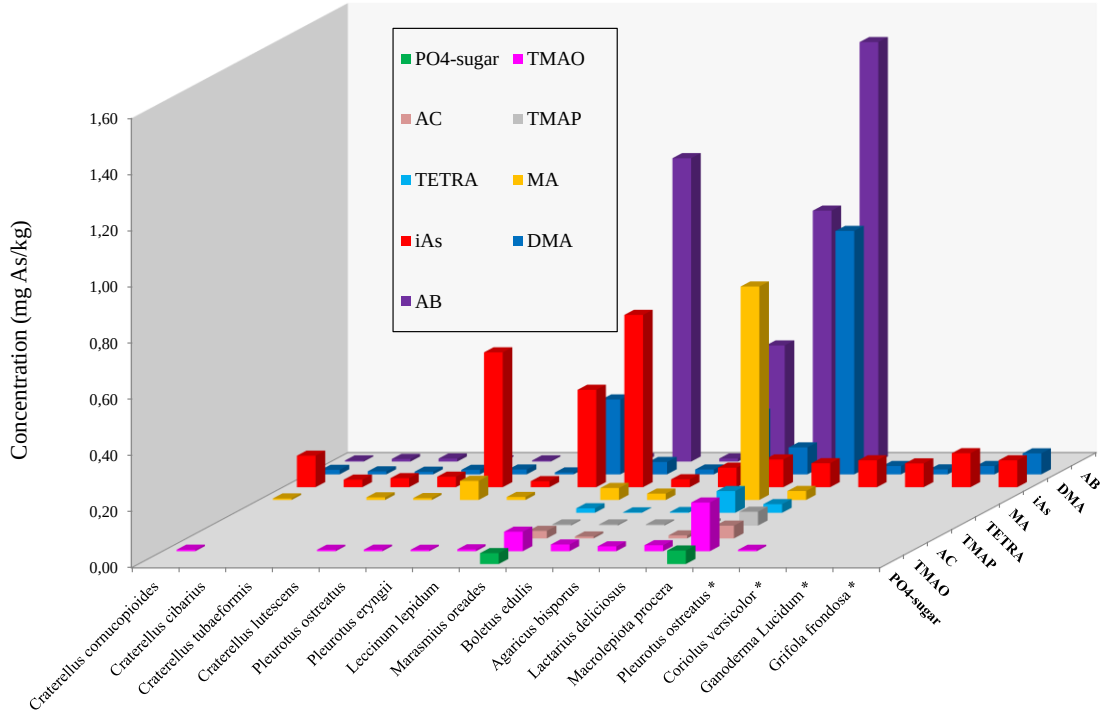


Figure 40. Concentration of arsenic species in edible mushroom and mushroom supplements. * Indicates mushroom supplements

7.2.4 Relationship between As species and tAs in mushrooms

As expected, considering all the studied mushrooms there was no relationship between iAs and tAs, highlighting that iAs content is independent of tAs and depends on the species of mushroom. However, considering only *L .edodes* products (n=32) the concentration of iAs was positively correlated with total arsenic as is shown in **Figure 41**. Meanwhile, as is commented, no relationship between iAs and tAs was for the "other mushroom species", but the concentration of AB in the mushrooms containing this compound (n=13) was positively correlated with tAs (**Article V)**.

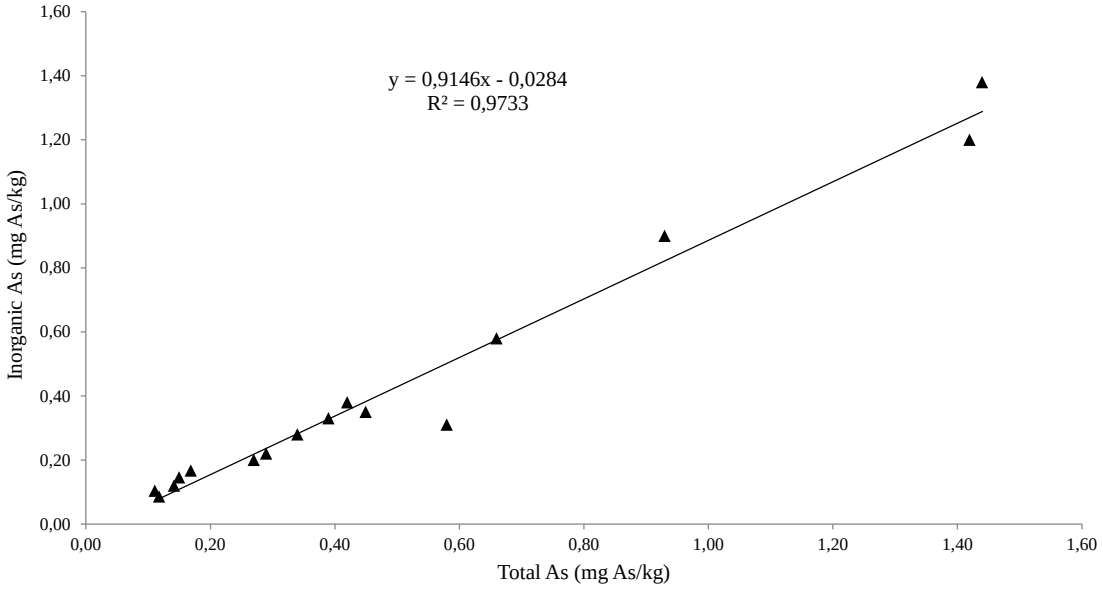


Figure 41. Relationship between inorganic and total arsenic for *L. edodes* products (represented by triangles)

7.3 Arsenic levels in seaweeds

Two arsenic speciation studies in several seaweeds species are presented in **Chapter 5**. Summarising, a study focused on marine seaweeds from the Catalan coast (Western Mediterranean) was performed (**Article VI**). Furthermore, tAs and As species were determined in various commercial edible seaweeds purchased in some food markets in Barcelona (**Article VII**). Moreover, an additional research study based on arsenic speciation in other edible seaweed species was carried out in the present thesis. This investigation was not included in the publications shown before. Thus, these unpublished results are presented and discussed in the following **section 7.3.1**. Furthermore, an overall discussion of all analysed seaweed samples is presented in the following sections. This discussion includes results published in **Articles VI** and **VII** as well as this abovementioned unpublished results.

7.3.1 Arsenic speciation study in *Sargassum fusiforme, Himanthalia elongate* and *Undaria pinnatifida*

An arsenic speciation study is performed in some of the most consumed seaweed worldwide. *Sargassum fusiforme* (also known as Hijiki or Hiziki) is brown seaweed growing wild on rocky coastlines around Japan, Korea, and China; *Himanthalia elongata* (also known by the common names sea thong and sea spaghetti) is a brown alga. It is commonly found in the north east Atlantic Ocean and the North Sea and *Undaria pinnatifida* (also called by its Japanese name Wakame) is brown seaweed and Japanese and Korean sea-farmers have grown wakame for centuries and they still are the main producers and eaters.

Total arsenic content in these samples was determined by ICPMS after a microwave-assisted acid digestion procedure (method A, **Table 6**). For arsenic speciation, seaweeds were

extracted applying an acidic oxidising MW-assisted extraction (method B, **Table 26**), and arsenic species were determined by anion and cation exchange chromatography coupled to ICPMS (HPLC-ICPMS). Total arsenic, total extracted arsenic and arsenic species results as well as column recovery and extraction efficiency are shown in **Table 26.** Several arsenic species were determined in these commercial edible seaweeds, iAs was predominant in the three *H. fusiforme* samples (named *H. fusiforme-I, II and III*) while arsenosugars were the main arsenic compounds in *H. elongata and U. pinnatifida.* Other arsenic species such as AB, TMAO and AC contents were below the LOD in extracts of these samples. Furthermore, a quality control study was carried out throughout the sample analysis. For this, two seaweed CRMs, NMIJ 7405-a Hijiki and BCR-279 *U. lactuca* were analysed to assess the accuracy. Total arsenic and arsenic species results in these CRMs are shown in **Table 26** and as can be observed, measured values are in agreement with certified values. Furthermore, our speciation results in BCR-279 are consistent with those reported in the literature [182]. As an example, HPLC-ICPMS chromatograms of *Sargassum fusiforme-I* extract are shown in **Figure 42a and b.** As is observed, iAs was identified as the main arsenic species and other species such as PO_4-sug, Gly-sug, SO_4-sug were also clearly detected and DMA in minor proportion.

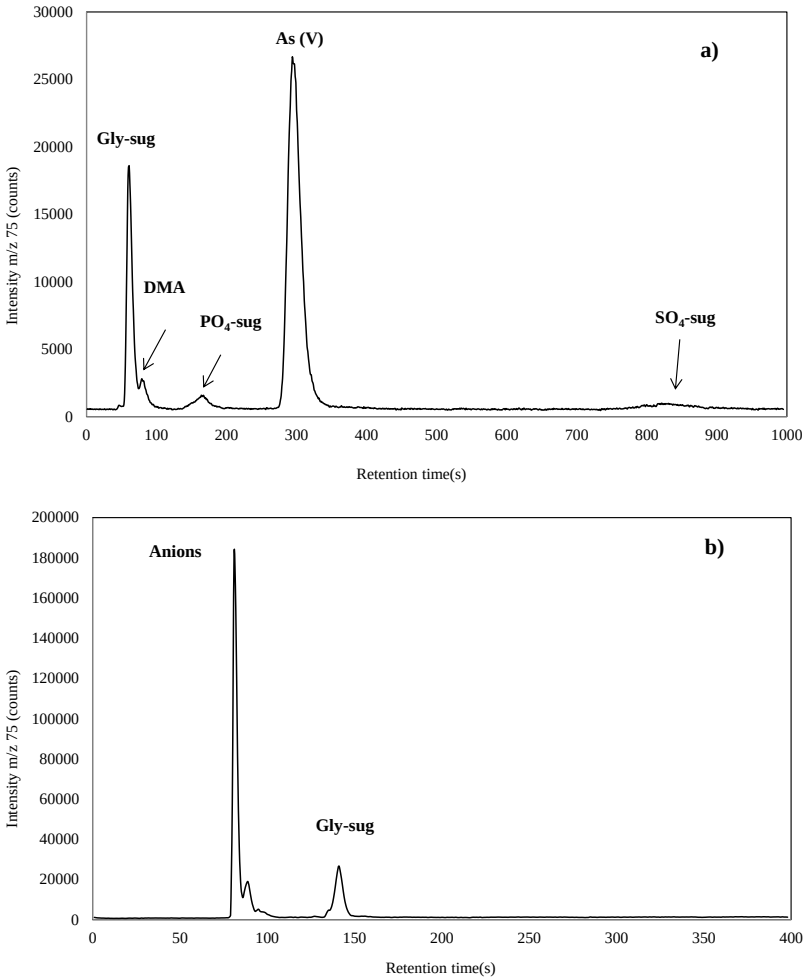


Figure 42. Chromatograms of *Sargassum fusiforme-I* extract (Hijiki) by anion exchange (a) and cation exchange (b) HPLC-ICPMS

Table 26. Concentration of total arsenic, total extracted arsenic and arsenic species in CRMs and seaweed samples. Results expressed as mg As kg^{-1} (mean ± SD, n=3, dry mass) [a].

Samples	Total As	Total extracted As	Arsenic species									Sum of species	EE (%) [b]	CR (%) [c]
			DMA	MA	PO_4-sug	As (V)	SO_3-sug	SO_4-sug	Gly-sug	TMAP	UC [b]			
Commercial seaweeds														
Sargassum fusiforme-I	120.8 ± 3.5	115.8 ± 1.8	3.83 ± 0.04	<LOD	2.06 ± 0.05	71.2 ± 0.9	<LOD	5.87 ± 0.1	11.1 ± 0.1	<LOD	<LOD	94.06 ± 0.91	96	81
Sargassum fusiforme-II	110.8 ± 4.2	100.8 ± 1.5	3.14 ± 0.03	<LOD	2.50 ± 0.10	69.4 ± 0.2	<LOD	7.3 ± 0.8	9.2 ± 0.5	<LOD	<LOD	91.54 ± 0.98	91	91
Sargassum fusiforme-III	105.2 ± 3.4	95.2 ± 1.7	2.2 ± 0.05	<LOD	3.5 ± 0.17	62.3 ± 0.8	<LOD	6.7 ± 0.12	10.4 ± 0.7	<LOD	<LOD	85.1 ± 1.08	90	89
Himanthalia elongata	39.0 ± 0.6	33.6 ± 2.3	4.7 ± 0.2	<LOQ	<LOD	<LOD	4.6 ± 0.1	<LOD	16.0 ± 0.8	<LOD	<LOD	25.3 ± 0.83	86	75
Undaria pinnatífida	39.9 ± 1.9	31.3 ± 2.8	1.19 ± 0.06	<LOD	1.27 ± 0.06	<LOD	3.2 ± 0.1	<LOD	15.5 ± 0.6	<LOD	<LOD	21.16 ± 0.61	78	68
Certified Reference Materials														
NMIJ 7405-a *Sargassum fusiforme*	35.5 ± 1.5	31.6 ± 4.9	2.45 ± 0.08	0.47 ± 0.03	4.2 ± 0.1	10.21 ± 0.32	<LOD	2.07 ± 0.06	4.6 ± 0.1	<LOD	<LOD	24.0 ± 0.37	89	76
certified value	35.8 ± 0.9					10.1 ± 0.5								
BCR-279 *Ulva lactuca*	3.04 ± 0.22	2.38 ± 0.09	0.15 ± 0.01	0.30 ± 0.02	<LOD	1.17 ± 0.02	<LOD	<LOD	0.30 ± 0.01	0.058 ± 0.005	0.17 ± 0.02	2.15 ± 0.04	78	90
certified value	3.09 ± 0.21													

[a] AB, TMAO and AC are below the corresponding LODs

[b] UC is an unknown cationic arsenic species that does not match any available standards. [b] EE means Extraction efficiency. [c] CR means Column recovery.

7.3.2 Total arsenic and arsenic species in all the studied seaweeds

The average tAs content in all analysed seaweeds was 34.4 mg As kg^{-1} (median=23.7, n=24) and ranged from 2.0 to 120.8 mg As kg^{-1}. The highest tAs content was found in the bioaccumulator species of *S. fusiforme* (Hijiki), for instance 120.8, 110.8 and 105.2 mg As kg^{-1} for *S. fusiforme*-I, *S. fusiforme*-II and *S. fusiforme*-III, respectively. These high As levels are in agreement with those reported in the literature and contents up to 100 mg As kg^{-1} have been published in *S. fusiforme* [21–29]. Levels of tAs in the present seaweeds are in the range of previously reported in the literature [21–23]. In general, brown algae accumulate higher arsenic levels than green or red algae [21, 22]. Our data are in agreement with this finding; in addition to *S. fusiforme* samples other brown algae had the high tAs levels: 41.0, 52.4 and 56.8, mg As kg^{-1} for *Undaria pinnatifida, Laminaria saccharina and Laminaria ochroleuca,* respectively. Furthermore, the following relationship between tAs content and algae type it has been proposed: brown > red > green [21, 22, 201]. Sorting samples for their origin, samples could be divided into two groups: "Seaweeds collected from the Catalan coast" (n=13) and "commercial seaweeds purchased in a food market" (n=11). Total As content was higher in "commercial seaweeds" than and "collected seaweeds", average content was: 57.3 vs 14.9 mg As kg^{-1} respectively, meanwhile median value was 41.0 and 11.0 mg As kg^{-1} respectively.

Arsenic species distribution (%) for the assayed seaweed samples (n=24) is shown in **Figure 43.** Several arsenic species were determined and high variability in their proportions depending on the species of seaweeds was found. The most commonly arsenosugars found in seaweeds were detected, i.e: Gly-sug, PO_4-sug, SO_3-sug and SO_4-sug. As is clearly evident in **Figure 43,** arsenosugars (green bars represents sum of Gly-sug, PO_4-sug, SO_3-sug and SO_4-sug species) were the predominant compounds in all seaweeds except in *S. fusiforme, Posidonia oceanica* and *Codium vermilara* samples where iAs (red bars) was predominant. Considering all seaweeds, arsenosugars accounted for 63.2% of the sum of species, meanwhile iAs accounted for 27.4% and the other species were found in low proportions (**Figure 43)**.

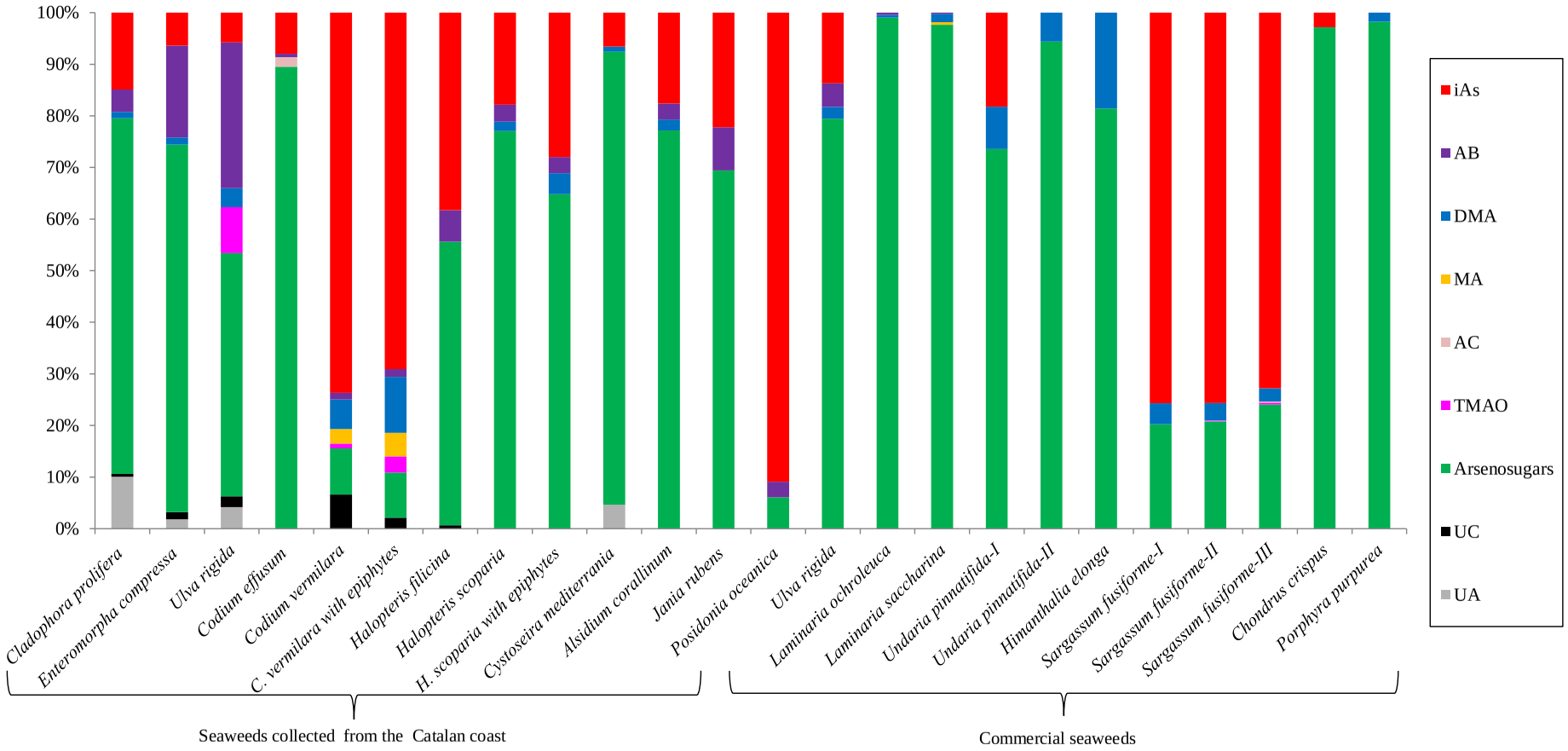


Figure 43. . Proportions of arsenic species (%) in seaweed samples extracts with respect to the sum of arsenic species. Arsenosugars means the sum of the following species: Gly-sug, PO_4-sug, SO_3-sug and SO_4-sug

7.3.3 Seaweeds collected in the Catalan coast

Total arsenic in seaweeds collected on the Catalan coast (Lloret de mar) (n=14) ranged from 2.0 to 39.0 mg As kg^{-1}. The concentration of arsenic species is shown in **Figure 44** and arsenosugars were the predominant As species (>50%) in most of the seaweed samples. For instance, high proportions of SO_3-sug (yellow bars) were found in *Halopteris scoparia*, *H. scoparia* with epiphytes, *Cystoseira mediterrania and Alsidium corallinum* and Gly-sug (cyan bars) was predominant in *Jania rubens, Cladophora prolifera, Enteromorpha compressa Ulva rigida* and *Codium effusum.* On the other hand, in *P. oceanica, C. vermilara* and *C. vermilara* (with epiphytes), iAs accounted for 91%, 74% and 69% of the sum of species (red bars), respectively. It is noted that, arsenobetaine (purple), which is not a common compound in seaweeds, was determined in two samples at unusual levels and accounted for 17.8% and 28.3% in *Enteromorpha compressa* and *Ulva rigida*, respectively. As is widely discussed in (**Article VI**), these significant percentages of AB cannot be attributed to the presence of epiphytes and is probably due to the presence of microorganisms that transform arsenosugars into AB.

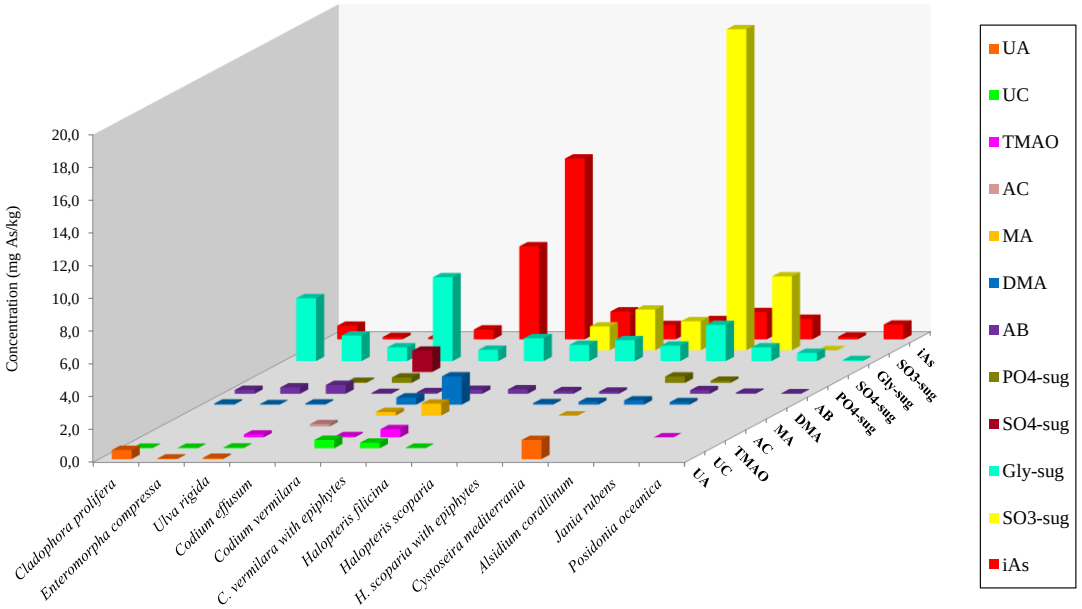


Figure 44. Concentration of arsenic species in seaweeds collected in the Catalan coast

7.3.4 Commercial seaweeds

Arsenic levels in seaweeds purchased in food markets ranged from 5.8 to 120.8 mg As kg^{-1} (n=11). The contents of arsenic species are shown in **Figure 45** and as can be observed several arsenic species were determined in these edible seaweeds products. It should be noted that very high iAs content (red bars) was found in three *S. fusiforme* samples: 71.2, 69.4 and 62.3 mg As kg^{-1} corresponding for more than 70% of the sum of species. For the other edible seaweeds, arsenosugars were the predominant compounds. For instance: SO_3-sug (yellow bars) accounted for 81% and 74% of the sum of species in *Laminaria* sp. samples, meanwhile Gly-sug (cyan bars) was predominant in *Ulva rigida*, in two *U. pinnatifida* and in *H. elonga*

(proportions > 50%); on the other hand, in *Chondrus crispus* and *Porphyra purpurea* PO_4-sug (olive green bars) was the major compound corresponding to 51% and 92% of the sum of species, respectively.

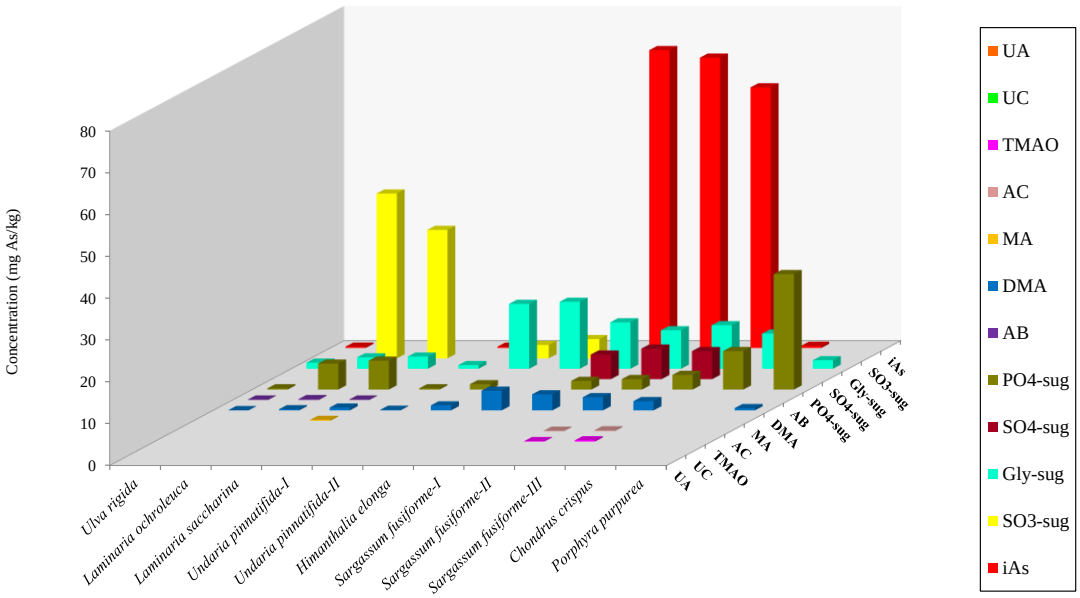


Figure 45. Concentration of arsenic species in commercial seaweeds

7.3.5 Relationship between As species and tAs in seaweeds

The arsenic content of seaweeds is regulated by genetic factors and water natural conditions (habitat and environmental factors). Seaweeds can accumulate iAs from seawater and biotransform it to arsenosugars as a detoxification mechanism [72, 202]. Thus, the relationship between iAs and tAs and as well as arsenosugars (as sum of all them) and tAs in both collected and commercially available seaweeds (n=24) is represented in **Figure 46.** As expected for the high variability of seaweed species analysed from different origins, there was no relationship between the sum of arsenosugars (red squares) and tAs ($y = 0.2505x + 4.1212$, $R^2 = 0.3799$). However, a soft relationship between iAs and tAs was found (regression analysis $y = 0.5032x - 8.5871$, $R^2 = 0.7845$) highlighting that iAs content is independent of tAs and depends on the species of seaweeds. For instance, the high iAs is correlated with a high tAs in the three *S. fusiforme* samples, but this relationship cannot be made for other species of seaweeds.

7.3.6 Regulation of maximum levels of arsenic in seaweeds

Arsenic, either as total or inorganic, in seaweeds for human consumption is currently not regulated by the European Union (EU) [108]. However, the EU established a maximum level (ML) for iAs in animal feeds, contents of below 2 mg kg^{-1} are recommended, especially those based on the seaweed species *H. fusiforme* [106].

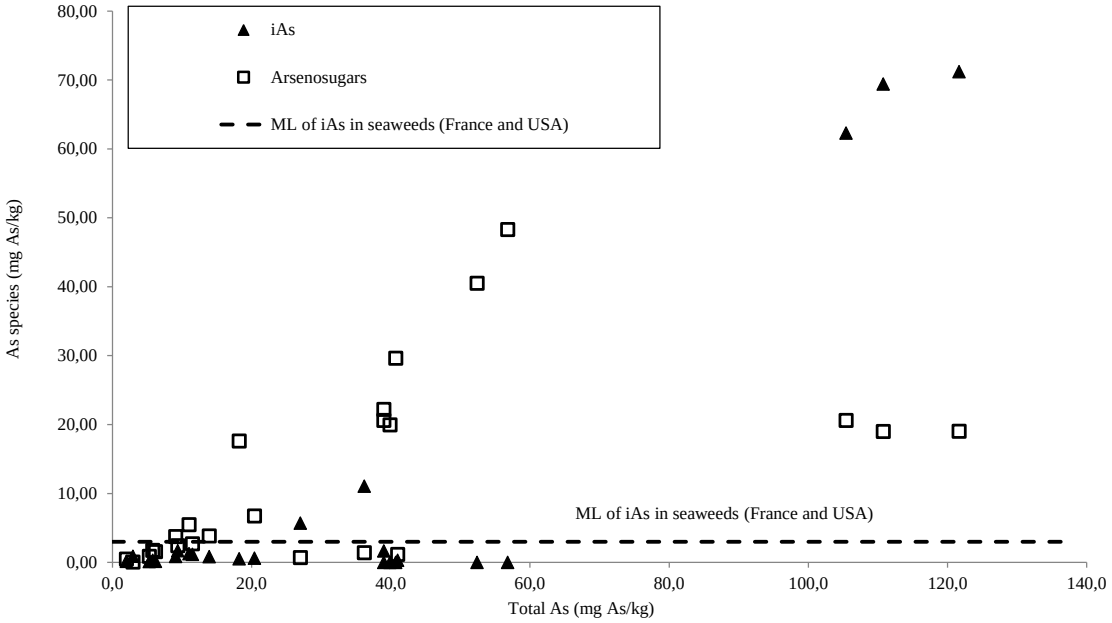


Figure 46. Plot of tAs concentration versus iAs concentration in the 24 assayed seaweeds from the present study. Inorganic arsenic is represented by dark triangles and arsenosugars by squares. Maximum level of iAs in seaweeds established by USA and France is shown.

Specific maximum levels (ML) of iAs in seaweeds have been established in some countries. France was the first European country to regulate the human consumption of seaweed as a non-traditional food substance, and the French limit for iAs in edible seaweed is 3 mg As kg^{-1}. Currently, 12 macroalgae are authorized in France for human consumptionare [203]: six brown seaweeds (*Ascophyllum nodosum, Fucus vesiculosus. Fucus serratus, Himanthalia elongata, Undaria pinnatifida),* four red seaweeds (*Porphyra umbilicalis, Palmaria palmata, Cracilaria verrucosa* and *Chondrus crispus*), two green seaweeds (*Ulva* spp. and *Enteromorpha* spp.) and two microalgae (*Spirulina* sp. and *Odontella aurita).* Furthermore, countries such as New Zealand and Australia have legislation for the maximum levels of iAs in seaweed and established a value of 1 mg As kg^{-1} [196]. Moreover, specific regulations for iAs contents in edible seaweed have been established in the United States with 3 mg As kg^{-1} as the maximum level [204]. The maximum level of 3 mg As kg^{-1} for the content of iAs in seaweeds established by France and USA is represented **Figure 46.** As can be observed, iAs content in five seaweed samples (blue triangles) is above this ML: *C. vermilara* and *C. vermilara* with epiphytes, and in three *S. fusiforme* samples. Considering the more restrictive legislation established by Australia and New Zealand; the abovementioned samples and other four seaweeds exceeded the ML of iAs of 1 mg As kg^{-1}: *Halopteris filicina, Halopteris scoparia* with epiphytes, *Cystoseira mediterrania* and *Alsidium corallinum.* Although the edibility of the collected seaweeds is unknown to us, it should be taken into account that levels of iAs in some samples are above the established ML and therefore should not be ignored as potential contributors to dietary iAs exposure.

Special care should be taken in case of high consumers of *S. fusiforme* samples, we found high arsenic levels: tAs > 100 mg As kg^{-1} and iAs > 60 mg As kg^{-1}. As commented before, our levels are in agreement with recent studies which have reported that *S. fusiforme* contains high toxic quantities of iAs and were significantly higher than in other types of seaweed. In addition, several studies warned about the risk to consume this algae and the high exposure to iAs and the possible incidence on some type of cancers [21–29]. All this facts made that some food safety agencies have advised against its consumption. Thereby, the Canadian Food Inspection Agency [205] and the Food Standards Agency (FSA) of the United Kingdom [206] advise consumers to avoid the consumption of Hijiki seaweed. Notwithstanding all these facts, there is no specific legislation regarding Hijiki seaweed in Spain, the only legislation concerns seaweed for animal consumption; it establishes a maximum level of 2 mg As kg^{-1} (dw) for iAs and warns of the possible risk of *H. fusiforme* as animal feeding. Although the consumption of Hijiki in Spain has increased in recent years, they cannot be considered a staple food and their inclusion in the diet remains at low levels. However, Hijiki should not be ignored as a potential high contributor to dietary iAs exposure and their consumption should be regulated in EU legislation.

7.4 Arsenic levels in fish and shellfish

To evaluate the applicability of the established method for the determination of arsenic species in seafood, several samples were analysed: fish, crustaceans and bivalves. First, a global discussion of the results obtained in all these seafood samples is presented in this section. Second, samples are sorted into two groups: "fish", "shellfish" and the discussion for each food group is presented in the following paragraphs.

7.4.1 Total arsenic and arsenic species in fish and shellfish samples

The average tAs content in fish and shellfish was 10.2 mg As kg^{-1} (median= 4.8, n=22) and ranged from 1.2 to 35.2 mg As kg^{-1}. The present results in fish and shellfish are in agreement with the literature [113, 207–209]. Furthermore, fish species with the highest tAs levels were white fish, red porgy and forkbeard and tAs content was: 35.2, 35.0 and 31.8 mg As kg^{-1}, respectively. The concentration of tAs increases in the following order: bivalves (n=5) > fish (n=14) > crustaceans (n=3) with an average content of 15.0, 10.2 and 2.2 mg As kg^{-1} in bivalves, fish and crustaceans, respectively. Arsenic species distribution (%) for the assayed fish and shellfish samples is shown in **Figure 47.**

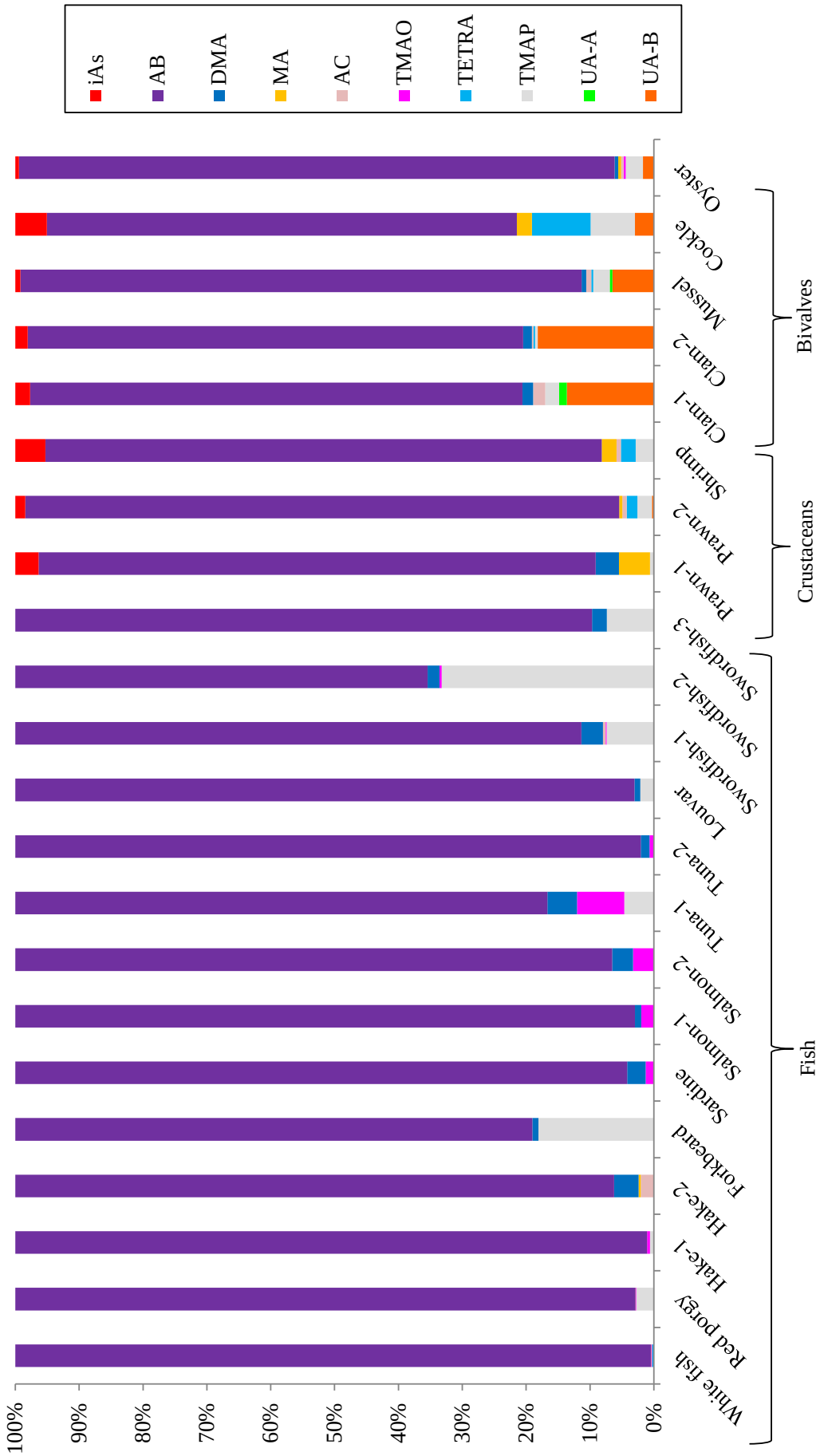


Figure 47. Proportions (%) of arsenic species in fish and shellfish extracts with respect to the sum of arsenic species.

Ten different arsenic species were determined in the fish and shellfish extracts. As can be clearly observed, arsenobetaine (purple bars) was the predominant arsenic species in these two food groups and accounted for 88.9% of the sum of arsenic species, meanwhile iAs (red bars), DMA (blue bars), MA (yellow bars), AC (light pink bars), TMAO (hot pink bars), TMAP (grey bars), TETRA (cyan bars) and two unknowns were found as minor compounds (below 5%) (**Figure 47)**.

7.4.2 Fish samples

Total arsenic in fish samples ranged from 1.4 to 35.2 mg As kg^{-1} and the average content was 10.2 mg As kg^{-1} (median=4.3, n=14). The concentration of arsenic species in fish samples is shown in **Figure 48**. As expected, AB (purple bars) was the predominant As species with an average content of 8.46 mg As kg^{-1} corresponding to 91% of the sum As species and ranged from 0.86 to 33.5 mg As kg^{-1} meanwhile iAs (red bars) was not detected in all fish samples. The iAs contents were in accordance with previously reported results for fish samples [113, 207, 210]. Furthermore, other arsenic compounds such as: TMAO, DMA, MA, TMAP and TETRA were found in minor proportions.

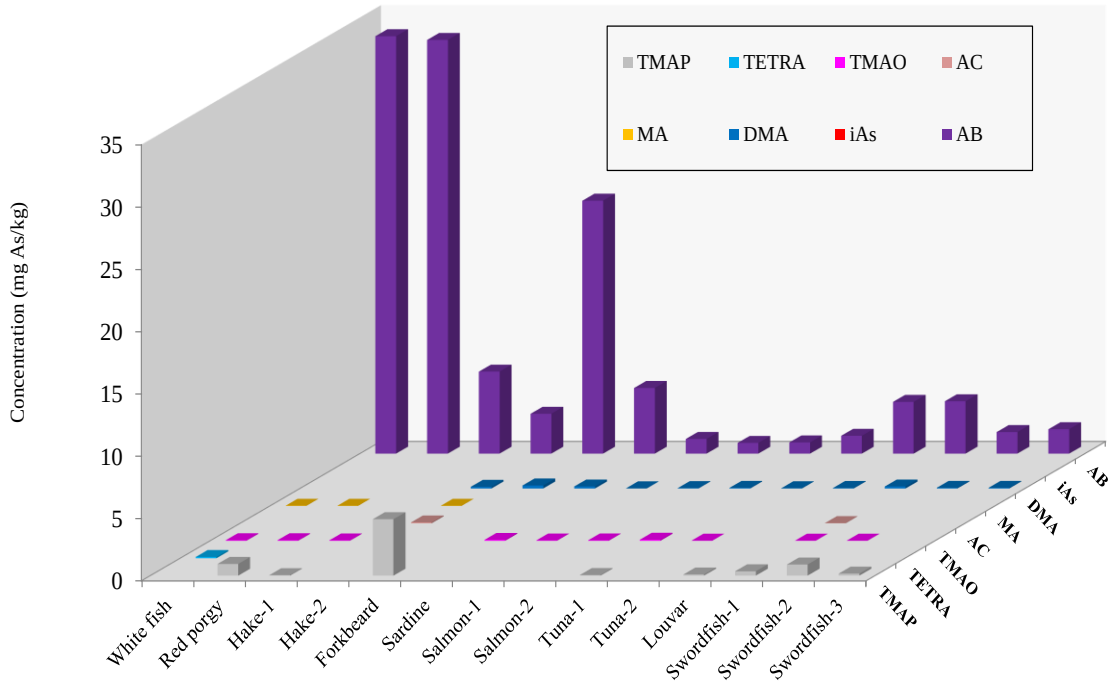


Figure 48. Concentration of arsenic species in fish samples.

7.4.3 Shellfish samples

Total arsenic shellfish samples ranged from 1.2 to 24.6 mg As kg^{-1} and the average content was 10.2 mg As kg^{-1} (median=10.3, n=8). Ten arsenic compounds were determined in the studied shellfish samples (**Figure 49)** and AB (purple bars) was the main As species found with an average content of 6.57 mg As kg^{-1} corresponding to 85% of the sum As species and ranged from 0.61 to 15.90 mg As kg^{-1} meanwhile low contents of iAs were found and the

average content (red bars) was 0.141 mg As kg^{-1} and ranged from 0.033 to 0.350 mg As kg^{-1} . Our results on iAs content are in the range of previously reported results for shellfish samples (0.001–0.26 mg As kg^{-1}) [113].

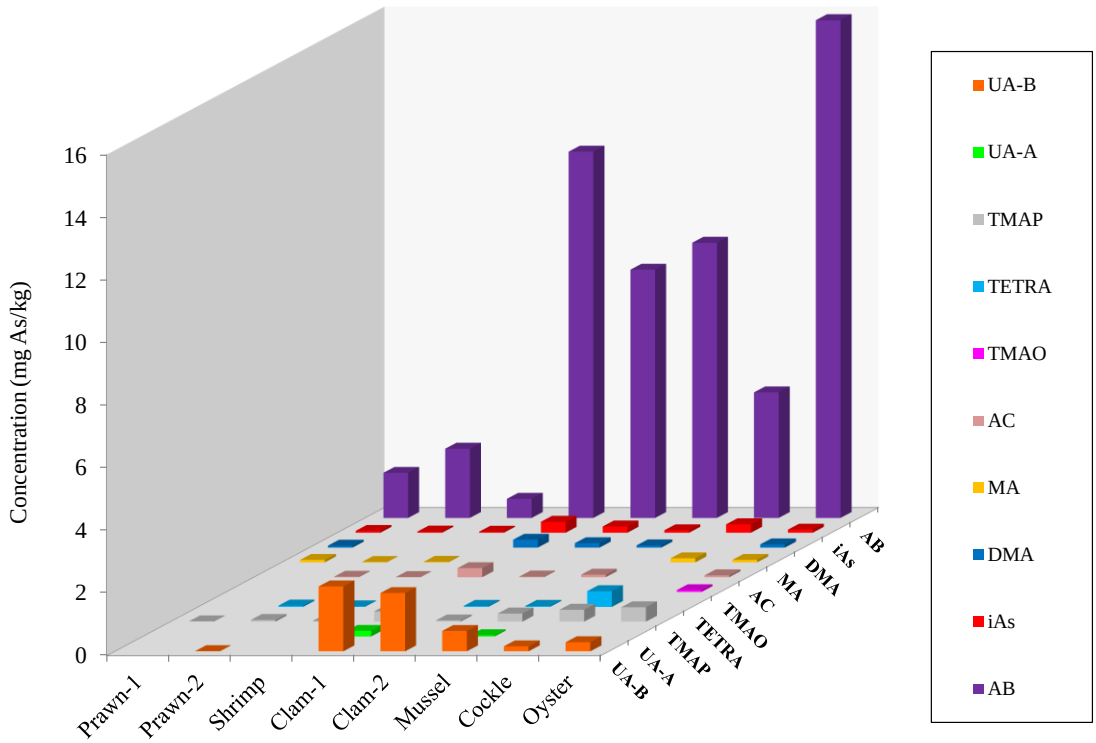


Figure 49. Concentration of arsenic species in shellfish samples

7.4.4 Relationship between As species and tAs

In contrast to terrestrial foods, low proportions of inorganic arsenic were found in tfish and shellfish samples: below the LOD in fish samples and on average below 3% in shellfish samples. **Figure 50** shows a plot of the iAs versus the tAs concentration for fish (n=14), crustaceans (n=3) and bivalves (n=5). As can be observed, there was no relationship between iAs and tAs for the assayed samples (**Figure 50).** The concentration of iAs was higher in bivalves (green triangles) than in crustaceans (blue squares) and the average content of iAs was 0.043 (ranged from 0.033 to 0.060) and 0.200 (ranged from 0.080 to 0.350) mg As kg^{-1} for crustaceans and bivalves, respectively. The number of samples analysed of each group is too low to generalise but seems that the three groups of samples are differentiated and quite separated between and there is no clear relationship between iAs and tAs. Sloth and Julshamn [30] reported that for tAs contents above 3.0 mg kg^{-1} a significant linear relationship between the iAs and tAs was found. They stated that the data indicate the presence of a biotransformation threshold value for iAs: at low iAs exposure the animals were capable of biotransforming the iAs to organoarsenic species, presumably as a detoxification process, but at higher exposure to iAs the biotransformation threshold was exceeded and the animals deposit

and accumulated the iAs in their tissues [30]. From our results and given the number of samples analysed in the present study we cannot be generalised to indicate any relationship between iAs and tAs in shellfish. On the other hand and as expected, a positive correlation between AB and tAs for fish, crustaceans and bivalves samples (n=22) is observed (**Figure 51).** This relationship could be predictable since AB is the predominant arsenic compound in these food samples (>80% of the sum As species).

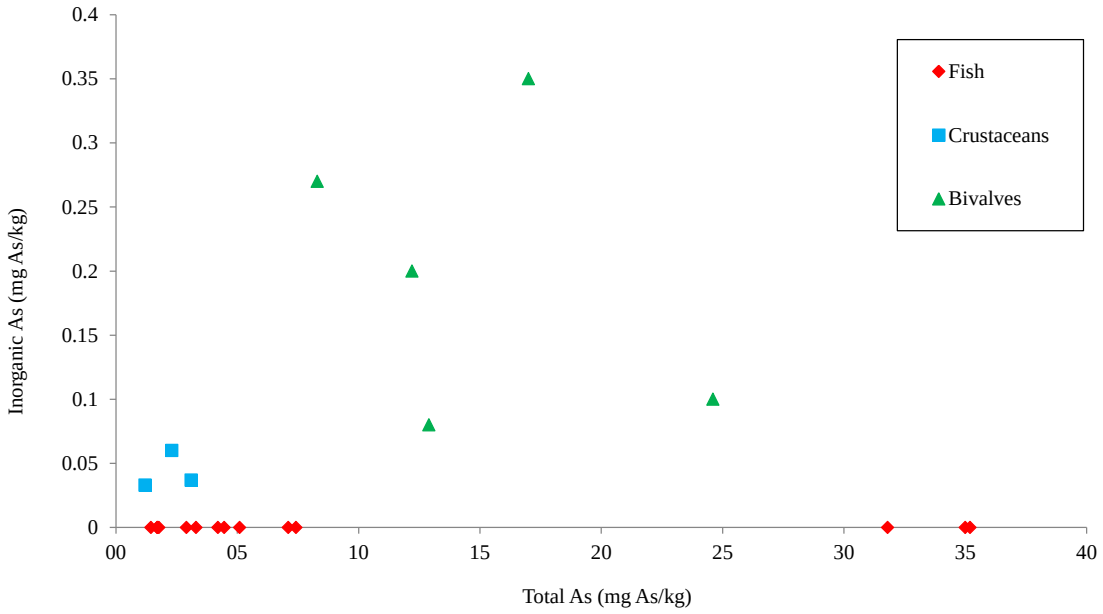


Figure 50. Plot of tAs concentration versus iAs concentration in the 22 fish (red rhombus), crustaceans (red rhombus) and bivalves (green triangles) samples from the present study

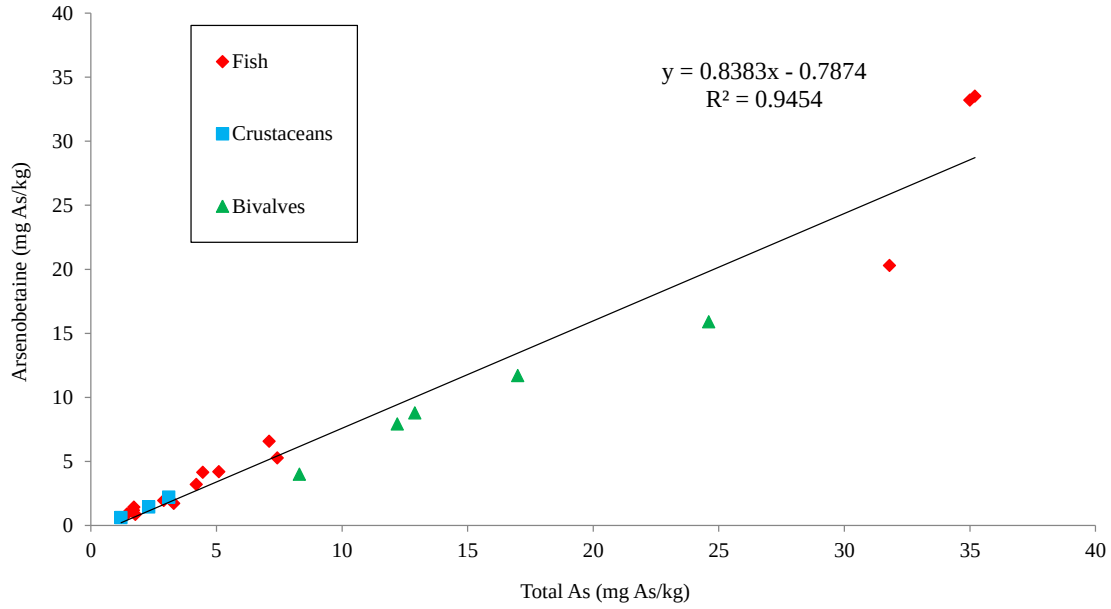


Figure 51. Relationship between AB and tAs for fish (red rhombus), crustaceans (red rhombus) and bivalves (green triangles) samples.

7.4.5 Regulation of maximum levels of arsenic in fish and shellfish

Maximum levels of inorganic arsenic in fish and aquatic animal and its products have been established by some countries and are summarised in **Table 27.** However, the European Union has not established a limit for tAs or iAs in fish and seafood in its legislation [108]. Inorganic arsenic content was not detected (LOD=2.4 µg As kg) in all fish samples (n=14) and in crustaceans (n=3) and bivalves (n=5) samples, the iAs concentration was up to 0.350 mg As kg^{-1}. Therefore the iAs levels detected in all the studied fish and shellfish samples is lower than the MLs for crustaceans and molluscs established by Australia and New Zealand [196] and China [190] (**Table 27**).

Table 27. Maximum levels of inorganic arsenic in crustaceans, fish and molluscs established by Australia and New Zealand and China .

	Maximum levels of inorganic arsenic (mg As kg^{-1})	
	Australia and New Zealand	**China**
Crustaceans	2	0.5
Fish	2	0.1
Molluscs	1	0.5

7.5 Arsenic contents in all studied food groups

In order to compare the presence and distribution of arsenic in all studied foodstuffs in this thesis, an overall discussion is summarised in the following section.

7.5.1 Total arsenic

The total arsenic content in the analysed foods is shown in **Table 28** and ranged from 0.004 to 121.7 mg As kg^{-1} (**Figure 52**). The mean arsenic concentration of all assayed foodstuffs was 7.7 mg As kg^{-1} (median= 0.20, n=137) with a high variability of As contents depending on the group of food. Low levels of arsenic were found in the assayed terrestrial foods (i.e. rice, cereal-based products, infant cereals and mushrooms) and average value was 0.26 mg As kg^{-1} (median= 0.11, n=91) and ranged from 0.004 to 2.83 mg As kg^{-1}. Arsenic content was below 0.32 mg As kg^{-1} in rice, cereal-based products and infant foods and below 2.8 mg As kg^{-1} in mushrooms. Our results are in agreement with those reported in the literature, terrestrial foods usually had levels below 0.3 mg As kg^{-1} dm [195], with the exception of some species of mushrooms which are arsenic bioaccumulator organisms and concentrations up to 146.9 mg As kg^{-1} have been reported [211].

On the other hand, high total arsenic contents were found in the analysed marine foods (i.e. fish, shellfish and seaweeds). Average concentration of all seafood samples was 22.6 mg As kg^{-1} (median= 9.1, n=46) and ranged from 1.2 to 121.7 mg As kg^{-1}. Among them, seaweeds

had higher tAs levels than fish or shellfish: tAs ranged from 2.0 to 121.7 and from 1.2 to 35.2 mg As kg^{-1} for seaweeds and, fish and shellfish, respectively. This results are concordant with the literature since marine foods are able to bioaccumulate high levels of arsenic from seawater, i,e arsenic content up to 150 and 75 mg As kg^{-1} have been reported in seaweeds and fish samples, respectively [195].

Sorting by food groups, arsenic content increases in the following order: Cereal-based products < Infant food < Rice and Rice products < Mushrooms < Shellfish < Fish < Seaweeds. This fact can be clearly observed in **Figure 52**.

Table 28. Mean and median total arsenic concentrations (mg As kg^{-1}, dry mass) in the assayed foods. Number of analysed samples (n) and range of values are indicated.

Food group	Number of samples (n)	Total Arsenic		
		Mean value	Median value	Range
Cereal-based products	21	0.007	0.007	0.004 to 0.023
Infant food	18	0.073	0.038	0.008 to 0.31
Rice and rice products	20	0.170	0.172	0.08 to 0.32
Mushrooms	32	0.588	0.280	0.05 to 2.8
Shellfish	8	10.2	10.3	1.2 to 24.6
Fish	14	10.2	4.3	1.4 to 35.2
Seaweeds	24	34.4	23.7	2.0 to 121.7

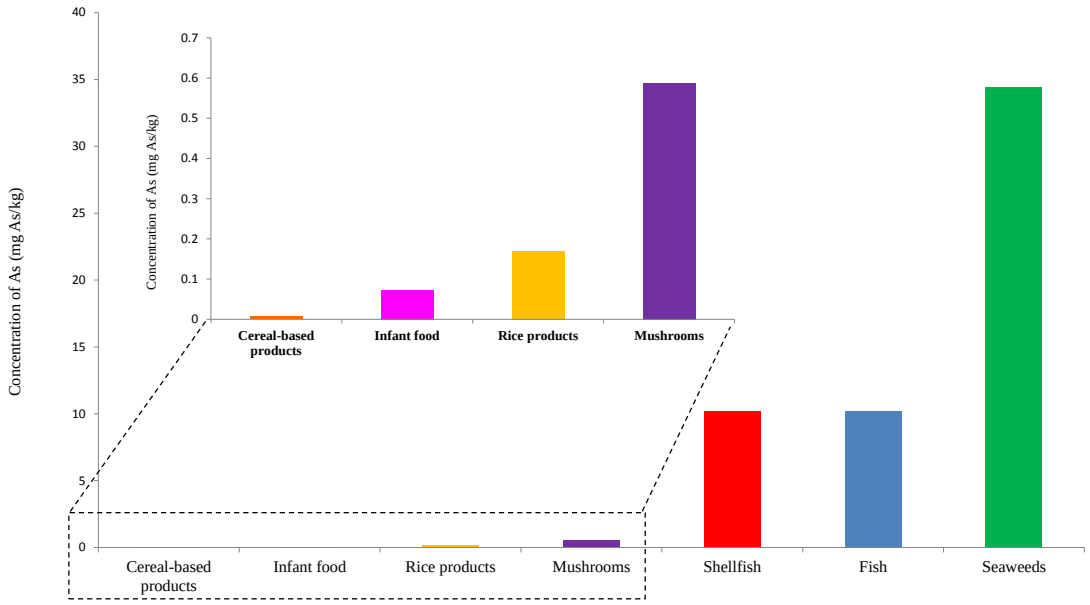


Figure 52. Total arsenic concentration in the studied food groups.

7.5.2 Arsenic species

Arsenic species concentration and distribution (%) for all studied food groups are shown in **Table 29** and **Figure 53,** respectively**.** The occurrence of arsenic species in foodstuffs differs according to type of food group, which is in agreement with the literature [100]. The differences between the speciation patterns are clearly highlighted in **Figure 53**, which shows the proportions of arsenic compounds found for each food groups analysed.

The toxic inorganic arsenic was determined in all food groups except in fish samples which was below the detection limits, iAs ranged from 2.6 to 100% of the sum of arsenic species depending of the type of food and is the predominant arsenic compound in cereal-based products, infant cereals, rice and mushrooms and accounted from 69.5 to 100% with a mean percentage of 79.8 % in these food groups. The non-toxic arsenobetaine was found in mushrooms, seaweeds, shellfish and fish; being a minor compound in the first two groups and the predominant compound in the last two accounting for 84.6 and 91.3% of the sum of arsenic species, respectively. AB was not detected in cereal-based products, infant cereals and rice samples. Our data clearly show that arsenosugars are the most abundant arsenic compounds in seaweeds, 63.8% of the sum of As species. Besides, these were quantified in some mushroom species but were not detected in cereal-based products, infant cereals, rice, shellfish and fish samples. Other organoarsenicals, which could be considered as potentially toxic [2, 100], such as DMA, MA, TMAO, AC, TMAP and TETRA were found in less proportion depending of the food group. DMA was detected in all studied food groups except in cereal-based products and ranged from 1.0 to 28.6% with an average value of 11.5%. MA, AC and TMAO were determined in some food groups and accounted for a few percent of the extracted species; MA accounted for 0.1 to 5.4%, AC accounted for 0.1 to 0.6% and TMAO accounted for 0.6 to 1.8% of sum of arsenic species. Due to the lack of appropriate standards, TMAP and TETRA were identified by comparison with the literature due to the matching of the retention time when using the same chromatographic conditions [212] (Kirby, 2004). However, these identifications need to be verified with detailed spiking and molecular mass spectrometry experiments with an authentic standard. TMAP was found in mushrooms, crustaceans and bivalves and fish and accounted for 0.2 to 5.4%. Besides, TETRA was quantified in mushrooms and crustaceans and bivalves and accounted for 0.2 and 1.7%, respectively.

Two anionic unknown compounds, UA-A and UA-B, were separated and determined by AEC-HPLC-ICPMS in some shellfish samples. These unknowns were well separated from other arsenic compounds and were found as minor species. These unknown peaks accounted for 0.2 and 5.4% for UA-A and UA-B, respectively. Besides, some unknown compounds (both anionic and cationic species) were found in several seaweeds. Unfortunately, its identification could not be performed due to the lack of appropriate standards. Therefore we decided to group these arsenic species as the sum of these anionic and cationic species, UA and UC, respectively (**Table 29 and Figure 53**) to check their contribution to the sum of all arsenic species. As can be observed, the proportion of these unknowns respect to the overall distribution of arsenic species in seaweeds is insignificant.

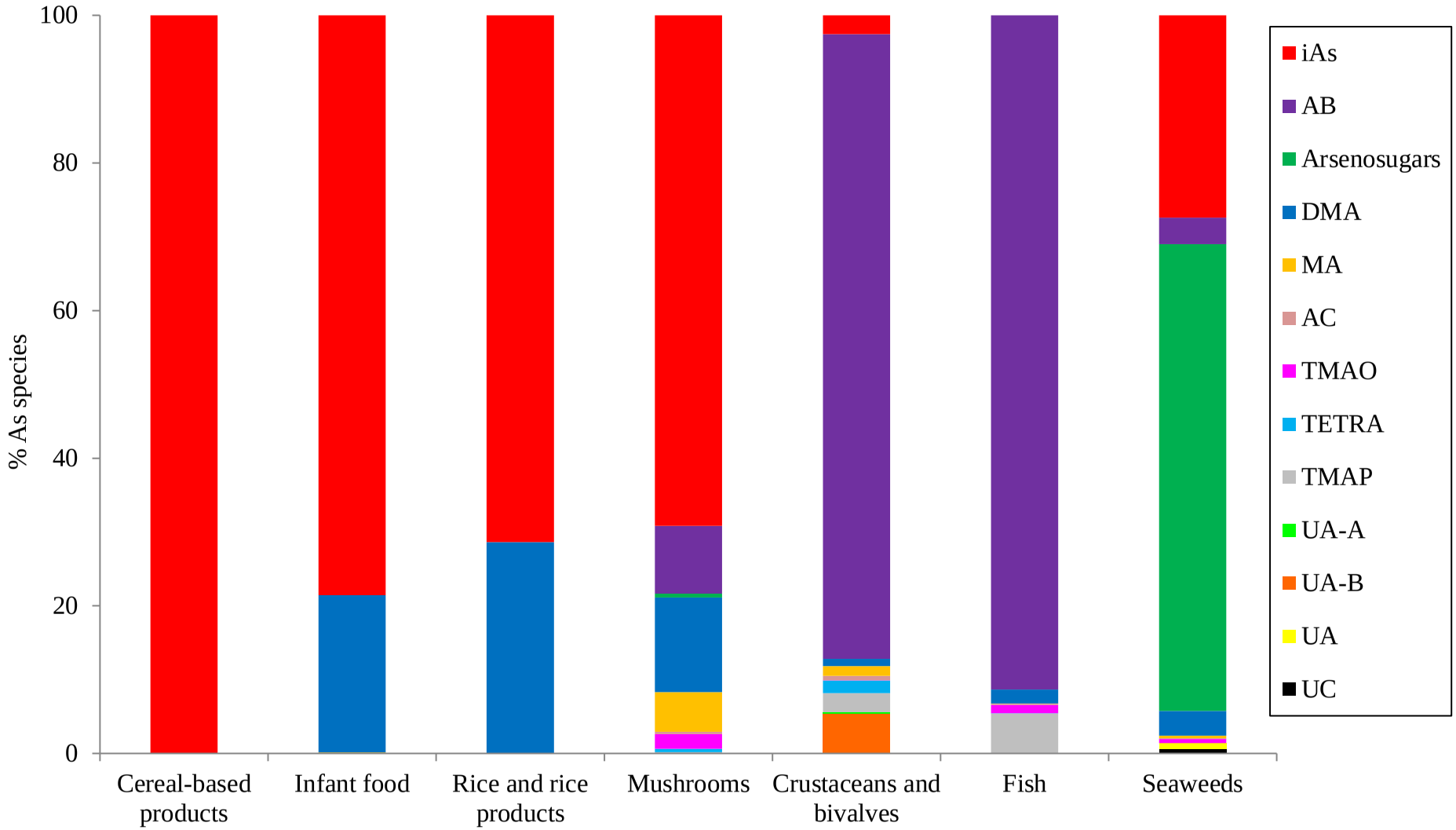


Figure 53. Proportions of arsenic species in extracts of studied foodstuffs with respect to the sum of arsenic species,. UA-A and UA-B are unknown anionic arsenic species found in some shellfish samples that does not match any available standards. UA and UB represent the sum of all anionic and cationic arsenic species found in seaweed samples, respectively

Table 29. Mean concentrations of arsenic species in foodstuffs (mg kg^{-1}). Range of values, number of samples (n) and number of times detected for each arsenic species (N) are shown.

Food type		DMA	MA	iAs	AB	TMAO	AC	TMAP	TETRA	PO_4-sugar	SO_3-sugar	SO_4-sugar	Gly-sug	Unknown anions [a]	Unknown cations [b]	UA-A [c]	UA-B [c]
Cereal-based products	**mean**	<LOD	<LOD	0.007	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
n=21	**range**	…	…	0.003-0.023	…	…	…	…	…	…	…	…	…	…	…	…	…
	N	0	0	21	0	0	0	0	0	0	0	0	0	0	0	0	0
Infant food	**mean**	0.041	0.006	0.044	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
n=18	**range**	0.000-0.183	0.000-0.0063	0.000-0.200	…	…	…	…	…	…	…	…	…	…	…	…	…
	N	12	1	18	0	0	0	0	0	0	0	0	0	0	0	0	0
Rice and rice products	**mean**	0.041	<LOD	0.112	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
n=20	**range**	0.016-0.081	…	0.034-0.239	…	…	…	…	…	…	…	…	…	…	…	…	…
	N	20	0	20	0	0	0	0	0	0	0	0	0	0	0	0	0
Mushrooms	**mean**	0.076	0.057	0.284	0.331	0.030	0.022	0.029	0.030	0.051	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
n=32	**range**	0-0.867	0-0.760	0-1.380	0-1.493	0-0.173	0-0.045	0-0.049	0-0.077	0-0.067	…	…	…	…	…	…	…
	N	27	21	32	12	12	4	2	5	3	0	0	0	0	0	0	0
Shellfish	**mean**	0.124	0.064	0.141	6.57	0.06	0.08	0.19	0.12	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.110	0.840
n=8	**range**	0-0.250	0-0.130	0-0.350	0-15.90	0.06	0-0.290	0-0.460	0-0.500	…	…	…	…	…	…	0-0.180	0-2.07
	N	5	5	8	8	1	6	8	5	0	0	0	0	0	0	2	6
Fish	**mean**	0.086	0.012	<LOD	8.46	0.03	0.05	0.88	0.10	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
n=14	**range**	0-0.240	0-0.014	…	0-33.5	0-0.080	0-0.070	0-4.53	0-0.100	…	…	…	…	…	…	…	…
	N	11	3	0	14	10	2	8	1	0	0	0	0	0	0	0	0
Seaweeds	**mean**	1.037	0.290	12.111	0.188	0.190	0.147	<LOD	<LOD	4.34	10.77	5.29	4.20	0.463	0.163	<LOD	<LOD
n=24	**range**	0-4.70	0-0.730	0-71.2	0-0.540	0-0.500	0-0.200	…	…	0-27.6	0-39.4	0-7.3	0-16.0	0-1.17	0-0.510	…	…
	N	19	4	19	15	6	3	0	0	14	10	4	24	4	6	n= 0	n= 0

[a] Sum of unknown anion arsenic species found in some seaweeds samples. [b] Sum of unknown anion arsenic species found in some seaweeds samples. [c] Unknowns anion arsenic species (UA-A and UA-B) found in some shellfish samples.

7.6 Assessment of arsenic exposure and associated health risks

To evaluate the toxicological implications from the ingestion of the all foodstuffs analysed in this thesis, i.e cereal-based products, infant food, rice and rice products, mushrooms, shellfish, fish and seaweeds a risk assessment study is performed and is presented in this section. Thus, dietary intake of tAs and iAs is estimated for an adult Catalan population and for infants and young children. Furthermore, iAs exposure is evaluated by comparison with the BMDL exposure risks range stated by the EFSA and JECFA. Moreover, as recommended by EFSA [5], margins of exposure are estimated and presented in the following paragraphs.

As commented above, the toxic effects of iAs forms led the Joint Commission FAO/WHO in 1989 to set a provisional tolerable weekly intake (PTWI) for iAs of 15 μg kg^{-1} of body weight (equivalent to 2.1 μg kg^{-1} bw per day) [102]. Recently, the EFSA [5, 104] and the FAO/WHO JECFA [103] evaluated dietary exposure to iAs. Both recommended that the dietary exposure to iAs should be reduced and reported the urgent need for further data on arsenic species, particularly iAs data, in food commodities, in order to improve the background data for future risk assessment analysis. Both concluded that the PTWI parameter was no longer appropriate and should no longer be used and it was thus withdrawn. The EFSA and JECFA evaluations provided estimates of toxicological intake limits for iAs as a BMDL of 0.3–8 μg kg^{-1} bw day^{-1} for a 1% increased risk of cancers of the lung, skin and bladder as well as skin lesions (EFSA $BMDL_{01}$) [5] and 2–7 μg kg^{-1} bw day^{-1} for a 0.5% increased risk of lung cancer (JECFA $BMDL_{0,5}$) [103].

7.6.1 Dietary exposure assessment

To assess dietary exposure to arsenic and the toxicological implications of the ingestion of foodstuffs, it is necessary to known the contribution of tAs as well as iAs in the diet of these foods. In this study, the assessment of dietary exposure of tAs and iAs is estimated following the guidelines exposed as follows. Thus, to estimate the health risk associated with food consumption, the average daily dietary tAs and iAs intake for the adult (20-65 years) Catalan population and for infant and young children (from 4 to 12 months) is estimated as the product between their mean concentration (mg As kg^{-1} wet mass) in each food group and the mean daily consumption of these food groups (g/day wet weight of uncooked food). To provide a more realistic scenario of the risk posed by iAs in food, daily intake of iAs is also calculated by considering populations with higher consumptions than average (75th, 90th, 95th, and 99th percentiles).

For dietary exposure calculations the WHO suggests a conservative estimation (upper bound) considering samples below the detection limit (LOD) with a value. In contrast, when the percentage of samples without quantifiable results is high, a lower bound approach may be used, assuming the analyte content as zero [213]. In this study both approaches (upper and lower bound) are conducted. For the upper bound estimation samples below the LOD were considered with a value of 1/2 LOD.

Food consumption data (g/day wet weight of uncooked food) for the following food groups : “cereals”, “industrial bakery” and “fish and shellfish” are obtained from the Catalan

Nutrition Survey (ENCAT) of the Health Department of the Catalan Government, [214]. Mushrooms were not included in the Catalan survey and its daily consumption is not available for the Catalan population. Therefore, daily consumption of edible mushrooms is obtained from the latest data published by the Spanish Agency for Food Safety and Nutrition [215] in ENIDE study. Seaweeds cannot be considered a staple food and their inclusion in the diet remains at low levels in Catalan and Spanish population. Daily consumption of seaweeds as a food is not available neither ENCAT nor ENIDE surveys. Furthermore, the edibility of the food group "collected fresh seaweeds" is unknown for us, thus they are not considered in the general study of Catalan population. Moreover, two specific studies of daily intake of tAs and iAs are performed considering high consumers of seaweeds ("commercial edible seaweeds") and specific consumers of mushroom supplements, respectively. Besides, daily intake of tAs and iAs is estimated in infants and young children considering the recommended amount of serving as stated in each label product.

The daily tAs and iAs intakes per body weight (b.w) are calculated using 70 kg for adults as the average weight of the Catalan population. The daily As and iAs intakes per b.w are also estimated for infants and young children at different ages: 4 months (6.65 kg), 6 months (7.75 kg), 8 months (8.30 kg) and 12 months (9.30 kg) [216].

7.6.2 Margins of Exposure (MOEs)

Margins of exposure (MOEs) have been adopted by the EFSA [5] and JECFA [103] as the preferred approach for evaluating genotoxic carcinogens in food, among them iAs [96]. For genotoxic and carcinogenic substances, for which no dose can be considered free of a potential effect, the MOE is defined as a dimensionless ratio between a reference point on the observed dose range from experimental studies and the estimated dietary exposure in humans. This reference point corresponds to the daily dose causing a low but measurable increase in the incidence of tumors. EFSA established a limit of the benchmark dose causing a 1% extra risk ($BMDL_{01}$) for distinct end points (including lung and bladder cancer) [5] meanwhile the JECFA established a limit on the benchmark dose for a 0.5% increase in the incidence of lung cancer ($BMDL_{0.5}$) [103]. In this study, an assessment of MOEs is made between the above-mentioned reference points and the estimated dietary intake for iAs.

7.6.3 Risk assessment estimation for adult population

If one considers all the above statements and criteria, daily tAs and iAs intake is estimated in cereals, industrial bakery, mushrooms, fish and shellfish samples for an adult Catalan population. Since the consumption of seaweeds is very low in Catalan and Spanish population, these cannot be considered a staple food and therefore seaweeds are not included in this global survey. Besides, considering that mushroom supplements are destined to a specific group of population and not to all population they are excluded to this overall evaluation of iAs exposure in Catalan population.

Daily intake of tAs and iAs is calculated by considering average iAs concentration for each food group and daily consumption of foodstuffs. Adult average daily tAs and iAs intake for terrestrial and marine food groups using the lower bound estimation is summarized in

Tables 30 and 31, respectively. Thus, **Table 30** shows the estimation for cereals, industrial bakery and mushrooms and **Table 31** for fish and shellfish. The overall result of each table is shown in both approaches, lower and upper bound estimations, since the WHO recommends showing both when possible. The total average dietary intake for an adult is 162.1 μg As day^{-1} $person^{-1}$ and is obtained by adding the overall result of intake through seafood consumption + intake through samples of terrestrial origin (**Table 32**). Dietary intake of tAs and iAs using both lower and upper bound estimations is shown in **Table 32.** There are no differences when both estimations are compared for tAs as only a breakfast cereal sample was below our LOD. For upper bound estimation of iAs, half of the LOD is considered in samples where iAs content was below LOD in all fish samples. However, almost no effect on iAs intake when both estimations are compared (14.97 and 15.00 μg As day^{-1} $person^{-1}$).

As previously reported [207, 217, 218], fish is the highest contributor to tAs intake (130.9 μg As day^{-1}; 81% contribution) due to both its high tAs concentration and high consumption (**Figure 54**). However, in fish more than 90% of tAs content is harmless arsenobetaine highlighting the importance of speciation studies, as is evident that the tAs concentration does not provide enough information about the toxicity of the food sample and is not adequate as an indicator for risk assessment [2]. In contrast to fish, cereals contributed only 13% of the dietary intake of tAs, bivalves and crustaceans accounted for 4% and 2% of the contribution, respectively. The contribution of industrial bakery and mushrooms is negligible due to both low consumption and its low tAs concentration compared to fish and shellfish samples. On the other hand and as expected, the main contributor to iAs dairy intake are the group of cereals (14.41 μg As day^{-1}; 96% contribution) (**Figure 54**). Among them, rice is the highest contributor to iAs intake (4.41 μg As day^{-1}) followed by pasta (0.735 μg As day^{-1}) and French bread (0.540 μg As day^{-1}). Only a relatively small proportion of dietary iAs intake came from mushrooms (2%) and industrial bakery (1%). As previously reported [207, 217, 218], the contribution of bivalves and crustaceans is negligible compared to cereal samples due to both low consumption and its low iAs concentration. Inversely to tAs (contribution is 81%), the contribution of fish to daily iAs intake is null (iAs<LOD).

Table 30. Mean concentrations of inorganic arsenic (iAs) and total arsenic (tAs), iAs contribution, mean consumption, and estimated daily intake (daily consumption x mean tAs or iAs concentration) by individual foods and food groups of terrestrial origin.

Food commodities	**N**	**Concentration (mg As kg^{-1} ww)**		**% iAs**	**Daily consumption (g/day)**	**As daily intake (µg As/kg)**	
		tAs	**iAs**			**tAs**	**iAs**
Cereals	**34**	**0.095**	**0.064**	**76**	**225.3** [a]	**21.50**	**14.41**
		(0-0.298)	(0.003-0.220)				
French Bread	3	0.006	0.005	89	107.5	0.622	0.540
Integral Bread	1	0.009	0.007	73	3.7	0.034	0.025
Sandwich Bread	1	0.012	0.010	84	5.2	0.063	0.053
Flour	3	0.006	0.006	93	n.a [b]		
Snack	3	0.005	0.004	79	n.a [b]		
Pasta	3	0.011	0.012	112	62.2	0.693	0.735
Rice	16	0.156	0.105	66	42.2	6.581	4.412
Rice crackers	4	0.161	0.101	57	0.04	0.006	0.004
Industrial bakery	**7**	**0.006**	**0.006**	**97**	**45.5** [a]	**0.25**	**0.26**
		(0-0.010)	(0.003-0.009)				
Cookies	3	0.005	0.005	107	4.3	0.020	0.021
Breakfast cereals	4	0.006	0.006	87	1.3	0.008	0.008
Mushrooms	**26**	**0.063**	**0.031**	**63**	**5.61** [c]	**0.36**	**0.17**
		(0.004-0.283)	(0.002-0.138)				
Fresh mushrooms	19	0.076	0.033	54	5.61	0.42	0.18
Canned mushrooms	3	0.033	0.030	96	0.60	0.18	0.17
Dehydrated mushrooms [d]	4	0.029	0.023	79	n.a [b]		
Total samples of terrestrial food (Lb/Ub) [e]	**67**					**22.1/22.1**	**14.8/14.8**

[a] These mean consumptions correspond to the total consumption for the Catalan population according to the Catalan Nutrition Survey (ENCAT [214]) and not only to individual consumption of the individual products shown in the table.
[b] Individual consumption estimates of these food samples are not available.
[c] These mean mushroom consumption correspond to the total consumption for the Spanish population according to the latest data published by the Spanish Agency for Food Safety and Nutrition [215] and not only to individual consumption of the individual products shown in the table.
[d] Concentrations of tAs and iAs in dehydrated mushrooms are calculated on the basis of the mass of the food prior to drying by applying a dehydration rate of the food raw material.
[e] Lb, lower bound estimate (considering samples <LOD = 0); Ub, upper bound estimate (considering samples <LOD = 1/2*LOD.

Table 31. Mean concentrations of inorganic arsenic (iAs) and total arsenic (tAs), iAs contribution, mean consumption, and estimated daily intake (daily consumption x mean tAs or iAs concentration) by individual foods and food groups of marine origin.

Samples	N	Concentration (mg As kg^{-1} ww)		% iAs	Daily consumption	As daily intake (µg As/kg)	
		tAs	iAs		(g/day)	tAs	iAs
Fish	**14**	**3.30 (0.43-12.72)**	**0.00**	**0**	**39.7**	**130.9**	**0**
Sardine		2.97	<LOD	0	2.8	8.3	0
White fish		10.56	<LOD	0	n.a [b]	0.0	0
Red porgy		10.50	<LOD	0	n.a [b]	0.0	0
Hake-1		2.13	<LOD	0	15.8	33.7	0
Hake-2		1.68	<LOD	0	15.8	26.5	0
Forkbeard		12.72	<LOD	0	n.a [b]	0.0	0
Salmon-1		0.53	<LOD	0	2.0	1.1	0
Salmon-2		0.55	<LOD	0	2.0	1.1	0
Tuna-1		0.43	<LOD	0	1.5	0.6	0
Tuna-2		0.51	<LOD	0	1.5	0.8	0
Louvar		1.34	<LOD	0	n.a [b]	0.0	0
Swordfish-1		1.02	<LOD	0	0.1	0.1	0
Swordfish-2		0.66	<LOD	0	0.1	0.1	0
Swordfish-3		0.58	<LOD	0	0.1	0.1	0
Bivalves	**5**	**2.56 (1.42-4.92)**	**0.035 (0.009-0.058)**	**1.6**	**2.49**	**6.4**	**0.086**
Clam-1		2.81	0.058	2	0.46	1.29	0.027
Clam-2		2.01	0.033	2	0.46	0.93	0.015
Mussel		1.42	0.009	1	1.40	1.99	0.012
Cockle		1.66	0.054	3	0.18	0.30	0.010
Oyster		4.92	0.020	0	0.01	0.05	0.000
Crustaceans	**3**	**0.57 (0.31-0.81)**	**0.011 (0.010-0.016)**	**2.2**	**4.67**	**2.7**	**0.053**
Prawn-1		0.60	0.016	3	3.5	2.09	0.055
Prawn-2		0.81	0.010	1	3.5	2.82	0.034
Shrimp		0.31	0.009	3	1.62	0.51	0.014
Total samples of marine food (Lb/Ub) [c]	**22**					**140.0/140.0**	**0.14/0.17**

[a] This mean consumption correspond to the total consumption "fish and shellfish" for the Catalan population according to the Catalan Nutrition Survey (ENCAT [214]) and not only to individual consumption of the individual products shown in the table.

[b] Daily consumption of these foods are not available for the Catalan population; therefore As daily intake cannot be estimated.

[c] Lb, lower bound estimate (considering samples <LOD = 0); Ub, upper bound estimate (considering samples <LOD = 1/2*LOD

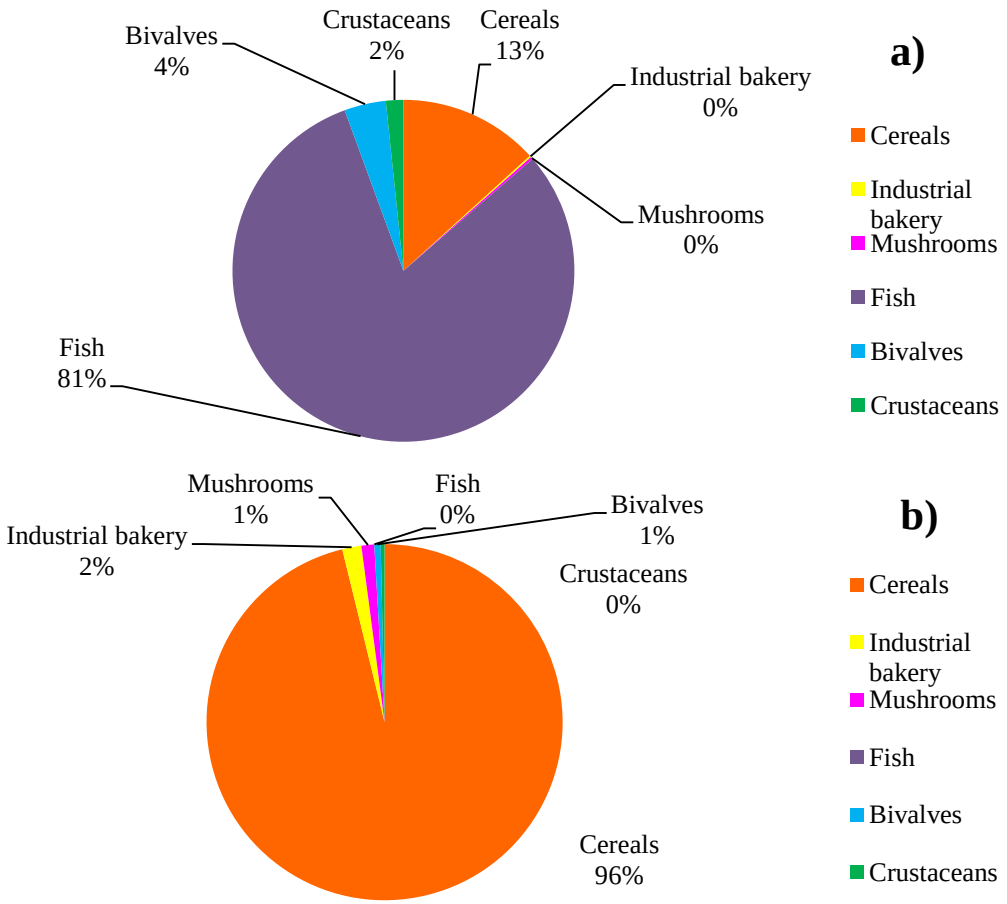


Figure 54. Contribution of food groups to daily intake of total (a) and inorganic arsenic (b) for adult population.

Table 32. The average tAs and iAs dietary intake for adult Catalan population by groups of terrestrial and marine origin and by all food samples using the lower and upper bound estimations.

	N	Daily consumption (g/day)	As daily intake (µg As/day)			
			Lower bound estimation		Upper bound estimation	
			tAs	iAs	tAs	iAs
Total samples of terrestrial food	67	276.4	22.1	14.8	22.1	14.8
Total samples of marine food	22	46.9	140.0	0.14	140.0	0.17
Total food samples	89	323.3	162.1	14.97	162.1	15.00

Data from total diet studies conducted in several countries worldwide are presented in **Table 33.** Compared to total diet studies, the number and variety of studied samples in this thesis is limited. If we focus on dietary studies on Catalan population that are similar than our study, the present results for tAs dietary As intake are in the range to those estimated in some previous studies for the same population [209, 219, 220] meanwhile are lower than other similar studies [207, 221]. The comparison between different diet studies may be difficult for design and methodological reasons and high variability is found from data reported in other countries. Our results are in the range of values reported by other countries such as USA [222] and India [223]. Meanwhile, reported data for dietary iAs intake for an adult male in the Catalan population adult ranged from 2.2 to 27.45 μg As day^{-1}. Our results are in this range of values and similar to previous estimation in the European Union [104] and lower than those estimated in other countries such as Thailand [224] and Vietnam [225] (**Table 33**). Results of dietary iAs intake are difficult to compare as some studies extrapolated iAs content considering fixed proportions of iAs from tAs for each food category instead of considering analytical iAs results.

Table 33. Daily intake of tAs and iAs from total diet studies conducted in several countries worldwide.

Country	As daily intake (μg As day^{-1})		Reference
	tAs	iAs	
Catalonia	217	3.48	EDT CAT 2015 [218]
Catalonia	171	2.2	Perello et al., 2014 [209]
Catalonia	259	14.03	Martorell et al., 2011[226]
Catalonia	199	n.e	Domingo et al., 2012 [220]
Catalonia	354	6.1	Fontcuberta et al., 2011 [207]
Catalonia	344	27.4	EDT CAT 2008 [221]
Catalonia	206	27.45	Marti-Cid et al., 2008 [219]
Catalonia	223.6	n.e	Llobet et al., 2003 [227]
Bangladesh	214	n.e	Khan et al., 2009 [228]
Belgium	285-649	n.e	Baeyens et al., 2009 [229]
China	n.e	42	Li et al., 2011 [230]
China	4.24	n.e	Chen et al., 2011 [231]
France	54.88	16.94	Arnich et al., 2012 [232]
India	150	n.e	Signes et al., 2008 [223]
Japan	27	3.8	Oguri et al., 2012 [233]
New Zealand	211	n.e	Whyte et al., 2009 [234]
Thailand	73-390	n.e	Ruangwises and Saipna 2010 [235]
Thailand	262	62	Ruangwises et al., 2011 [224]
USA	195	n.e	Cleland et al., 2009 [222]
Vietnam		28-102	Hanh et al., 2011 [225]

n.e means not evaluated

Taking the EFSA and JECFA BMDL parameters as a reference points and considering a mean body weight of 70 kg , the consumption of the studied foodstuffs would lead to an exposure to 0.21 μg kg^{-1} bw day^{-1} for adult average consumers. The contribution of each group of food is represented in **Figure 55** and is clearly observed cereals (rice bar) are the main

contributor. Despite the high occurrence levels of tAs in fish, its contribution is imperceptible. Furthermore, the contribution of the remaining groups (mushrooms, industrial bakery, bivalves and crustaceans) is negligible. Our estimated exposure to iAs is below the benchmark doses: $BMDL_{01}$ of 0.3-8 µg kg^{-1} b.w. per day (**Figure 55)** established by EFSA [5] and also below $BMDL_{05}$ of 2-7 µg kg^{-1} b.w. per day by JECFA [103]. Our results are in the range estimated by EFSA from food and water across several European countries (0.09 to 0.38 µg kg^{-1} bw day^{-1}) for the average adult consumer [104].

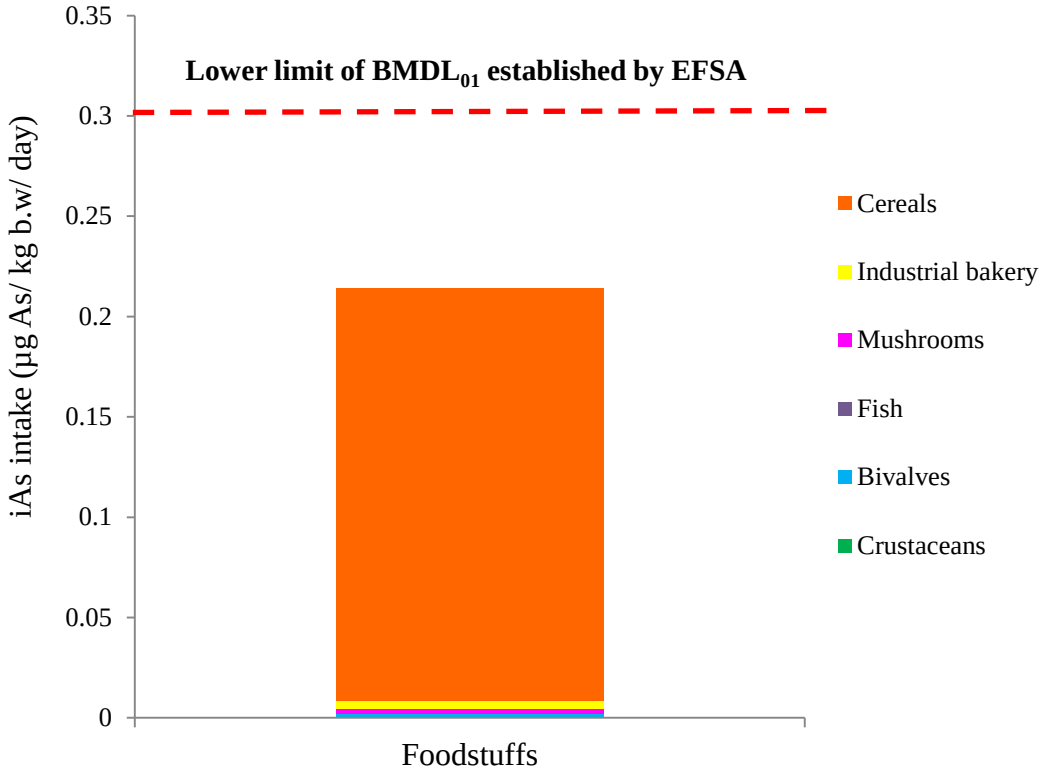


Figure 55. Estimated exposure to iAs for adult Catalan population consuming the studied foodstuffs. Red dotted line represents the lower limit of $BMDL_{01}$ established by EFSA [5]

As commented above, daily intake of iAs is calculated by considering average iAs concentration for each food group and average consumption of foodstuffs. The estimation of 75th, 90th, 95th, and 99th percentiles for food consumption by the Catalan population and estimated iAs intake for fresh fish, bivalves, crustaceans, rice and rice crackers using the lower bound estimation is shown in **Table 34**. The overall dietary intake of iAs is shown in both approaches, lower and upper bound estimations. There are no differences when both estimations are applied for iAs as only fish samples were below LOD (<0.0014) and almost no effect on iAs intake when both estimations are compared for each percentiles for food consumption (**Table 34).** As expected and as can be noted, considering populations with higher consumption than the average (75th, 90th, 95th, and 99th percentiles) would lead a higher iAs daily intake than those estimated previously (**Tables 30. 31, 32)**. For instance, when adult Catalans among high consumers (95th percentile) of rice their iAs mean dietary exposure is 0.30 µg kg^{-1} bw day^{-1}.

Considering high consumers (95th percentile) of all selected food groups (fish, bivalves, crustaceans, rice and rice crackers) their iAs mean dietary exposure (0.31 μg kg^{-1} bw day^{-1}) is within the BMDL range (0.3 and 8) proposed by the EFSA [5] but could fall below the interval identified by the JECFA (2 and 7 μg/kg bw/day) [103].

Table 34. 75th, 90th, 95th, and 99th percentiles for food consumption by the Catalan population and estimated inorganic arsenic intake.

Food group	Daily consumption (g day^{-1} wet weight)					iAs daily intake (μg As day^{-1})			
	P75	P90	P95	P99		P75	P90	P95	P99
Fresh fish	75	147	167	250		0	0	0	0
Bivalves	0	0	15	60		0	0	0.52	2.08
Crustaceans	0	13	30	80		0	0.15	0.34	0.90
Rice	28	135	200	299		2.93	14.11	20.91	31.26
Rice crackers	0	0	0	0		0	0	0	0
Total food samples					**Lb [a]**	**2.93**	**14.26**	**21.77**	**34.24**
					Ub [a]	**2.98**	**14.36**	**21.88**	**34.42**

[a] Lb, lower bound estimate (considering samples <LOD = 0); Ub, upper bound estimate (considering samples <LOD = 1/2LOD)

Risk assessment is carried out following the recommendations of the EFSA for the exposure to genotoxic and carcinogenic substances using the margin of exposure (MOE). The MOE is defined as a ratio between a reference point on the observed dose range from experimental studies and the estimated dietary exposure in a particular situation or population. Thus, MOE is calculated as a ratio between $BMDL_{01}$ and the estimated iAs dietary exposure (0.21 μg kg^{-1} bw day^{-1}). Therefore, in a particular situation or population is desirable a MOE value as high as possible which corresponds to exposure of low potential risk. Estimated MOEs for the average adult Catalan consumer ranged between 1.4 and 5.7 considering the lower and higher limits of BMDL01 (0.3 and 8 μg kg^{-1} b.w. per day), respectively. The lower end of these MOEs indicates that the possibility of a potential risk to average adult consumers is excluded as MOE> 1. Nevertheless, considering the adults with high consumptions (95th percentile) of fresh fish, bivalves, crustaceans, rice and rice crackers, estimated MOEs ranged between 0.96 and 8.29. The lower end of this interval is low enough to indicate that the possibility of a risk to some consumers cannot be excluded (MOE< 1) [5].

7.6.4 Dietary exposure to arsenic for high consumers of selected foods

Mushroom supplements and seaweeds are destined to a specific group of population and are excluded to the overall evaluation of iAs exposure in Catalan population presented before. Therefore in this section, a specific dietary exposure to arsenic for consumers of this selected food groups is shown.

Consumers of mushroom supplements

Dietary supplements have beneficial health effects and are popular due to their easy accessibility, therapeutic efficacy and relatively low cost. Some dietary supplements are made from edible mushrooms and may contain elevated concentrations of arsenic, contributing to the overall exposure to this contaminant. Although the consumption of mushrooms and their supplements has increased considerably in recent years due to their nutritional properties, few studies of daily intake of tAs and iAs considering the consumption of these supplements have been reported.

The exposure to iAs from the consumption of the mushroom supplements in the present study is calculated by multiplying the recommended amount of consumption (g/day) with the iAs content (mg As kg^{-1} wet mass) of each sample (**Table 35**). The recommended daily consumption of mushroom supplements was derived from the recommended number of pills per day as stated on the product label (2 to 6 pills per day; 4 per day is used for calculations) and the mass of the pills. The daily exposure to tAs and iAs range from 0.29 to 1.26 μg iAs day^{-1} and from 0.23 to 0.98 μg iAs day^{-1}, respectively depending on the mushroom supplement (**Table 35**). The highest exposure of tAs and iAs came from one mushroom supplements based on *L .edodes,* and the lowest from *C. versicolor* supplement. This finding is clearly observed in **Figure 56** where the contribution to tAs intake for each mushroom supplement is represented. *L.edodes*-I is the highest contributor to tAs intake (39% contribution) as well as iAs intake (43% contribution) (**Figure 56**) due to both its high As concentrations (**Table 35**).

Furthermore, considering average tAs and iAs concentration for the group of "mushroom supplements", average daily intake of tAs and iAs is: 0.53 and 0.39 μg iAs day^{-1}, respectively (**Table 35)**. The overall result is calculated in both approaches, lower and upper bound estimations, and there are no differences when both estimations are compared for tAs or iAs as samples are above the established LODs.

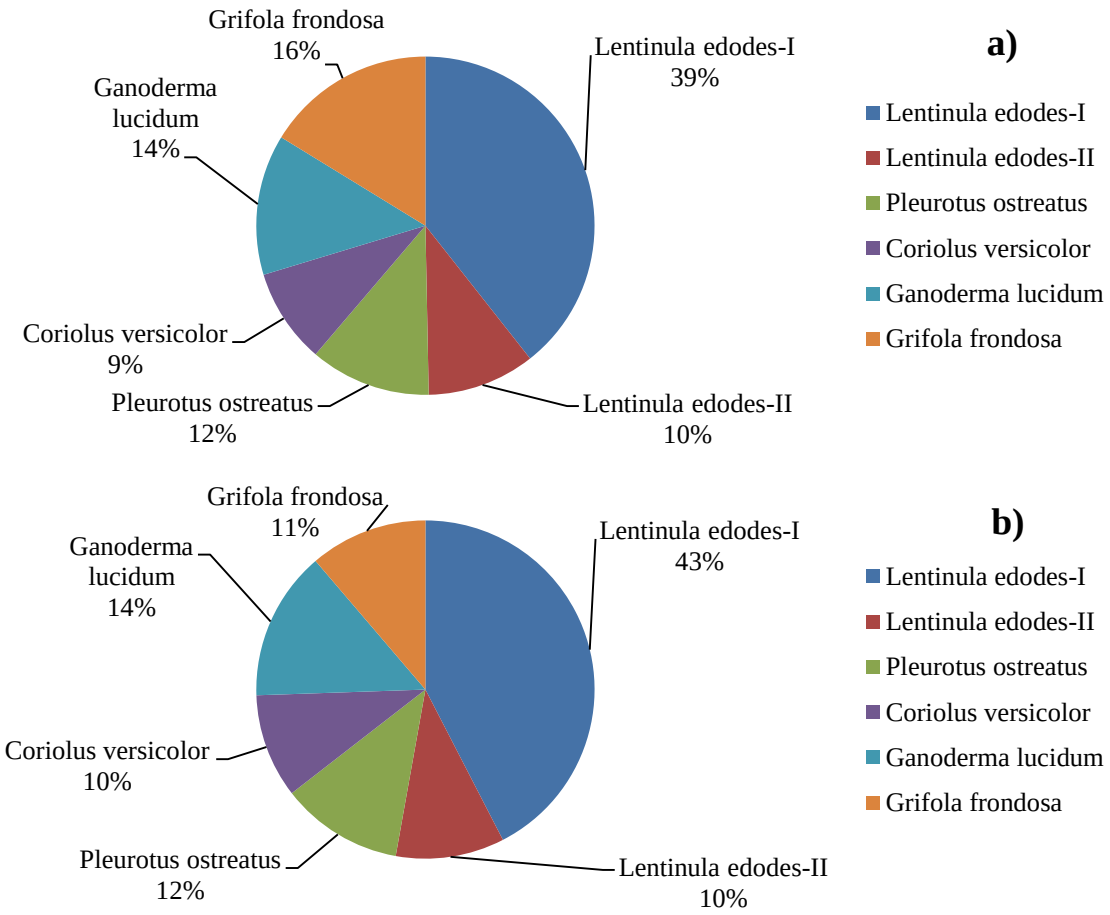


Figure 56. Contribution of each mushroom supplement to total (a) and inorganic arsenic (b) daily intake for specific consumers

Taking the BMDL parameters established by EFSA and JECFA as a reference points and for an adult of 70 kg body weight, the consumption of the analysed samples would lead to an exposure ranging from 0.003 to 0.0014 μg kg^{-1} bw day^{-1} from this dietary source alone, depending on the mushroom supplement ingested. Considering average tAs and iAs concentration, the consumption of these food supplements would lead to an average exposure of 0,006 μg kg^{-1} bw day^{-1}. The contribution to the present exposure to iAs is quite low relative to the estimated exposure for the average consumer in European countries [104] and is considerably lower than the limits recommended by EFSA and JECFA. Considering the average case scenario (0.006 μg kg^{-1} bw day^{-1}), the present exposure corresponds to 1.9% and 0.3% of the lower value of the EFSA and JECFA BMDL ranges, respectively. This estimation is made considering the number of pills per day as stated on the product label: 2 to 6 pills per day and 4 pills per day is used for calculations. Even considering the ingestion of 10 pills per day the exposure to iAs would be quite low compared to BMDL ranges and the toxicological risk could be considered low

Table 35. Mean concentrations of inorganic arsenic (iAs) and total arsenic (tAs), iAs contribution, recommended consumption, and estimated daily intake (recommended consumption x mean tAs or iAs concentration) by individual mushroom supplements.

Mushroom supplements	N	Concentration (mg As kg^{-1} ww)		% iAs	Recommended amount of consumption [a]	As daily intake (µg As/kg)	
		tAs	iAs		(g/day)	tAs	iAs
Mushroom supplements [b]	**6**	**0.175**	**0.127**	**72**	**3.04**	**0.53/0.53**	**0.39/0.39**
Lentinula edodes-I [c]		0.413	0.321	78	3.05	1.26	0.98
Lentinula edodes-II [c]		0.108	0.079	73	3.05	0.33	0.24
Pleurotus ostreatus		0.120	0.087	73	3.05	0.37	0.27
Coriolus versicolor		0.097	0.077	79	3.04	0.29	0.23
Ganoderma lucidum		0.140	0.110	79	3.04	0.43	0.33
Grifola frondosa		0.170	0.087	51	3.04	0.52	0.26

[a] Recommended amount of consumption of mushroom supplements is derived from the recommended number of pills per day as stated on the product label (2 to 6 pills per day; 4 per day is used for calculations) and the mass of the pills.
[b] Lb, lower bound estimate (considering samples <LOD = 0); Ub, upper bound estimate (considering samples <LOD = 1/2*LOD. [c] Two *Lentinula edodes*-based supplements from different brand are analysed.

Consumers of edible seaweeds

Since the consumption of seaweeds is very low in Catalan and Spanish population and cannot be considered a staple food, seaweeds are not included in the global survey of Catalan population abovementioned. The edibility of seaweeds collected from the Catalan coast (LLoret de mar Beach) is unknown for us, thus this are not considered to estimate daily intake of tAs and iAs. Therefore in the following paragraph, a specific dietary exposure to arsenic for consumers of commercially edible seaweeds is presented.

The maximum exposure to iAs from the consumption of the seaweeds in the present study is calculated by multiplying the recommended amount of consumption (g/day) with the iAs content (mg As kg^{-1} wet mass) of each sample (**Table 36**). Daily consumption of seaweeds as a food was not available neither in the Catalan and Spanish surveys. The recommended daily consumption of seaweeds is derived from the recommended amount of serving as stated on the product label of *S. fusiforme* (Hijiki) (4 g per week). No indications of the recommended dose are available on the product label of the other seaweeds analysed.

Table 36. Mean concentrations of inorganic arsenic (iAs) and total arsenic (tAs), iAs contribution, recommended consumption, and estimated daily intake (recommended consumption x mean tAs or iAs concentration) by individual edible seaweeds.

Seaweeds	N	Concentration (mg As kg^{-1} ww) [a]		% iAs	Recommended amount of consumption [b]	As daily intake (µg As/kg)	
		tAs	iAs		(g/day)	tAs	iAs
Edible seaweeds [c]	**11**	**8.61**	**2.78**	**17**	**0.57**	**4.92**	**1.59**
Chondrus crispus		2.73	0.08	3	0.57	1.56	0.04
Porphyra purpurea		6.11	<LOD	-	0.57	3.49	0
Ulva rigida		0.87	0.05	5	0.57	0.50	0.03
Laminaria ochroleuca		8.52	<LOD	-	0.57	4.87	0
Laminaria saccharina		7.86	<LOD	-	0.57	4.49	0
Undaria pinnatifida-I [d]		6.15	0.04	1	0.57	3.51	0.02
Undaria pinnatifida-II [d]		5.99	<LOD	-	0.57	3.42	0
Sargassum fusiforme -I [e]		18.25	10.68	59	0.57	10.43	6.10
Sargassum fusiforme –II [e]		16.61	10.41	63	0.57	9.49	5.95
Sargassum fusiforme –III [e]		15.81	9.34	59	0.57	9.03	5.34
Himanthalia elongate		5.85	<LOD	-	0.57	3.34	0

[a] Concentrations of tAs and iAs in dehydrated seaweeds are calculated on the basis of the mass of the food prior to drying by applying a dehydration rate of the food raw material.
[b] Recommended daily consumption of "edible seaweeds" is estimated from the recommended serving size as stated on the product label of Hijiki seaweed (4 g per week).
[c] Lb, lower bound estimate (considering samples <LOD = 0); Ub, upper bound estimate (considering samples <LOD = 1/2*LOD.
[d] Two individual *Undaria pinnatifida* from different brands are analysed. [e] Three individual *Sargassum fusiforme* from different brands are analysed.

The daily exposure to tAs and iAs range from 0.5 to 10.43 µg As day^{-1} and from 0.03 to 6.09 µg As day^{-1}, respectively depending on the seaweed species (**Table 36**). The highest exposure of tAs came from the three *S. fusiforme* (contribution of 17, 18 and 19%, respectively), and the lowest from *Chondrus crispus* and *Ulva rigida*: 3% and 1%, respectively This fact is observed in **Figure 57a** where the contribution to tAs intake for each seaweed is represented. As expected, the main contributor to iAs dairy intake are *S. fusiforme* seaweeds: 35%, 34%, and 30% for each one named I, II and III, respectively, thus the contribution of *S. fusiforme* species accounting of 99% contribution (**Figure 57b**). The contribution of the other seaweeds is negligible (1%) compared to Hijiki due to low iAs concentration

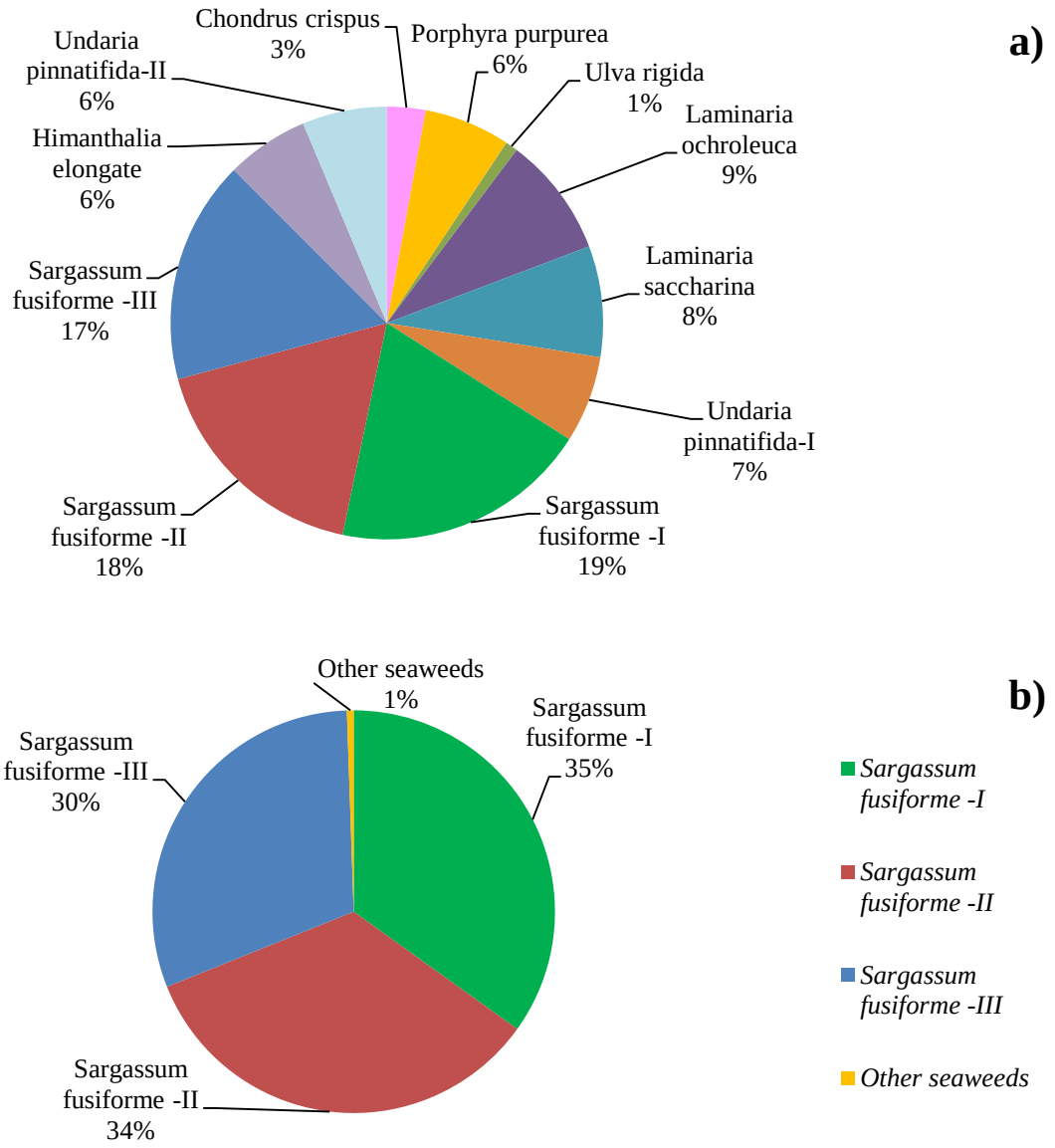


Figure 57. Contribution of each edible seaweeds to total (a) and inorganic arsenic (b) daily intake for specific consumers of this food commodity. The group of "others seaweeds" included the following species: *Chondrus crispus, Porphyra purpurea, Ulva rigida, Laminaria ochroleuca, Laminaria saccharina, Undaria pinnatifida-I, Himanthalia elongate* and *Undaria pinnatifida*-II

Furthermore, considering average tAs and iAs concentration and a consumption of 0.57g per day as a recoomended dose for the group of "edible seaweeds ", average daily intake of tAs and iAs is: 4,91 and 1,58 µg iAs day^{-1} , respectively (**Table 36)**. The overall result is calculated in both approaches, lower and upper bound estimations (**Table 36).** Although iAs content in some seaweed samples was below LOD (<0.017) there are no differences when both estimations are compared for tAs or iAs as samples are above the established LODs

For an average adult (using 70 kg body weight), the consumption of the seaweeds in the present study would lead to an exposure from 0.0004 to 0.087 µg kg^{-1} bw day^{-1} from this dietary source alone, depending on the seaweed ingested. Considering the average iAs concentration, the consumption of these seaweeds would lead to an average exposure of 0.023

μg kg^{-1} bw day^{-1} which is below the BMDL01 range identified by EFSA. Considering the worst case scenario of *S. fusiforme*-I (0.087 μg kg^{-1} bw day^{-1}), the present exposure corresponds to 29% and 4.3% of the lower value of the EFSA and JECFA BMDL ranges, respectively. Therefore, the iAs exposure is below the BMDL01 range identified by EFSA, which highlights that the risk can be excluded for adults consuming the recommended dose of seaweeds.

As is commented, the main contributor to iAs dairy intake are *S. fusiforme* seaweeds (Hijiki). Although this alga is particularly consumed in the Asian market, it is also commercialised in Europe and can be found in restaurants, supermarkets and as part of food supplements of dietary fibre and/or minerals [104]. Considering high consumers of alga Hijiki, the daily intake of iAs from the consumption of Hijiki in the present study is calculated by multiplying the average iAs content (mg As kg^{-1} wet mass) with the an estimated high consumption value (10g per day) [236, 237]. High dietary exposure to iAs is found, especially for people that regularly consume algae as food in their diet, whereby the exposure to iAs can be 1.45 μg kg^{-1} bw day^{-1} which is within the BMDL01 range identified by EFSA (2009), which highlights that the risk cannot be excluded for high consumers of *S. fusiforme* (Hijiki). Our estimation of iAs exposure is in agreement with those reported by EFSA in 2014 for high consumers: about 1.57 μg kg^{-1} b.w. per day [104] but lower than those previously advised: 4 μg/kg b.w. per day [5]. Obviously, the additional amount of consumption of alga Hijiki than those considered in this study may lead to an overall increased exposure to iAs at levels, which are considered potentially to lead to adverse health effects.

7.6.5 Risk assessment estimation for infants and young children consumers

The EFSA [5] has reviewed the diet of the European Union population and has recommended that dietary exposure to iAs in rice and young children should be reduced. The EFSA report stated that iAs risk of toxicity cannot be excluded, especially for children whose dietary exposure has been estimated to be from 2 to 3-fold (0.50–2.66 lg/kg b.wt. per day) respect to adult population [5]. Additionally, in the recent EFSA report [104], the highest dietary exposure to iAs was estimated in the younger population, mean dietary exposure among infants, toddlers and other children ranged from 0.20 to 1.37 μg kg^{-1} b.w. per day, while the 95th percentile dietary exposure estimates ranged from 0.36 to 2.09 μg kg^{-1} b.w. per day. The main contributors were 'Milk and dairy products' followed by 'Drinking water', 'Grain-based processed products (non rice-based)' and 'Food for infants and young children'. EFSA Consumption of three portions (90 grams/day) of rice-based infant food could represent an important source of iAs (1.59-1.96 μg/kg b.w. per day) [5].

Several infant food samples destined to infant and young children, i.e. infant rice, infant cereals and rice crackers were analysed in this thesis. EFSA advised that cereal and cereal-based products have been identified as high contributors to daily iAs exposure in young children (<3 years of age) which have been categorised as the most exposed to iAs. Therefore, in the following section daily tAs and iAs daily intake is estimated in infant cereals, infant rice and rice crackers for infant and young children population. The evaluation is made taking into account the statements and criteria abovementioned and the estimation is presented as follows.

Therefore, we estimated the daily tAs and iAs intake of infants and young children consuming the studied infant food products and results using the lower bound estimation are shown in **Table 37**.

Table 37. Concentrations of inorganic arsenic (iAs) and total arsenic (tAs), iAs contribution, mean consumption, and estimated daily intake (recommended serving x mean tAs or iAs concentration) by individual foods of infant food.

Samples	Concentration (mg As kg^{-1} ww)		% iAs	Recommended amount of serving	As daily intake (µg As/serving)	
	tAs	iAs		(g ww) [a]	tAs	iAs
Infant rice						
Infant rice (multigrain)	0.043	0.022	52	30	1.29	0.67
Infant rice (multigrain with fruits)	0.036	0.024	66	30	1.09	0.72
Infant rice (organic whole-wheat rice)	0.215	0.186	86	30	6.46	5.58
Infant rice (100% rice)	0.282	0.117	41	30	8.45	3.50
Infant rice (rice based-1)	0.088	0.063	72	30	2.64	1.89
Infant rice (rice based-2)	0.075	0.059	79	30	2.25	1.77
Infant rice (rice based with fruits)	0.040	0.028	69	30	1.19	0.83
Infant rice (cereals without gluten)	0.060	0.041	68	30	1.79	1.22
Infant rice (rice based-3)	0.251	0.070	28	30	7.54	2.10
Infant Cereals						
Infant cereals (corn)	<LOD	<LOD	-	30	0	0
Infant cereals (honey and fruits)	0.013	0.013	97	30	0.40	0.39
Infant cereals (organic spelt)	0.007	0.007	106	30	0.21	0.22
Infant cereals (fruits)	0.015	0.015	100	30	0.45	0.45
Infant cereals-1	0.020	0.020	101	30	0.60	0.61
Infant cereals (honey)	0.009	0.010	106	30	0.27	0.29
Infant cereals-2	0.014	0.013	94	30	0.41	0.38
Infant cereals (honey)	0.022	0.021	93	30	0.66	0.62
Infant cereals (fruits)	0.033	0.024	73	30	0.98	0.72
Rice crackers						
Rice crackers (sesame)	0.076	0.033	43	30	2.27	0.98
Rice crackers (original)	0.079	0.039	49	30	2.36	1.16
Brown rice crackers	0.249	0.152	61	30	7.46	4.56
Brown rice crackers	0.239	0.180	75	30	7.18	5.41

[a] Recommended serving correspond to the consumption of one serving (30g) of an "infant food" as stated on the product label).

Average concentrations and average intakes of tAs and iAs for each infant food group (infant food, infant cereals and rice crackers) are summarised in **Table 38**. Average dietary intake of tAs and iAs considering all groups is shown in both approaches, lower and upper bound estimations (**Table 38)**. The average content of all infant foods is 0.085 and 0.052 mg As kg^{-1} wet weight for tAs and iAs, respectively. For an infant or young children consuming one serving per day (30g/serving), tAs and iAs average dietary intake is 2.54 and 1.55 μg As day^{-1}, respectively**.** There are no differences when lower and upper bound estimations are compared for tAs and iAs as only an infant cereals (corn-based) sample was below our LODs. For upper bound estimation of tAs and iAs, half of the LOD is considered in this sample and, almost no effect on tAs and iAs intakes when both estimations are compared.

Table 38. Mean concentrations of inorganic arsenic (iAs) and total arsenic (tAs), iAs contribution, mean consumption, and estimated daily intake (recommended serving x mean tAs or iAs concentration) by infant food and by each group: infant rice, infant rice and rice crackers.

Samples	**N**	**Concentration (mg As kg^{-1} ww)**		**% iAs**	**Recommended amount of serving (g ww)** [b]	**As daily intake (μg As/serving)**	
		tAs	**iAs**			**tAs**	**iAs**
Infant cereals	9	0.015	0.014	86	30	0.44	0.41
Infant rice	9	0.121	0.067	62	30	3.63	2.03
Rice crackers	4	0.161	0.101	57	30	4.82	3.03
All infant food samples (Lb/Ub) [b]	22	0.085 (0-0.282)	0.052 (0-0.186)	69	30	2.54/2.55	1.55/1.55

[a] Recommended serving correspond to the consumption of each infant food sample as stated on the product label .
[b] Lb, lower bound estimate (considering samples <LOD = 0); Ub, upper bound estimate (considering samples <LOD = 1/2*LOD.

As expected, infant products containing rice are the highest contributor to tAs and iAs intake. For tAs daily intake, 95% of the contribution correspond to rice crackers and infant rice samples due to its high tAs concentration. Estimated tAs daily intake is 4.82 μg As $serving^{-1}$ (54%) and 3.63 μg As $serving^{-1}$ (41%) for rice crackers and infant rice, respectively (**Figure 58**). In contrast, infant cereals contributed only 5% of the dietary intake of tAs, due to its low tAs concentration compared to rice crackers and infant rice. The same pattern for iAs daily intake can be observed in **Figure 58**, the main contributor are rice crackers and infant rice: 3.03 μg As $serving^{-1}$ (55% contribution) and 2.03 μg As $serving^{-1}$ (37% contribution). Only a relatively small proportion of dietary iAs intake came from infant cereals (8%).

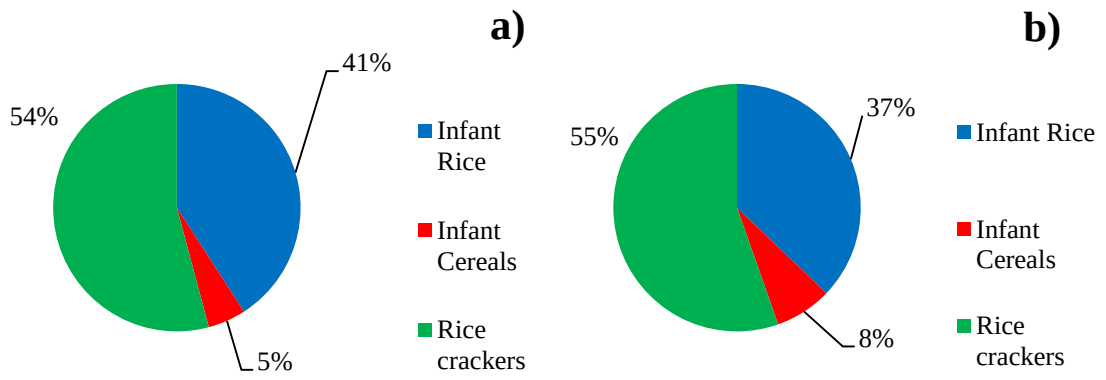


Figure 58. Contribution of infant food groups to total (a) and inorganic arsenic (b) daily intake for infants and young children

Taking as a reference point the EFSA BMDL, we estimated dietary iAs daily intake in the same units as the BMDL01 values. Considering an average iAs daily intake of 1.55 µg As serving^{-1} (n=22), an infant of 12 months (mean body weight of 9.30 kg, [216] and based on the assumption that one serving (30 g) is consumed per day, this would lead to an exposure to 0.17 µg kg^{-1} bw day^{-1}. This exposure is below the $BMDL_{01}$ range identified by EFSA [5], however, if additional servings are given, inorganic arsenic consumption is simply multiplied by the number of servings. For an infant of 12 months (mean b.w. of 9.30 kg) eating 3 servings per day their iAs daily intake would be 0.50 µg kg^{-1} bw day^{-1} which is within the $BMDL_{01}$ range identified by EFSA, so the risk cannot be excluded for infants consuming the assayed infant foods.

According to the World Health Organization [238] after 6 months of age, it becomes increasingly difficult to breastfeed infants to meet their nutrient needs from human milk alone. Thus, 6 months is the recommended appropriate age at which to introduce complementary foods. However and despite these guidelines, weaning occurs at an early age in most countries [239, 240]; for example, Santamaria-Orleans et al. [241] reported that Spanish infants started with weaning foods at 4.4 months of life. Briefel et al. [242] reported that in the period from 4 to 6 months US infants are introduced to infant cereals and pureed infant foods. During the period of 6-8 months, the consumption of infant cereals reached its highest values. Therefore, to provide a more realistic scenario of the risk posed by iAs in infant food, the iAs exposure according to the infant nutrition (three types of infant foods consumed), at four different ages: 4, 6, 8 and 12 months and considering different infant nutrition conditions (number of serving per day) is evaluated. Thus, the daily intake of iAs is calculated by considering the average iAs concentration for each infant food group individually (infant rice, infant cereals and rice crackers) and a specific discussion for each one is presented in the following paragraphs.

Infant cereals

The daily exposure to inorganic arsenic ranged from 0.00 to 0.72 µg As serving^{-1} (median=0.39, n=9) depending on the infant cereal sample (**Table 37**). The highest exposure came from an infant cereal with fruits and the lowest from an infant cereal based on organic

spelt. Average contents of tAs and iAs as well as estimation of tAs and iAs daily intake per serving (recommended serving of 30g) from infant cereals are shown in **Table 38**. Our estimation of iAs daily intake are in agreement with those reported in the literature for infant cereals [243, 244].

The plot A included in **Figure 59**, shows the iAs exposure for infants and young children consuming different servings of the studied infant cereals. The average iAs daily intake of 0.41 µg As serving^{-1} (**Table 38**) is used for calculations in the plot A at four different ages: 4 months (6.65 kg), 6 months (7.75 kg), 8 months (8.30 kg) and 12 months (9.30 kg) [216]. Taking into account all the scenarios evaluated for infants (4 to 12 months) and depending on the number of servings, the consumption of these infant cereals would lead and iAs exposure ranging from 0.04 to 0.25 µg kg^{-1} bw day^{-1} from this dietary source alone. As is illustrated in **Figure 59**, these values are below the reference limit recommended by EFSA (represented by red dotted line) and considering the worst case scenario (0.25 µg kg^{-1} bw day^{-1}), the present exposure corresponds to 82% of the lower value of the EFSA BMDL01 range [5].

Infant rice

The daily exposure to inorganic arsenic ranged from 0.67 to 5.58 µg As serving^{-1} (median= 1.77, n=9) depending on the infant rice sample (**Table 37**). The highest exposure came from organic whole-wheat rice and the lowest from infant rice (multigrain). Average contents of tAs and iAs as well as estimation of tAs and iAs daily intake per serving (recommended serving of 30g) from infant rice are shown in **Table 38**. Our estimation of iAs daily intake are in agreement with those reported in the literature for infant rice [243–248].

The iAs exposure for infants and young children consuming different servings of the studied infant rice is presented in the plot B included in **Figure 59.** The average iAs daily intake of 2.03 µg As serving^{-1} (**Table 38**) is used for calculations in the plot B at four different ages: 4, 6, 8 and 12 months considering the same body weights abovementioned [216]. Depending all the variables evaluated, i.e infants from 4 to 12 months and depending on the number of servings per day, the consumption of these infant rice would lead and iAs exposure ranging from 0.22 to 1,22 µg kg^{-1} bw day^{-1} from this assayed infant rice alone. As can be noted in **Figure 59**, the consumption of two or more serving per day (30g of infant rice) lead to an exposure above the lowest reference point stated by EFSA (>0.3 µg kg^{-1} bw day^{-1}) (represented by red dotted line), which highlights that, according to EFSA, the risk cannot be excluded for infants consuming rice cereals [5].

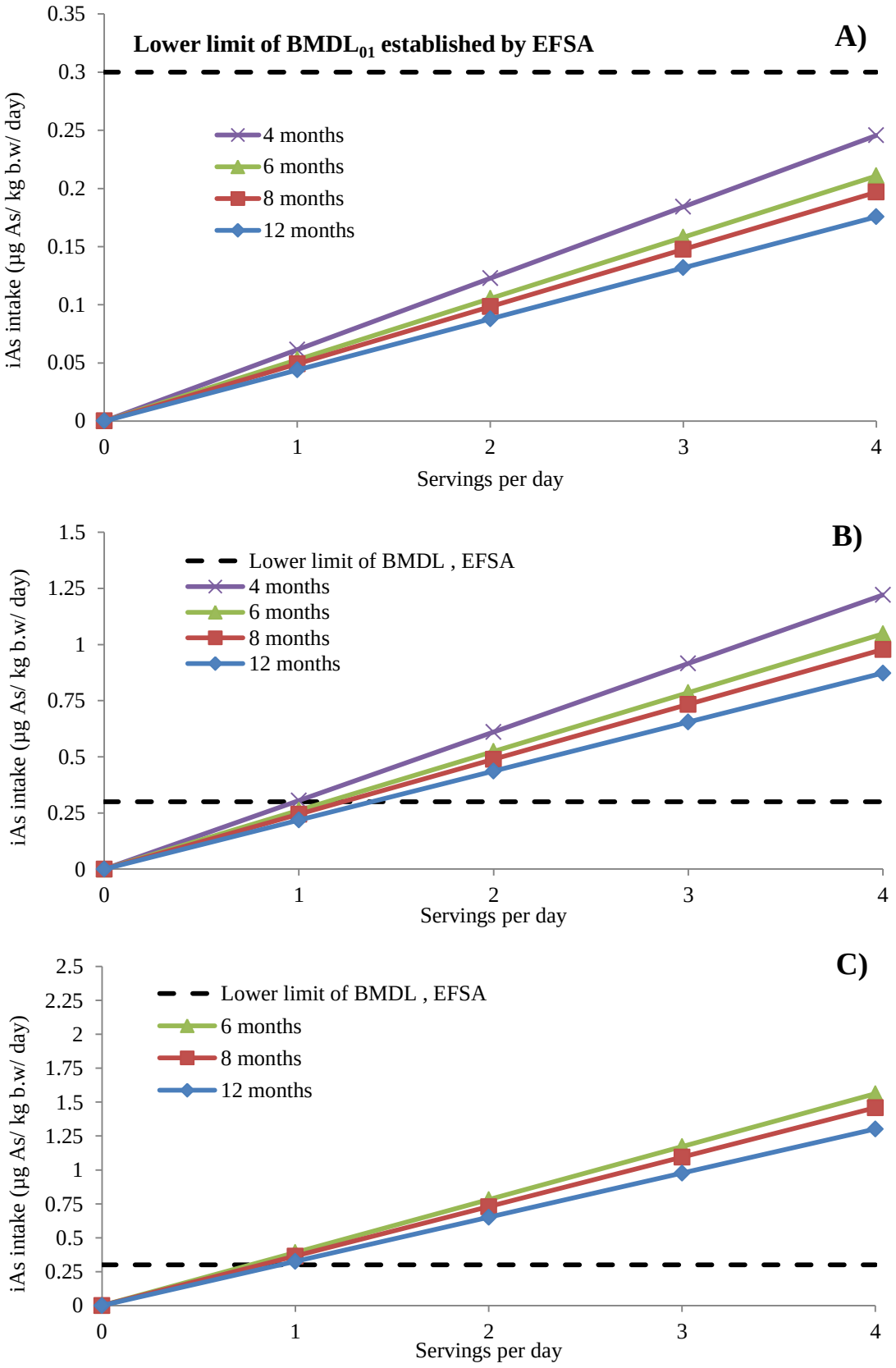


Figure 59. Estimation of iAs exposure for infants and young children (ranging from 4 to 12 months) from infant cereals (A), infant rice (B) and rice crackers (C). The average iAs daily intake of 0.41, 2.03 and 3.03 µg As serving^{-1} from infant cereals, infant rice and rice crackers, respectively is used for calculations. Infant and young children body weights are based on WHO [216] and recommended serving of 30g is used for calculation.

Rice crackers

Infants of 4 months are excluded of the study since the consumption of rice crackers is improbable at this age. In the period from 6 to 11 months, infants are introduced to rice crackers [240], so iAs daily intake is estimated at three ages: 6, 8 and 12 months. The daily exposure to inorganic arsenic ranged from 0.98 to 5.41 μg As serving^{-1} (median=2.86, n=4) and the highest exposure came from brown rice crackers (**Table 37**). Average contents of tAs and iAs as well as estimation of tAs and iAs daily intake per serving from rice crackers are shown in **Table 38**. Few studies evaluated the contribution of rice crackers to the iAs daily intake of infants and young children. Our estimation of iAs daily intake is in the range with those reported by Signes-Pastor et al. [245]: iAs ranged between 0.5 and 9.1 μg As serving^{-1} with a median value of 3.5 μg As serving^{-1}.

The iAs exposure for infants and young children consuming different servings of the studied rice crackers is shown in the plot C included in **Figure 59.** The average iAs daily intake of 3.03 μg As/serving (**Table 38**) is used for calculations in the plot C at four different ages: 4, 6, 8 and 12 months considering the same body weights abovementioned [216]. As can be noted in **Figure 59**, the consumption of one or more serving per day of these rice crackers would lead to an iAs exposure ranging from 0.33 to 1,82 μg kg^{-1} bw day^{-1} from this assayed samples alone. These values are within the $BMDL_{01}$ range identified by EFSA [5] (represented by red dotted line, Figure 28), which highlights that the risk cannot be excluded for infants consuming rice crackers.

Comments on the risk assessment study

In summary, the main contributor to total dietary As intake are fish and shellfish which contains high proportions of the non-toxic arsenobetaine showing that tAs concentration is not adequate as an indicator for risk assessment. On the other hand, cereals are the main contributor for iAs in adult Catalan population. The estimated iAs daily intake is below the EFSA and JECFA BMDL ranges, but considering the ratio between iAs intake and some BMDL for distinct end points (MOEs), the possibility of risk for high consumers of rice and rice products cannot be excluded.

The daily exposure to iAs for consumers of mushroom supplements would be quite low compared to BMDL ranges and the toxicological risk could be considered imperceptible compared to those contribution of other sources of iAs in the diet.

Considering the recommended amount of *S. fusiforme*, the iAs exposure would be below the $BMDL_{01}$ range identified by EFSA, which highlights that the risk can be excluded for the consumption of the recommended serving of this seaweed. However, high dietary exposure to iAs is found especially for people that regularly consume this algae as food in their diet, whereby the exposure to iAs can be within the $BMDL_{01}$ range identified by EFSA which highlights that the risk cannot be excluded for high consumers of *S. fusiforme* (Hijiki). However, our estimation of iAs exposure would be below the $BMDL_{05}$ interval identified by the JECFA.

The concentration of iAs in infant foods is higher in products based on rice than in similar products prepared using mixtures of other cereals with gluten (wheat, barley and oat).

Even considering high consumption of infant cereals, the daily intake of iAs is below the reference limit recommended by EFSA. The maximum daily intake of iAs is estimated for infants consuming high amounts rice-based products, i.e.: infant rice and rice crackers. Thus, the consumption of two or more serving per day of infant rice or rice crackers would lead to an exposure above the lowest reference point stated by EFSA which highlights that the risk cannot be excluded for infants consuming these infant products.

The finding of elevated contents of iAs in infant rice products and consequently elevated intakes of iAs in infants older than 4 months is of concern and deserves further attention. Furthermore, a wide range of rice-based products are fed to babies, increasing the risk of dietary exposure to iAs. In addition, special attention should be paid to infants with celiac disease which is an autoimmune disease that affects the villi of the small intestine causing abdominal pain, gas, diarrhea, or bad absorption due to gluten intolerance. The only treatment for this disease consists of a lifelong gluten free diet; this is, celiac people cannot consume products containing gluten, such as wheat, barley, and rye, but they can consume rice and corn. Thereby, infants with celiac disease have to eat gluten-free food that is mainly based on rice and are the most exposed to iAs.

Therefore, as iAs is a class one, non-threshold and carcinogen [96] , there is a fundamental need to reduce the iAs content in infant rice products that would reduce the infant exposure to iAs. Some initiatives to address this issue that deserve further investigation are: (i) the elimination of rice from infant products; (ii) the diversification of diets by including other cereals such as oat, barley, maize and wheat as a cereal carbohydrate/protein source; (iii) screening of As levels in rice to identify varieties that have low iAs contents; (iv) selecting and sourcing rice from low arsenic regions.

Furthermore, other potential options to reduce the high As contents in rice are proposed in the literature [243, 249]. Briefly, this research is focused on: (i) breeding rice to get rice cultivars with restricted As uptake and upward transport to the edible grain; (ii) modifying the current anaerobic growing practices (flooding of fields) for rice, moving towards more aerobic conditions, which will reduce As availability to rice plants. However, these approaches are at least theoretically and these potential options deserve further investigation.

The EFSA scientific opinions [5, 104] identified children under three years of age as the most exposed to iAs dietary exposure. Dietary exposure iAs for children under three years old, including from rice-based foods, is in general estimated to be about 2 to 3-fold that of adults. In view that iAs in rice-based infant products deserve special attention, the EU have recently published ML in rice and rice products. Since rice is an important ingredient in a broad variety of food for infants and young children, a specific ML is established for this commodity when used as an ingredient for the production of such food. Thus, a specific ML of iAs of 0.1 mg As kg^{-1} is established for "rice destined for the production of food for infants and young children" [107]. Furthermore, the new regulation stated that since rice waffles, rice wafers, rice crackers and rice cakes can contain high levels of iAs and these commodities can make an important contribution to the dietary exposure, a specific ML for these commodities should be envisaged.

7.6.5 Bioaccessibility of arsenic in foodstuffs

The exposure levels calculated in the section presented above are the maximum daily intake values because the estimates presupposed that all the iAs present in the food commodities eventually reaches the blood stream. However, there are many cooking treatments that could reduce the content of arsenic ingested via food. For instance, washing and/or cooking with large volumes of water significantly reduces the As content of cooked rice [207, 250, 251].

From our study focused on arsenic bioaccessibility in cooked mushrooms, tAs decreased by 9% and 11% in griddled *A. bisporus* and *P. ostreatus*, respectively with respect to raw mushroom. Boiling, meanwhile, decreased tAs content by 53% and 71% in *A. bisporus* and *P. ostreatus*, respectively producing high differences with respect to the tAs content of the raw mushroom assayed (**Article IX)**. Although, there are no data on arsenic content in the other mushroom species following cooking treatment, several studies have been published on other foods in which high percentages of arsenic were released from food into the cooking water e.g. seaweeds [130] and pasta samples [252]. In addition, the bioaccessibility of arsenic should be assessed to refine and improve the toxicological risk process. We found high bioaccessibility of tAs in our study in mushrooms (**Article IX**). For instance, bioaccessibility of tAs in raw mushrooms varied between 74% and 88% for the G fraction and 86% and 97% for the GI fraction. Even when a cooking process led to a decrease in tAs content in these mushrooms, the bioaccessibility of tAs remained high (>76%) for both G and GI fractions. Therefore, the real levels of iAs exposure that are bioaccessible for potential consumers of the mushrooms studied in this thesis are likely to be quite lower than the values reported in section presented above in **Table 30**.

From the results found in the literature, high variability of arsenic bioaccessibility depending on the type of food and on the approach used to estimate bioaccessibility was found. For instance, Signes-Pastor [253] found that the percentage of As bioaccessibility depended on the rice type: parboiled (59%–99%), and nonparboiled (36%–69%). Similar results were previously reported where bioaccessibility of iAs reached 63%–99% [132]. Furthermore, arsenic bioaccessibility studies have been conducted in other foodstuffs items: fish and shellfish [128, 129], edible seaweeds [24, 128, 130, 131, 254], vegetables [133, 134] and in country foods from contaminated sites in Canada [255]. Considering the bioaccessibility values of iAs reported in the literature, the real arsenic daily intake are likely to be few lower than those results estimated in the section discussed above. The consideration of iAs bioaccessibility data in the risk assessment can further refine and improve the dietary exposure assessment and the margins of exposure. For this, further studies on the bioaccessibility of arsenic species, especially iAs, in food commodities which consider the effect of cooking should be conducted.

PART V: CONCLUSIONS

Chapter 8

Conclusions

Conclusions derived from the work conducted in this doctoral thesis are divided into four parts: the first concerns the review that focused on method developments and quality assessment of inorganic arsenic determination in food, the second focuses on the development and validation of methods for the determination of arsenic species, the third concerns the occurrence and distribution of arsenic species in food commodities and the fourth relates to the assessment of arsenic exposure and associated health risks.

State of the art on recent method developments and quality assessment of inorganic arsenic determination in food

- ✓ Several chromatographic and non-chromatographic methods are available to determine iAs in food. Among them, chemical extraction of iAs species and further determination by HPLC-ICPMS is undoubtedly the most popular approach used in iAs analysis in food.

- ✓ Although several arsenic speciation methods have been reported, there is a need for the development of robust analytical methods for the extraction, separation and determination of inorganic arsenic in matrices with a complex distribution of arsenic species, i.e. food of marine origin.

- ✓ Certified reference materials (CRMs), especially for iAs in seafood products, are required. The production of such CRMs as well as participation in interlaboratory comparison exercises would help in the validation of iAs methods and facilitate future surveys of the iAs content of foods.

- ✓ There is a need for full validation of selective and sensitive methods for the determination of iAs to allow their future implementation in routine analysis in food control laboratories according to the ISO/IEC 17025 standard.

- ✓ There is a need to produce reliable speciation data, especially on iAs, for different food commodities in order to refine risk assessment studies. For this, the establishment and validation of methods for iAs determination are of paramount importance, and will allow consistent conclusions in dietary exposure studies.

Development and validation of methods for the determination of arsenic species in foodstuffs

- ✓ The use of microwave acidic digestion in a closed vessel for sample preparation and the ICPMS for detection provided an accurate determination of tAs in several types of food samples (matrices of marine or terrestrial origin and infant food products).

- ✓ The proposed method for tAs determination was successfully evaluated by appropriate selected validation parameters (linearity, LOD, LOQ, accuracy, precision, external QC), showing the suitability of the method. Furthermore, internal QC and external QC assessment was carried out to achieve a satisfactory level of quality for routine analyses.

- ✓ From the extraction methods assayed for arsenic speciation, microwave-assisted extraction with HNO_3/H_2O_2 solvent is the most suitable procedure for the quantitative extraction of all arsenic species in foodstuffs as well as the toxic iAs species, without degradation of other arsenocompounds. The use of both anionic and cationic exchange chromatography coupled to ICPMS (HPLC-ICPMS) provided a satisfactory separation of arsenic species as well as sensitive and selective measurement.

- ✓ A method for the determination of arsenic species in rice and rice products by HPLC-ICPMS was developed and fully validated. Linearity, LOD, LOQ, repeatability, intermediate precision, trueness, accuracy, selectivity, as well as expanded uncertainty were established for iAs, MA, and DMA. The method was used to analyse arsenic species in several samples including several types of rice, rice products, and infant rice products, showing the broad applicability of the method.

- ✓ In order to use the rice method to determine arsenic content in cereal-based products (As<0.010 mg As kg^{-1}), the LODs were improved by modifying instrumental conditions and HPLC-ICPMS parameters. Then, the main validation parameters were established for iAs, MA, and DMA. To assess the applicability of the method, several cereal-based samples were analysed: bread, biscuits, breakfast cereals, wheat flour, corn snacks, pasta and infant cereals. This optimised method can be used as a substitute for the previous method validated in rice samples and expands the applicability of the method to cereal-based products.

- ✓ The proposed speciation method was successfully validated according to the ISO/IEC 17025:2005 standard, is sensitive and selective and could be considered fit for purpose, i.e. iAs determination in food. This method is straightforward enough for routine analysis for the determination of toxic iAs and other arsenic species in several foodstuffs, even in challenging matrices such as those of marine origin. Furthermore, since maximum limits of iAs in rice and rice products have recently been established by the European Union, the method could be useful in food control laboratories.

- ✓ In terms of external quality control, the method was satisfactorily tested in proficiency tests (IMEPs and FAPAS), which showed the suitability of the developed method to provide reliable and accurate results about inorganic arsenic in foodstuffs.

- ✓ Participation in certification studies of CRMs (characterization, stability and confirmation studies) showed that the speciation method is suitable for the determination of arsenic speciation in food matrices.

- ✓ The collaborative trial on the determination of iAs in foodstuffs of marine and plant origin by HPLC-ICPMS (CEN TC275/WG10) produced results that were consistent with the assigned values. A method for the determination of iAs in foodstuffs of marine and plant origin was developed and the main performance characteristics of the method were assessed. This method is suitable for the quantitative analysis of iAs in foodstuffs of marine and plant origin and the standardization process is ongoing.

- ✓ An *in vitro* PBET method to estimate arsenic bioaccessibility was established and the main quality control parameters were evaluated. The method was applied in raw and cooked mushrooms and *a priori*, could be applied to assess arsenic bioaccessibility in other food samples; however further investigation on this application should be carried out.

Occurrence and distribution of arsenic species in food commodities

- ✓ Only iAs species were found in cereal products and iAs was the major As compound found in rice products, highlighting the importance of these food groups as a possible source of iAs in rice and cereal-based diets. There was a positive correlation between tAs and iAs in rice and cereal products, with iAs content dependent on tAs in the assayed samples.

- ✓ The concentration of tAs and iAs was higher in rice-based products than in products prepared using other cereals (wheat, barley, oat and maize) or mixtures of them. Concentrations of iAs in the assayed cereal-based products, rice, and infant-food samples were below the established MLs. Only one brown rice (whole medium grain rice) and an infant food sample (organic whole-wheat rice) exceeded the MLs established by China.

- ✓ The speciation study in mushrooms identified much variability in the content of both iAs and organoarsenic compounds. Given the small number of mushroom species analysed, we are not able to make any generalizations about the arsenic speciation

pattern in mushrooms. Furthermore, for reliable As speciation data, mushrooms should be sampled from each specific origin and analysed individually.

- ✓ High proportions of iAs were found in *L. edodes* (Shiitake) products, including the cultivated and purchased mushrooms. However, it is not entirely clear whether Shiitake mushrooms accumulate iAs from the substrate, or produce it through biotransformation.

- ✓ The potentially toxic arsenosugars were the predominant arsenic compounds found in the majority of seaweeds studied, while iAs was found at low proportions with a few exceptions, along with other arsenic species. Significant percentages of AB, which is not a common compound in seaweeds, were detected in *U. rigida* and *E. compressa.* The results for the commercially edible seaweeds showed that iAs was below the maximum limits established by France, the USA, Australia and New Zealand, with the exception of *S. fusiforme.*

- ✓ Special care should be taken in the consumption of *S. fusiforme* (Hijiki), since we found high total and inorganic arsenic levels: tAs > 100 mg As kg^{-1} and iAs > 60 mg As kg^{-1} that exceeded the maximum limits established by France, the USA, Australia and New Zealand.

- ✓ Ten arsenic species were determined in the fish and shellfish extracts and arsenobetaine was the major compound identified (approx. 90% of the tAs). Inorganic arsenic was not detected in fish samples and was below 0.4 mg As kg^{-1} in crustaceans and bivalves. Based on our results on marine foods, wide variability in arsenic species can be expected when dealing with seafood such as seaweeds, bivalves and crustaceans, highlighting the need to carry out speciation to discern the toxic from the non-toxic species.

Assessment of arsenic exposure and associated health risks

- ✓ All arsenic speciation data obtained in this thesis are useful for assessing the daily intake of arsenic in the Catalan population. Furthermore, these results may contribute to on-going discussions regarding establishing and implementing maximum levels in inorganic arsenic in food commodities, as recommended within the European Union.

- ✓ The main contributors to tAs dietary intake are fish and shellfish, which contain high proportions of the non-toxic arsenobetaine. On the other hand, cereals are the main contributor iAs in the adult Catalan population.

- ✓ The average estimated iAs daily intake in the adult Catalan population is below the EFSA and JECFA BMDL ranges, but given the ratio between iAs intake and some

BMDL for distinct end points, the possibility of risk for high consumers of rice and rice products cannot be excluded.

- ✓ The daily exposure to iAs for consumers of mushroom supplements is relatively low compared to BMDL ranges and the toxicological risk could be considered insignificant compared to the contribution from other sources of iAs in the diet. However, high consumers of *S. fusiforme* (Hijiki) would be exposed to iAs intake within the $BMDL_{01}$ range identified by EFSA, so the possibility of risk cannot be excluded.

- ✓ The consumption of infant cereals would lead to a daily intake of iAs below the reference limit recommended by EFSA. A high daily intake of iAs is estimated for infants consuming high amounts of rice-based products, i.e. infant rice and rice crackers. The consumption of two or more servings per day of these food commodities would lead to exposure above the lowest reference point stated by EFSA, so the possibility of risk cannot be excluded for infants consuming these rice-based products.

- ✓ In view of our estimated exposure of infants to iAs, there is a fundamental need to reduce the iAs content in infant rice products in order to reduce their exposure to iAs, a non-threshold, class one human carcinogen.

- ✓ High arsenic bioaccessibility values were found in raw and cooked mushrooms and even when cooking led to a decrease in tAs content, the bioaccessibility of tAs remained high (>76%) for both gastric and gastrointestinal fractions.

Future trends

- ➢ There is a need for robust methods for the determination of inorganic arsenic in a wide range of food items, particularly those of marine origin, which need to be properly validated and further implemented in routine analysis by food control laboratories.

- ➢ In order to validate methods for iAs determination, a certified value of iAs in CRMs must be available since this would be crucial to assure the accuracy of the method. Furthermore, since to date, proficiency testing (PTs) for iAs determination in seafood has been unsatisfactory, further PTs for iAs in seafood matrices are urgently needed.

- ➢ In order to refine risk assessment, there is a need to produce reliable inorganic arsenic data for different food commodities that would support dietary exposure assessment and indicate possible health effects.

- ➢ The inclusion of iAs bioaccessibility data in risk assessment would further refine and improve the assessment of dietary exposure and the margins of exposure. Thus further

studies on the bioaccessibility of arsenic species, especially iAs, in food commodities that also consider the effect of cooking should be conducted.

- The production of CRMs with a bioaccessible arsenic content in food matrices would be useful for quality control purposes and allow comparisons between the established bioaccessibility methods.

- *In vivo* arsenic bioavailability studies would be desirable to demonstrate the suitability of and validate *in vitro* bioaccessibility methods.

- Since existing studies of arsenosugar toxicity are not conclusive, there is a need for improved understanding of the human metabolism of arsenosugars in foods and the human health implications. Thus, further studies on arsenosugar toxicity should be performed to refine the risk assessment and further legislation on arsenic.

- As the arsenolipids have been found to be particularly abundant in marine oils and fats, analytical methods are needed for further investigation of these compounds in marine fish, fish feed and seafood in general. Further investigation of the abundance and toxicity of these compounds will also be important for risk assessments and legislation on arsenic relating to food safety.

- The establishment of standardised methods for the determination of iAs in foodstuffs is of paramount importance and a necessary tool for the implementation of future directives regarding maximum levels of iAs in food commodities.

PART VI: REFERENCES

Chapter 9

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ANNEX I

Summary in Spanish

PARTE I: INTRODUCCIÓN

Capítulo 1
Arsénico: propiedades, especies y ocurrencia

1.1. Propiedades y química del arsénico

El arsénico es un metaloide con una química compleja que demuestra las propiedades de los metales y no metales. El arsénico elemental tiene el número atómico de 33 y el peso atómico de 74,92 g mol^{-1}. El arsénico se encuentra en el grupo 15 de la tabla periódica, el mismo grupo como nitrógeno y fósforo, en consecuencia, la química del arsénico es similar en muchos aspectos a estos elementos. Debido a estas similitudes químicas, el arsénico a menudo puede sustituir a fósforo en los sistemas biológicos siendo una de las razones de la ocurrencia de arsénico a niveles elevados en muchos organismos marinos, y por lo tanto en muchos mariscos. El arsénico exhibe varias formas alotrópicas conocida donde el alótropo más estable de arsénico es la forma gris, similar a la forma romboédrica de fósforo. El arsénico se encuentra comúnmente en los minerales de ricos en sulfuros y el más abundante es la arsenopirita (FeAsS).

El único isótopo de arsénico natural es ^{75}As. El arsénico se encuentra ampliamente distribuido en la corteza terrestre y puede existir en cuatro estados de oxidación; -3, 0, 3, 5 y en una variedad de formas inorgánicas y orgánicas. La mayoría de las especies de arsénico conocidas en los organismos y alimentos contienen arsénico en estados de oxidación +3 y +5. Ambos estos dos arsenicales inorgánicos son tóxicos y pueden interconvertirse entre ellos según las condiciones redox y de pH. Además, la existencia del estado -3 en el medio ambiente ha sido cuestionado. El arsénico también se puede encontrar en compuestos orgánicos y, definidos como aquellos que contienen enlaces de carbono con arsénico. Además de estos compuestos, el arsénico puede formar compuestos de base lipídica. La afinidad del arsénico con el azufre significa que también pueden existir compuestos con componentes As-S con los enlaces As-S; por ejemplo, As (III) puede vincularse con los grupos sulfhidrilo de las proteínas. Actualmente se estima que hay más de 50 compuestos de arsénico que se encuentran en el medio ambiente.

Arsénico se moviliza en el ambiente acuoso y la atmósfera de forma natural a través de la erosión de las rocas y minerales, actividades volcánicas y los procesos biológicos. El arsénico puede entrar en el medio ambiente a través de procesos antropogénicos como la minería, la fundición, la combustión, la producción y el uso de pesticidas, herbicidas, insecticidas y los procesos naturales como la erosión, volcanismo, y la disolución de suelos y sedimentos ricos en arsénico. El arsénico se utiliza principalmente en la agricultura (pesticidas, agentes de conservación de la madera) y como aditivo para piensos (Roxarsona) para mejorar el crecimiento de las aves de corral, aunque el uso de arsénico en estas aplicaciones se han

reducido en los últimos años debido a problemas de salud. Además, el trióxido de arsénico se utiliza en medicina para el tratamiento de cierto tipo de leucemia.

1.2 Especiación química

La especiación es una palabra de importancia primordial en los trabajos presentados en esta tesis. A pesar de que el término especiación se ha utilizado durante mucho tiempo no fue hasta el año 2000 que la comunidad internacional acordó la nomenclatura y definición, y la IUPAC introdujo una guía para los términos relacionados con el fraccionamiento de los elementos y la especiación química (TEMPLETON et al., 2000). Las aplicaciones modernas de análisis de especiación son de gran alcance dentro de los campos de la química de los alimentos, química ambiental, la salud y la higiene, así como la geología. La especiación es, además, una herramienta importante en la investigación de la toxicidad y biodisponibilidad de elementos, donde la información de la concentración total del elemento puede ser insuficiente.
Las definiciones de la IUPAC:

I. *Especies químicas*. Los elementos químicos: forma específica de un elemento definida como a la composición isotópica, estado electrónico u oxidación, y/o complejo o estructura molecular.
II. *Análisis de especiación*. Química analítica: actividades analíticas de identificación y/o medición de las cantidades de una o más especies químicas individuales en una muestra.
III. *Especiación de un elemento; especiación*. Distribución de un elemento entre las especies químicas definidas en un sistema.
IV. *Fraccionamiento*. Proceso de clasificación de un analito o un grupo de analitos de una muestra determinada de acuerdo con la física (por ejemplo, tamaño, solubilidad) o propiedades químicas (por ejemplo, la unión, la reactividad).

1.3 Especies de arsénico

Más de 50 compuestos de arsénico diferentes de origen natural se han identificado, que comprenden formas tanto orgánicas como inorgánicas. Las siguientes secciones presentan los compuestos de interés para esta tesis y que son de relevancia en el campo de la alimentación.

Las estructuras de las especies de arsénico más relevantes estudiados en esta tesis se presentan en la **Figura 1**. Además la **Tabla 1** muestra el nombre común de cada especie, las abreviaturas, fórmulas, peso molecular y CAS para las principales especies de arsénico estudiadas en esta tesis y que son prioritarias en el campo de la alimentación. En esto la nomenclatura propuesta en los artículos de revisión de Maher, y Francesconi y Kuehnelt se sigue a lo largo de la tesis. En estos artículos de revisión, los autores proponen la adopción de la nomenclatura, así como de las abreviaciones de compuestos de arsénico, teniendo en cuenta que no existe acuerdo sobre este tema en la amplia bibliografía dedicada a la presencia, evolución y análisis de los compuestos de arsénico. La propuesta de los autores es claramente razonada y contribuye sin duda a disminuir la confusión existente en la bibliografía.

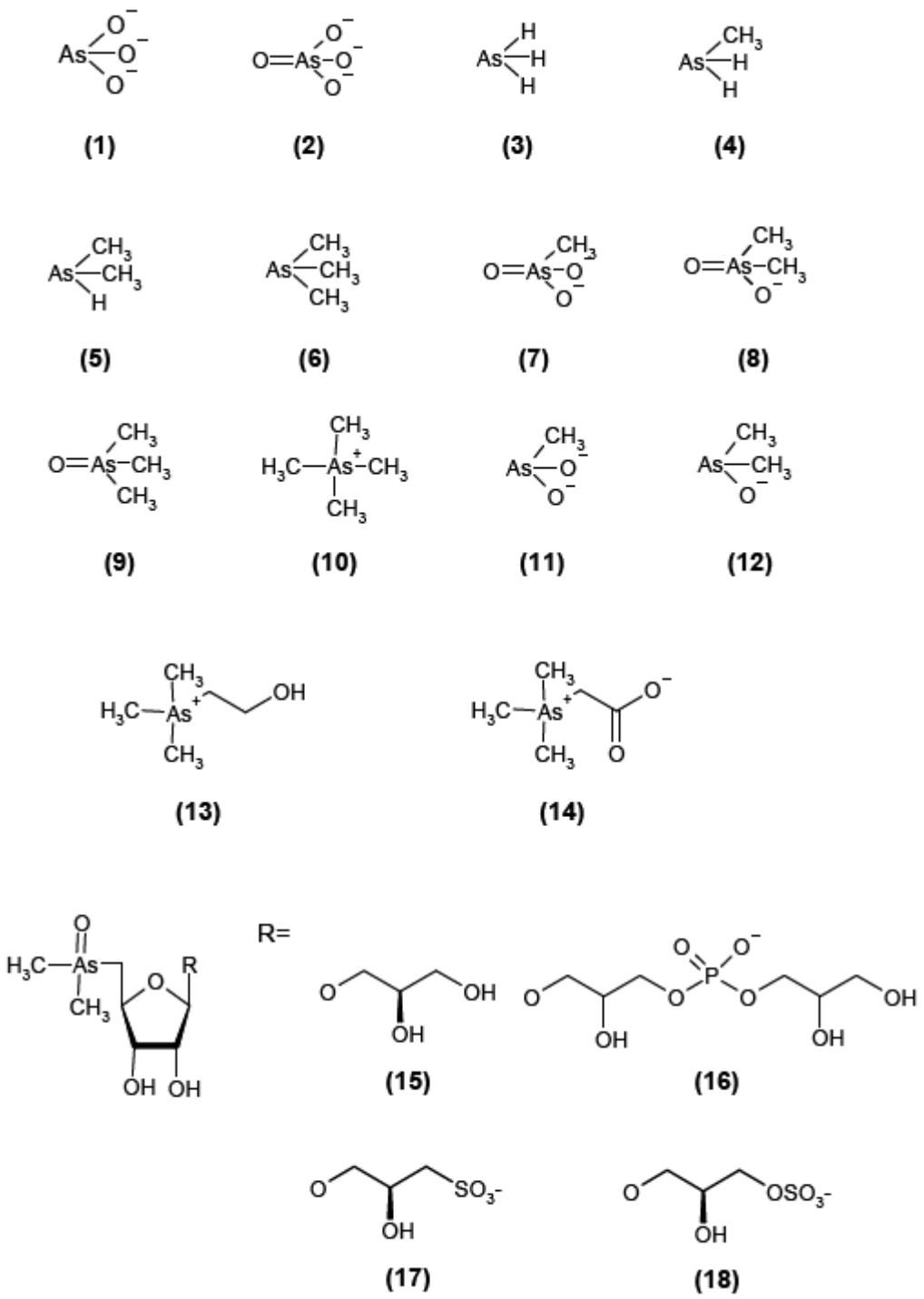


Figura 1. Estructuras de las principales especies de arsénico estudiados en esta tesis

Tabla 1. Nombre común, abreviaturas, fórmula, el peso molecular, y CAS para las principales especies de arsénico en los alimentos.

Compuesto	Nombre común	Abreviación	Fórmula	Peso molecular	Numero CAS	Comentario
1	Arsenite	As(III)	$As(OH)_3$	125.94	13464-58-9	Traza y niveles bajos en la mayoría de los alimentos; altamente tóxico
2	Arsenate	As(V)	$AsO(OH)_3$	141.94	7778-39-4	Traza y niveles bajos en la mayoría de los alimentos; una forma importante en agua; altamente tóxico
3	Arsine	-	AsH_3	77.94	7784-42-1	Detectado en aguas termales
4	Methylarsine	-	(CH_3AsH_2)			Detectados en nivel traza en gambas y langostas, también en los lodos de depuradora de los tratamiento de aguas residuales
5	Dimethylarsine	-	$((CH_3)_2AsH)$			
6	Trimethylarsine	-	$(CH_3)_3As)$			
7	Methylarsenate	MA	CH_5AsO_3	139.97	124-58-3	Especie de arsénico traza en algunos mariscos y alimentos terrestres; un importante metabolito de orina humana de la iAs.
8	Dimethylarsenate	DMA	$C_2H_7AsO_2$	138	75-60-5	Especies de arsénico menores en mariscos y en algunos alimentos terrestres; el principal metabolito de iAs en orina humana, arsenoazúcares y arsenolípidos
9	Trimethylarsine oxide	TMAO	C_3H_9AsO	136.02	4964-14-1	Especie de arsénico minoritaria en pescados y mariscos.
10	Tetramethyl arsonium	TETRA	$C_4H_{12}As$	135.06	27742-38-7	Especie de arsénico minoritaria en pescados y mariscos.
11	Methylarsenite	MA(III)	CH_5AsO_2	123.97	25400-23-1	No suele ser detectado en los alimentos; detectado en algunas muestras de orina humana como un metabolito de iAs; es una especie tóxica que se piensa que es importante para el modo de acción tóxica.
12	Dimethylarsenite	DMA(III)	C_2H_7AsO	122	55094-22-9	No detectado en los alimentos; detectado en algunas muestras de orina humana como un metabolito de iAs; una especie muy inestable que es muy difícil de medir; considerada por algunos investigadores como una especie altamente tóxica.
13	Arsenocholine	AC	$C_5H_{14}AsO$	165.09	39895-81-3	Especie traza de arsénico que se encuentra en pescados y mariscos; se oxida fácilmente a arsenobetaína en los sistemas biológicos
14	Arsenobetaine	AB	$C_5H_{11}AsO_2$	178.06	64436-13-1	Especies de arsénico importante en la mayoría de mariscos; no tóxica
15	Glycerol arsenosugar	Gly-sug	$C_{10}H_{21}AsO_7$	328.19	1227057-97-7	Especies de arsénico mayoritarias en alga comestibles o importantes en moluscos y en muchos mariscos
16	Phosphate arsenosugar	PO_4-sug	$C_{13}H_{28}AsO_{12}P$	482.25	88216-76-6	
17	Sulfonate arsenosugar	SO_3-sug	$C_{10}H_{20}AsO_9S$	391.25	123288-10-8	
18	Sulfate arsenosugar	SO_4-sug	$C_{10}H_{20}AsO_{10}S$	407.25	123257-94-3	

Arsénico inorgánico

El arsénico inorgánico (iAs) está ampliamente distribuido en el medio ambiente y se encuentra principalmente en el estado +3 o +5 de oxidación, ya sea atado en tio-complejos o como los dos oxianiones As (III) y As (V). Según los datos reportados, generalmente se encuentra arsenito y arseniato pesar de que el arsénico inorgánico es probable que esté ligado a tio-grupos en péptidos o proteínas en los alimentos. Bajo los niveles normales de oxígeno ambientales, As (V) está termodinámicamente favorecida. Sin embargo, son fácilmente interconvertibles y a menudo se encuentran juntos. En el agua de mar y de agua dulce, As (V) es la principal especie de arsénico y esencialmente todo el arsénico en el agua potable es arseniato. Las concentraciones de arsénico en aguas naturales son típicamente por debajo de 10 μg As L^{-1}, con frecuencia por debajo de 1 μg As L^{-1} y pueden alcanzar hasta 5000 μg As L^{-1}. Esta amplia gama se produce en condiciones naturales. En los casos excepcionales en los que se encuentran altas concentraciones de arsénico, en particular en las aguas subterráneas, los efectos para la salud son graves. Por ejemplo, en la Cuenca de Bengala donde el agua potable para millones de personas está altamente contaminada por arsénico.

Los productos alimenticios de origen terrestre son generalmente bajos en la concentración de arsénico total (tAs) y, consecuentemente, también bajo contenido de iAS, por lo general por debajo de 0,05 mg As kg^{-1}. La excepción a esto es el arroz, que contiene cantidades significativas de iAs, a menudo entre 0,05 y 0,4 mg As kg^{-1} y, a veces considerablemente mayor, hasta 1,9 mg As kg^{-1} en los solubles de salvado de arroz. Por otro lado, el pescados y el marisco tienen altas concentraciones de tAs, donde la mayoría de las muestras caen dentro de un rango de 5 a 100 mg As kg^{-1} , pero con niveles mucho más bajos de iAs, típicamente <0,2 mg As kg^{-1}. No se ha demostrado ninguna relación entre la concentración tAs y el nivel de iAs en pescados y mariscos (Edmonds, 1993). La mayoría de mariscos sólo tienen trazas de iAs, y los mariscos con alta concentración de iAs son las excepciones. Por ejemplo, el alga comestible Hijiki tiene alto nivel de iAs, valores superiores a 66 mg As kg^{-1} se han reportado. Por otra parte, también se han publicado niveles inusualmente altos de iAs en mejillones donde valores de iAs de hasta 5,8 mg As kg^{-1} fueron hallados.

Especies metiladas de arsénico

Las especies de arsénico que pertenecen a este grupo son methylarsonate (MA), dimetilarsinato (DMA), óxido de trimethylarsine (TMAO) y el ion tetramethylarsionium (TETRA). Las especies DMA y MA se producen de forma conjunta de acuerdo con la vía propuesta por Challanger para biotransformación arseniato que implica la reducción y metilación de As (V). Estos compuestos metilados se forman como resultado de biometilación, donde biometilación se refiere a una transferencia enzimática de un grupo metilo a partir de un átomo donador a un átomo aceptor dentro de un organismo vivo. La biometilación de compuestos de arsénico es descrita por la vía sugerida previamente, que se muestra en la **Figura 2**, que consiste en una serie alterna de reacciones de metilación de reducción y oxidación mediadas por enzimas de arsénico metiltransferasa y S-adenosilmetionina (SAM), un grupo

universal donante metilo en los sistemas biológicos. Dentro de este modelo As (V) se reduce primero a As (III) antes de ser metilado y se oxida para formar MA. Las medidas de reducción y oxidación de metilación se repiten produciendo las formas trivalentes y pentavalentes de MA y DMA y finalmente trimethylarsine.

Tanto MA y DMA generalmente se detectan en niveles bajos (<0,5 mg As kg^{-1}) en los organismos vivos, y también son metabolitos minoritarios de arsénico y se encuentran a menudo juntos. TMAO se encuentran generalmente sólo a niveles bajos o traza en organismos terrestres y marinos. A pesar de que es un metabolito en el proceso de biotransformación, TMAO generalmente se detecta a nivel de trazas, especialmente en el medio marino, y en algunos casos se encontró compuesto como importante. En el medio terrestre se detectó en niveles traza en muestras de plantas y líquenes. TETRA es generalmente una especie de menor importancia en el medio marino, pero se puede encontrar como especies principales en algunos moluscos. Además, TETRA ha sido reportado en ranas, hongos y algunas especies de plantas.

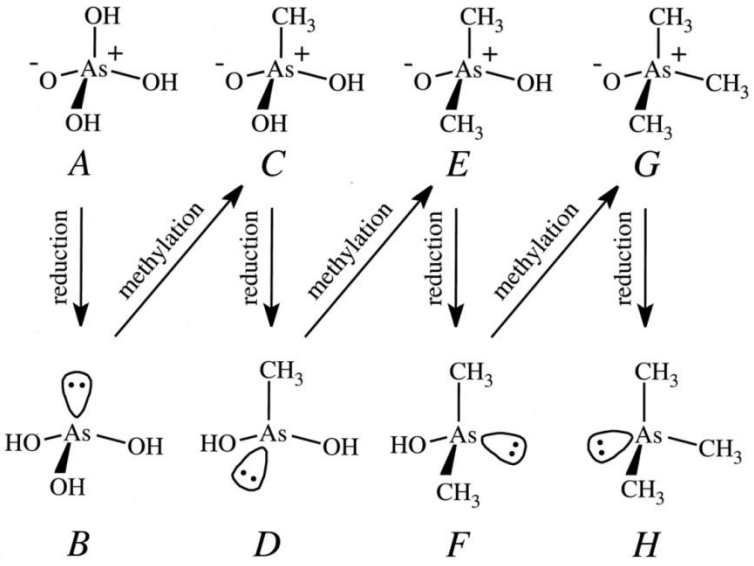


Figure 2. Esquema de la vía propuesta por Challenguer para la conversión de arseniato a trimethylarsine (adaptado de Challenger 1945). Arseniato de (A); Arsenito (B); (C) methylarsenate; (D) methylarsenite; (E) dimetilarsenato; Dimethylarsenite (F); (G) de óxido trimethylarsine; (H) trimethylarsine. La línea superior de las estructuras muestra los As (V) intermedios. Las flechas verticales indican las reacciones de reducción en el As (III) intermedios (línea inferior), y de las flechas diagonales indican los pasos de metilación por SAM.

Arsenocolina (AC)

Arsenocolina (AC) se encuentra comúnmente a nivel de trazas de organismos marinos típicamente <0,2 mg As kg^{-1}. Es un precursor metabólico de AB y se convierte rápidamente en este compuesto. En el medio terrestre se detectó por primera vez en las muestras de los hongos que crecen en el arsénico área contaminada; y se ha detectado a nivel de trazas en algunas muestras de las plantas terrestres.

Trimethylarsoniopropionate (TMAP)

Trimethylarsoniopropionate (TMAP), es un compuesto similar a arsenobetaína, se identificó por primera vez en 2000 en una especie de pez, y ahora se sabe que es un menor componente común de organismos marinos (típicamente en concentraciones de 0,2 a 2 mg kg^{-1}).

Arsenobetaína (AB)

Arsenobetaína (AB) fue identificada por primera vez por Edmonds y Francesconi en 1977. Actualmente, la hipótesis principal para la formación de AB es que se forma a partir de los productos de degradación de arsenoazúcares dimetilados (Figura 3, vía # 2). Se cree que arsenoazúcares son precursores para la formación de AB porque las fuentes dietéticas para los organismos marinos, como el fitoplancton y algas marinas, contienen niveles de arsenoazúcares elevados. Sin embargo otras rutas también se han mencionado en los ambientes terrestres o de aguas profundas, en biótico, y en ambientes abióticos. Las tres vías posibles de formación arsenobetaína mas mencionadas en la literatura se muestran en la **Figura 3**. Las vías # 1 y # 2 implican la degradación de arsenoazúcares y vía # 3 implica DMA (III) como precursor.Vía # 1 comienza con los arsenoazúcares trimetilados que se degradan en arsenocolina y luego se convierten en arsenobetaína. Vía # 1 se apoya en el hecho de que arsenoazúcares trimetilados se han identificado en el abulón (oreja de mar) como el 28% de tAs en los tejidos intestinales y en los gasterópodos de los ecosistemas de manglar, 6.8% de tAs. Sin embargo, arsenoazúcares trimetilados suelen estar presentes en concentraciones muy bajas en los organismos marinos, por lo que es poco probable que sean la fuente de arsenobetaína a una alta concentración en animales marinos. Al mismo tiempo, la absorción o transformación rápida pueden agotar las concentraciones de los compuestos intermedios y estudios controlados con arsenoazúcares trimetilados aún no se han llevado a cabo para evaluar estas posibilidades

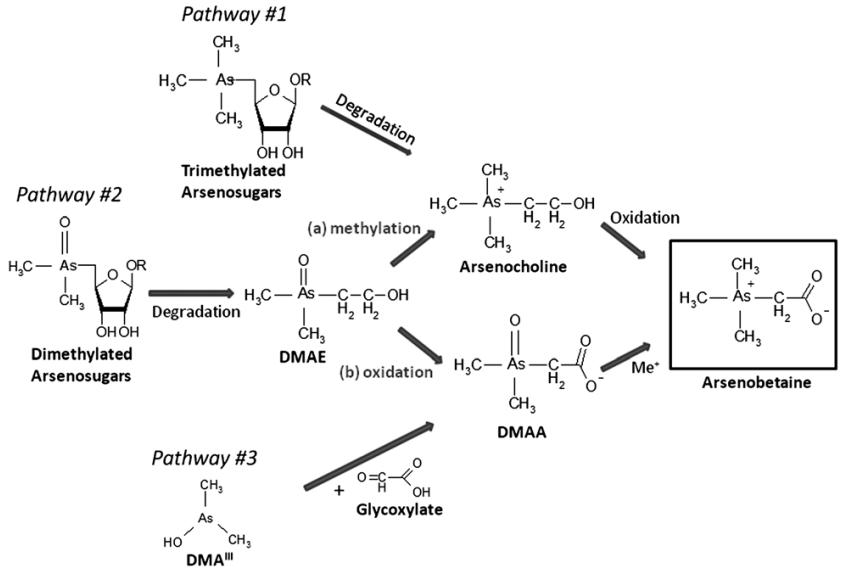


Figura 3. Vías propuestas de formación de AB (adaptado de Caumette 2012).

Vía #2 es la más ampliamente descrita en ambientes marinos, ya que los arsenoazúcares dimetilados se encuentran ampliamente distribuidos y disponibles en la base de las cadenas alimentarias acuáticas: en el fitoplancton, algas y tapetes microbianos. Vía #2 implica la degradación de los arsenoazúcares dimetilados. Dimethylarsinoylethanol (DMAE) es el primer producto de la degradación, y luego o bien (a) AC o (b) dimethylarsinoylacetate (DMAA) actuan como productos intermedios (**Figura 3**). La biotransformación se inicia con arsenoazúcares dimetilados que se degradan para dar DMAE, seguido por una oxidación y posterior metilación a través del intermedio DMAA propuesto para dar el producto final de arsenobetaína (**Figura 3,** vía 2b #**).** La formación a través de las arsenoazúcares fue apoyada por un estudio que muestra que el DMAE se formó después de la descomposición anaeróbica de un alga marrón; *Ecklonia radiata*. Además Duncan et al., mostraron la presencia del precursor arsenobetaína DMAE en el fitoplancton. DMAA se ha demostrado que es un importante producto de degradación de arsenoazúcares, segundo a DMA (V), en las ovejas. Se ha demostrado que DMAA también es un precursor en la formación de AB en estudios de laboratorio que implican extractos de bacterias lisadas. Además, se ha propuesto que la formación de AB se puede producir a partir de la degradación de arsenoazúcares dimetilados a DMAE intermedia y luego a arsenocolina (AC) que luego se convierte a AB (**Figura 3**, vía # 2a). La conversión de AC a AB se ha demostrado en muchos estudios de laboratorio con diferentes bacterias, ratones, ratas y conejos. Sin embargo, la degradación de arsenoazúcares a AC no ha sido así demostrada. Los estudios con camarones mostraron que arsenoazúcares permanecieron sin cambios o transformados para pequeñas cantidades de DMA (V) que sugiere la vía de la formación de arsenoazúcares a AB no implica AC como intermedio.

Otra vía propuesta (**Figura 3**, vía # 3) se basa en la síntesis de aminoácidos por los compuestos metilados simples que involucran ácido dimethylarsinous (DMA (III)) y ácidos 2-oxo, glioxilato y piruvato, para formar DMAA y luego AB.

Arsenobetaína es la principal forma de arsénico en peces y en la mayoría de otros mariscos. Arsenobetaína también se ha encontrado en algunos alimentos terrestres, en particular, en algunas especies de hongos, aunque generalmente como un compuesto menor. Más recientemente, se demostró que arsenobetaína también se produce en las algas marinas a bajas concentraciones. Arsenobetaína no se ha detectado aún en el agua de mar aunque es probable que presente niveles traza. Las concentraciones de arsenobetaína en organismos de agua dulce son generalmente mucho más bajos que en los organismos marinos, a menudo por debajo de 0,1 mg As kg^{-1}. Sin embargo, peces de agua dulce de piscifactoría (productos de la acuicultura) puede contener arsenobetaína en concentraciones más altas, ya que cuentan con de alimentos contienen ingredientes marinos.

Arsenoazúcares

Arsenoazúcares comprenden un dimethylarsinoyl o un derivado trimethylarsonium unido a un azúcar ribofuranósido. Más de 20 arsenoazúcares naturales han sido identificados, la mayoría de los cuales son dimethylarsinoylribosides y los trimethylarsonoribosides suelen ser constituyentes menores. Los principales arsenoazúcares que se encuentran comúnmente en las algas se muestran en la **Figura 1.** Los primeros arsenoazúcares fueron aislados del alga parda

Ecklonia radiata en 1981 y fueron sulfonato y glicerol arsenoazúcares. Arsenoazúcar sulfato fue aislado del riñón de una almeja gigante, *Tridacna maxima* en 1982. El último arsenoazúcar común, arsenoazúcar fosfato fue identificado en 1983 y también se aisló de *Ecklonia radiata*.

La absorción de arsénico del agua por organismos acuáticos inicialmente se produce debido a sus propiedades químicas similares a las del fósforo esencial de macronutrientes. Se supone que las algas absorben As (V) del agua de mar y lo bioacumulan como arsenoazúcares. Las algas tienen un sistema de transporte de membrana para asumir el fosfato esencial del agua de mar, pero esto no puede distinguir entre el fosfato y arsenato. Esta hipótesis está sustentada por un estudio donde la adición de fosfato en un medio de crecimiento del fitoplancton disminuyó la absorción de arsénico en las células de fitoplancton, lo que indica la competencia entre arseniato y fosfato para la captación celular. Por lo tanto, para eliminar el arseniato tóxico, las algas han desarrollado un proceso de conversión a arsenoazúcares. Este hallazgo ha sido apoyado por el estudio del proceso de desintoxicación para el alga parda *Fucus serratus*. El estudio muestra que a baja concentración de arseniato (20 mg As L^{-1}) el alga absorbe arseniato fácilmente y lo convierte de manera eficiente a los arsenoazúcares mientras que a alta exposición (100 mg As L^{-1}) el proceso de desintoxicación se sobrecarga, las especies arsénico tóxicas (presentados principalmente como arsenito y methylarsonate) se acumulan a niveles fatales para el alga y los arsenoazúcares no se produjeron significativamente.

Los arsenoazúcares parecen ser los intermedios clave en el ciclo bioquímico del arsénico. Estos pueden servir como precursores de arsenobetaína (**Figura 4**), la principal forma de arsénico en los animales marinos. La evidencia disponible indica que estos compuestos se forman a partir de arseniato, absorbido por las algas del agua de mar, en un proceso que implica la S-adenosilmetionina ya a ambos donantes de los grupos metilo y del grupo ribosil (azúcar) (**Figura 4**). La ruta biosintética propuesta, se basa en la vía de metilación de arsénico por microorganismos propuesta por Challanger (**Figure 3**). En esta vía el tercer paso de metilación se sustituye por un paso adenosylation seguido por glicosidación (**Figura 4**). Este esquema fue apoyado por la identificación del arsenosugar nucleósido intermediario clave en el riñón del almeja gigante *Tridacna maxima*.

Arsenoazúcares suelen ser los principales componentes arsenicales de algas marinas (típicamente 2-50 mg As kg^{-1}), y también se encuentran en concentraciones significativas en los animales que se alimentan de algas (por ejemplo, los mejillones y las ostras, típicamente 0,5-5 mg As kg^{-1}). En los organismos terrestres, los arsenoazúcares tienen lugar generalmente sólo a niveles traza, aunque se ha informado excepciones interesantes.

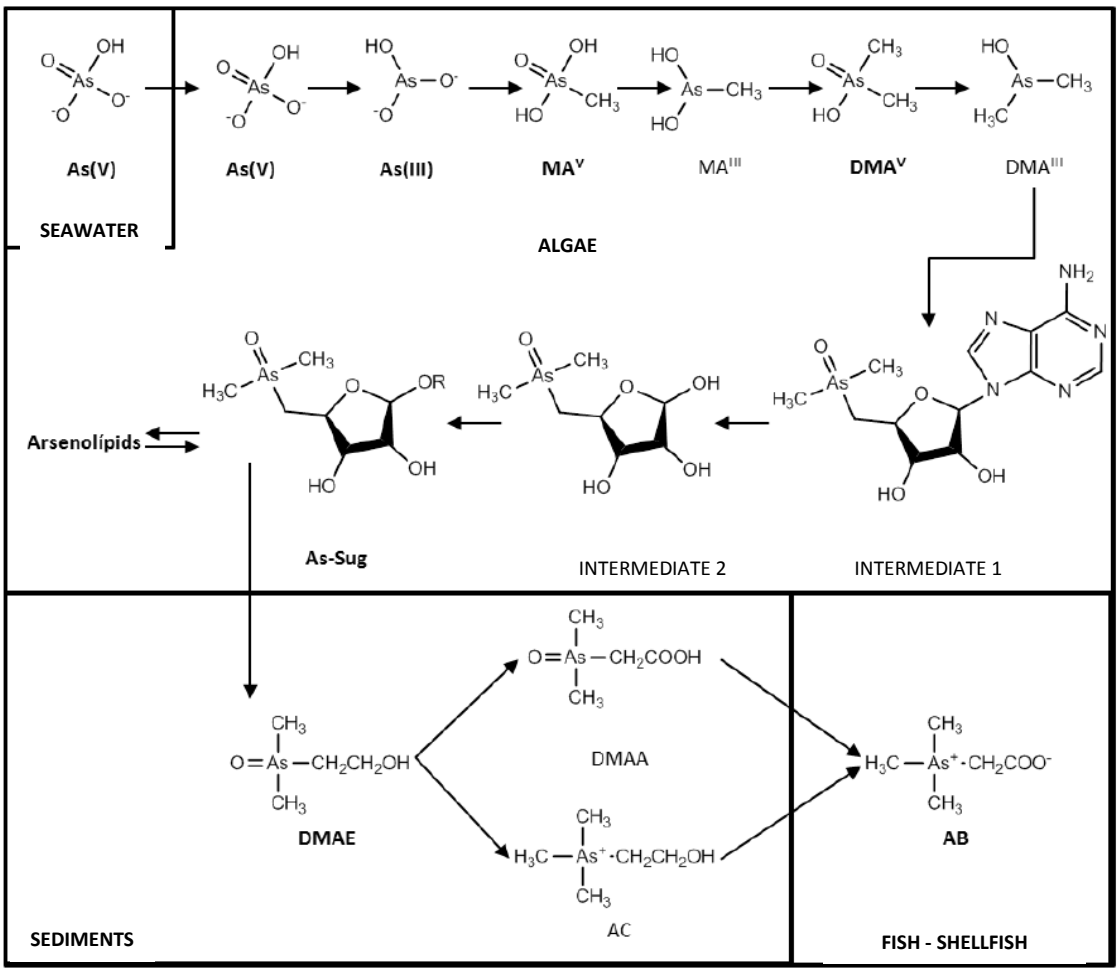


Figura 4. Propuesta ruta biosintética del metabólica para arsenoazúcares de arseniato de algas (adaptado de Edmonds, 1987 y 2003)

Tio-arsenoazúcares

Algunos arsenoazúcares pueden existir en dos formas diferentes, oxo y tio-arsenoazúcares. Estos compuestos son los análogos de azufre de oxo-arsenicales, donde el arsinoyl (As = O) grupo está sustituido por un grupo arsinothioyl (As = S). Las estructuras de los principales tio-arsenoazúcares se presentan en la **Figura 5** y la **Tabla 2**. El primero identificado tio-arsenical fue (tio-dimethylarsinoyl) DMAAS acetato en 2004 en la orina de las ovejas alimentadas con algas. Varias especies tio-arsenicales se han identificado en los moluscos, algas, y la orina humana.

Tabla 2. Nombre de la especie, abreviaturas, fórmula, peso molecular y CAS para las principales especies tio-arsenoazúcares

Compuesto	Nombre común	Abreviatura	Fórmula	Peso molecular	Numero CAS
19	Thio-arsenosugar glycerol	Thio-OH	$C_{10}H_{21}AsO_6S$	344.26	761458-55-3
20	Thio-arsenosugar phosphate	Thio-PO_4	$C_{13}H_{28}AsO_{11}PS$	498.32	761458-56-4
21	Thio-arsenosugar sulfonate	Thio-SO_3	$C_{10}H_{20}AsO_8S_2$	407.31	1227407-67-1
22	Thio-arsenosugar sulfate	Thio-SO_4	$C_{10}H_{20}AsO_9S_2$	423.31	1227407-68-2

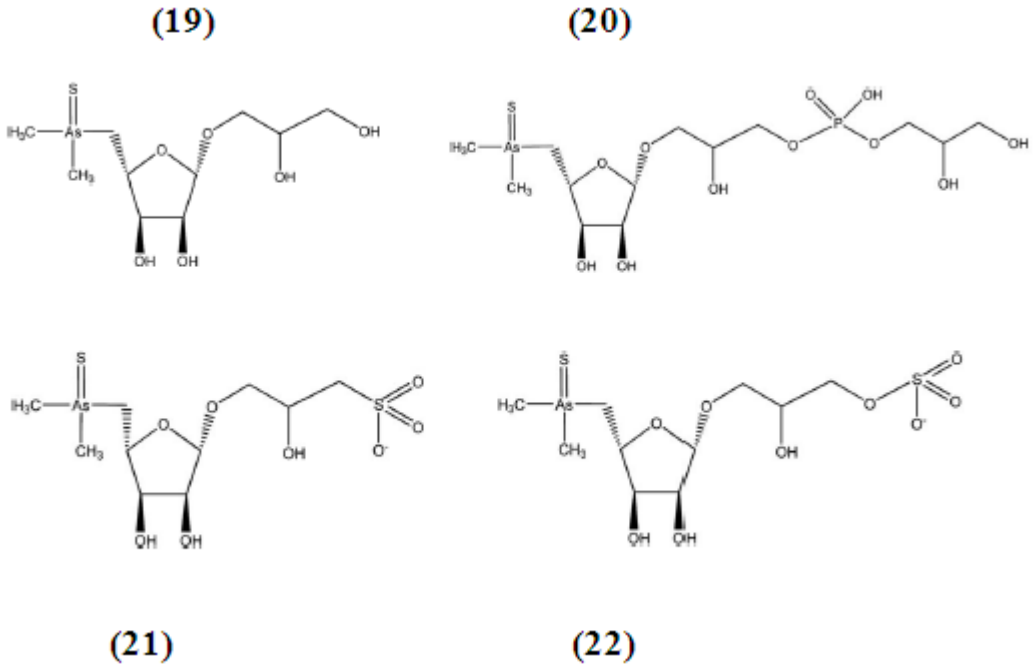


Figura 5. Estructuras de las principal especies tio-arsenoazúcares: tio-arsenosugar glicerol (19); fosfato tio-arsenosugar (20); sulfonato tio-arsenosugar (21); sulfato de tio-arsenosugar (22)

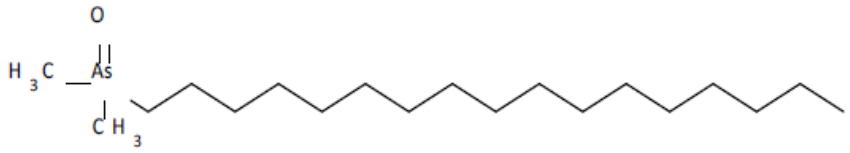
Arsenolípidos

Arsenolípidos es un término amplio para todos los compuestos naturales solubles en grasa que contienen arsénico (**Tabla 3 y Figura 6**). Arsenolípidos han sido mucho menos investigados en comparación con los arsenicales solubles en agua y están presentes en los aceites marinos, tales como aceites y aceites extraídos de algas de pescado. Sin embargo, la distribución de estos compuestos en diversos organismos marinos no está bien estudiada. Los compuestos arsenolipids han demostrado que varían de organismo a organismo.

Tabla 3. Nombre común, abreviaturas, fórmula, peso molecular y el número CAS para arsenolipidos: hidrocarburos arsénico, ácidos grasos de arsénico y fosfolípidos de arsénico

Compuesto	Nombre común	Abreviatura	Fórmula	Peso molecular	Numero CAS
23	**Arsenic hydrocarbons**	AsHC	$C_{19}H_{39}AsO$	358.44	1456610-45-9
			$C_{19}H_{41}AsO$	360.46	1083077-43-3
			$C_{20}H_{43}AsO$	374.48	1423745-42-9
			$C_{21}H_{45}AsO$	388.51	1393357-63-5
			$C_{22}H_{47}AsO$	402.54	1423745-43-0
			$C_{24}H_{39}AsO$	418.5	1456610-47-1
24	**Arsenic fatty acids**	AsFA	$C_{17}H_{35}AsO_3$	362.39	1032052-02-0
			$C_{22}H_{35}AsO_3$	422.44	1423745-44-1
			$C_{22}H_{37}AsO_3$	424.46	1423745-45-2
			$C_{23}H_{37}AsO_3$	436.47	1032052-10-0
			$C_{24}H_{37}AsO_3$	448.48	1296225-43-8
25	**Arsenic phospholipids**	AsPL	$C_{43}H_{84}AsO_{14}P$	931.03	1423745-30-5
			$C_{43}H_{84}AsO_{14}P$	931.03	1423745-46-3
			$C_{44}H_{86}AsO_{14}P$	945.05	1423745-31-6
			$C_{45}H_{88}AsO_{14}P$	959.08	115921-38-5
			$C_{45}H_{86}AsO_{14}P$	957.07	1423745-40-7
			$C_{45}H_{84}AsO_{14}P$	955.05	1393357-60-2
			$C_{46}H_{90}AsO_{14}P$	973.11	1423745-32-7
			$C_{47}H_{86}AsO_{14}P$	981.09	1423745-39-4
			$C_{47}H_{92}AsO_{14}P$	987.14	1423745-34-9
			$C_{47}H_{86}AsO_{14}P$	981.09	1423745-47-4
			$C_{47}H_{90}AsO_{14}P$	985.12	1423745-37-2
			$C_{47}H_{88}AsO_{14}P$	983.1	1423745-38-3
			$C_{48}H_{94}AsO_{14}P$	1001.16	1423745-35-0
			$C_{49}H_{96}AsO_{14}P$	1015.19	1393357-61-3
			$C_{51}H_{100}AsO_{14}P$	1043.24	1631038-74-8
			$C_{53}H_{104}AsO_{14}P$	1071.3	1393357-62-4

La presencia de compuestos de arsénico liposolubles se informó por primera vez en peces a finales de 1960. Varios años más tarde, en 2008 se reportaron algunas estructuras de los primeros compuestos de arsénico liposolubles y fueron finalmente dilucidados y seis ácidos grasos que contienen arsénico fueron identificados en el aceite de hígado de bacalao y tres hidrocarburos que contienen arsénico en el aceite de capelán. Además, varios otros arsenolípidos estaban presentes en los aceites cuyas estructuras aún se desconocen. Arsenolipids fueron encontrados recientemente en el atún, que es la primera identificación de arsenolipids en mariscos de consumo habitual y puede ocurrir en una amplia gama de muestras biológicas y pueden alcanzar concentraciones de más del 90% del contenido de tAs. Aunque los arsenolípidos parecen ser comunes, especialmente en los pescados grasos, los datos cuantitativos son escasos. En los aceites de pescado examinados hasta el momento, el contenido arsenolípidos varió entre alrededor de 4 a 12 mg As kg^{-1} de aceite. Aunque las primeras investigaciones sobre el arsénico soluble en lípidos en los aceites marinos fueron reportadas hace más de 40 años, el conocimiento acerca de su biosíntesis, estructuras químicas, los niveles y la toxicidad es aún limitada.



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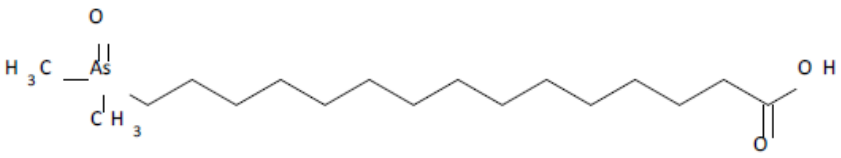

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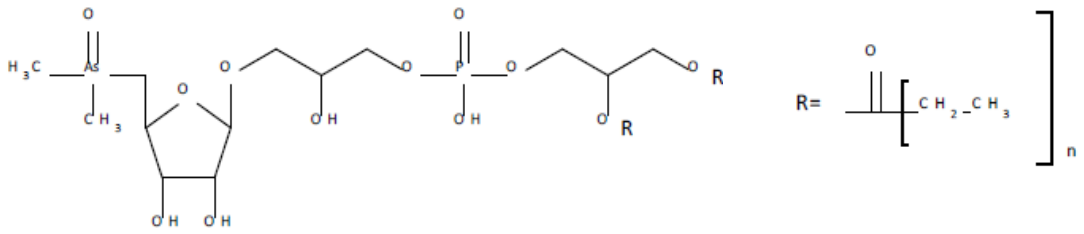

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Figura 6. Estructuras de los hidrocarburos de arsénico (23), los ácidos grasos de arsénico (24) y fosfolípidos arsénico (25). Estructuras son generalizadas y no muestran el grado de saturación de los ácidos grasos, hidrocarburos o fosfolípidos

1.4 Toxicidad de especies de arsénico

La toxicidad del arsénico depende de su forma química y el estado de oxidación y también en la forma en que se metabolizan en el cuerpo. El estado de oxidación y forma química del arsénico son factores importantes que afectan a la toxicidad y pueden ser cambiadas por procesos biológicos. Por lo tanto la vía metabólica de especies de arsénico debe ser considerado.

En la toxicología, la forma más común para evaluar la toxicidad aguda son LD_{50} y LC_{50}. La dosis letal media, LD_{50} (abreviatura de "dosis letal, 50%") o LC_{50} (“concentración letal, 50%”) es una medida de la dosis letal de una toxina, radiación, o patógeno. El valor de la LD_{50} para una sustancia es la dosis necesaria para matar a la mitad de los miembros de una población sometida a prueba después de duración de la prueba específica. Cifras LD_{50} se utilizan con frecuencia como un indicador general de toxicidad aguda de sustancias. Una LD_{50} inferior es indicativa de una mayor toxicidad. Se han descrito dos tipos de toxicidad del arsénico:

Aguda: provocada por la ingestión de grandes cantidades de formas inorgánicas de arsénico donde esto ha demostrado tener efecto en casi todos los sistemas fisiológicos del cuerpo y puede ser letal. La exposición aguda a algunos compuestos de arsénico puede causar la muerte. Tal y como se comentó, un parámetro común para evaluar la toxicidad aguda es la LD_{50} y los valores de varias especies de arsénico se muestran en la **Tabla 4**.

Crónica: los seres humanos pueden estar expuestos crónicamente al iAs principalmente a través del agua potable. Efectos de la exposición prolongada al arsénico puede provocar cáncer de la piel, vejiga, pulmón, así como otros tipos de cáncer, y también lesiones de la piel tales como la hiperpigmentación de la piel y queratosis. La exposición crónica puede incluir, además, efectos sobre el sistema nervioso periférico, el sistema nervioso central y se ha asociado con enfermedades cardiovasculares. Los efectos de la exposición crónica de arsénico orgánico no se conocen plenamente.

Estudios de especiación de arsénico han puesto de manifiesto la dependencia de la toxicidad de acuerdo con las especies de arsénico. La diferente toxicidad de las especies de arsénico refuerzan la importancia de su especiación química, ya que la cantidad total de arsénico no proporciona suficiente información sobre la toxicidad de la muestra analizada. En general, se puede considerar lo siguiente gradación de carácter tóxico de los compuestos de arsénico:

R_3As (R = H, Me, Cl)> As_2O_3 (As (III))> As_2O_5 (As (V))> RnAsO $(OH)_{3-n}$ (n = 1,2)> R_4 As +

Tabla 4. Toxicidad aguda (valores de LC_{50} y LD_{50}) de algunas especies de arsénico.

As species	LC_{50} [a] (μmol L^{-1})	LD_{50} [b] (mg kg^{-1})	Referencias LD_{50}
DMA (III)	2.16	-	
As(III)	5.49	14.0-42.9	(Petrick et al., 2000)
As(V)	571	20-800	(Shiomi, 1994)
DMA (V)	843	1.200-2.600	(Kaise et al., 1989)
MA (V)	-	700-1.800	(Hedegaard and Sloth, 2011)
MA (III)		3.5	(Petrick et al., 2000)
AC	-	6.500	Kaise et al., 1992)
AB	-	>10.000	(Kaise et al., 1985)
TETRA	-	890	(Hedegaard and Sloth, 2011)
TMAO		10600 [c]	(Kaise et al., 1989)

[a] valores de LC_{50} para las células humanas (Naranmandura 2007). [b] Ld_{50} para ratones (ingestión oral)

El arsénico inorgánico (arsenito o As (III) y el arseniato o As (V)) se considera la forma más peligrosa debido a su disponibilidad biológica, así como los efectos fisiológicos y toxicológicos (iAs se clasifica como un no-umbral, clase 1 carcinógeno humano). Compuestos inorgánicos de arsénico son generalmente más tóxicos que los compuestos orgánicos de arsénico y el arsénico trivalente se considera más tóxico que el arsénico pentavalente (Mandal, 2002). Se ha propuesto que la toxicidad aguda de arsenicales trivalentes es a causa de su unión a los grupos tiol de las proteínas biológicamente activas inhibiendo de este modo la función de varias enzimas metabólicas La toxicidad aguda generalmente disminuye con el aumento de grado de metilación (**Tabla 4**), con la excepción de TETRA, cuyo valor LD_{50} agudo es menor que para los otros compuestos metilados (MA, DMA, el TMAO). La toxicidad crónica de la mayoría de los arsenicales orgánicos, tales como MA y DMA, no se ha establecido de manera decisiva. AB es considerado no tóxico y puede ser consumido sin preocuparse, y AC es esencialmente no tóxico. Arsenoazúcares no son de toxicidad aguda, pero hay una posibilidad de que puedan tener efectos crónicos tóxicos ya que toxicidad y metabolismo se han estudiado escasamente. No se sabe mucho acerca de la toxicidad de arsenoazúcares, que se encuentran comúnmente en las algas y podrían ser considerado como potencialmente tóxicos, ya que son biotransformados por los seres humanos en organoarsenicales tóxicos. Además, se han reportado compuestos lipídicos de arsénico (arsenolipidos) como los principales compuestos de arsénico en los pescados grasos y su toxicidad aún es desconocida.

1.5 Exposición alimentaria de arsénico y evaluación del riesgo

En 1989, el Comité Mixto de Expertos en Aditivos Alimentarios (JECFA) perteneciente a la Organización de las Naciones Unidas para la Alimentación y la Agricultura /Organización

Mundial de la Salud (FAO/OMS) estableció una ingesta semanal tolerable provisional (PTWI) de 15 μg As kg^{-1} de peso corporal (pc) para iAs (equivalente a 2,1 μg As kg^{-1} de peso corporal por día). En 2009, la Comisión Técnica de Contaminantes de la Cadena Alimentaria (CONTAM) de Autoridad Europea de Seguridad Alimentaria (EFSA) evaluó los riesgos para la salud humana relacionados con la presencia de arsénico en los alimentos de la población europea. Se consideraron más de 100.000 datos de presencia de arsénico en los alimentos, pero aproximadamente el 98% se reportaron como arsénico total. Los niveles más altos de arsénico total se midieron en los siguientes productos alimenticios: pescados y mariscos, productos alimenticios o suplementos a base de algas, especialmente Hijiki y productos de cereales y cereales, con concentraciones particularmente altas en granos de arroz y los productos a base de arroz y salvado y germen.

La exposición al arsénico inorgánico de los alimentos y el agua a través de 19 países europeos se estimó en un rango 0,13-0,56 μg As kg^{-1} de peso corporal (pc) por día para el consumidor medio y 0,37-1,22 μg As kg^{-1} de peso corporal por día para los grandes consumidores (percentil 95th). Las subclases de alimentos: granos de cereales y productos a base de cereales, seguidos de los alimentos para usos especiales dietéticos, agua embotellada, café y cerveza, granos de arroz y los productos a base de arroz, pescado y verduras fueron identificados como los grandes contribuyentes a la exposición diaria de arsénico inorgánico en general población europea. Una visión simplificada de especies de arsénico en algunos productos alimenticios se muestra en la **Figura 7**. Feldmann y Krupp propusieron una estrategia para un enfoque analítico como rutina para clasificar los compuestos de arsénico en tres fracciones según su toxicidad: (i) arsénico inorgánico, tóxicos; (ii) AB según lo establecido arsenical no tóxico; (iii) la fracción organoarsenical sobrante, que puede contener arsenoazúcares y otros organoarsenicales, incluyendo compuestos no extraíbles con agua, compuestos de arsénico solubles en grasa o lipófilos extraíbles; quienes tendrían que ser reportado como la suma de arsenicales potencialmente tóxicos. Las proporciones esperadas de este arsénico fracciones en peces, algas, moluscos y el arroz se ilustran **Figura 7**. Como puede observarse, un alto contenido de arsénico total se encuentra en los productos alimenticios marinos en comparación con el arroz. El patrón de especiación revela que la iAs es predominante en el arroz y se espera por debajo del 5% en los productos alimenticios marinos. Mientras tanto, arsenobetaína, no tóxica, es el principal compuesto en los peces y moluscos. Además, se ilustra la importancia de la fracción potencialmente tóxica de los cuales los arsenoazúcares en algas y moluscos son las especies importantes.

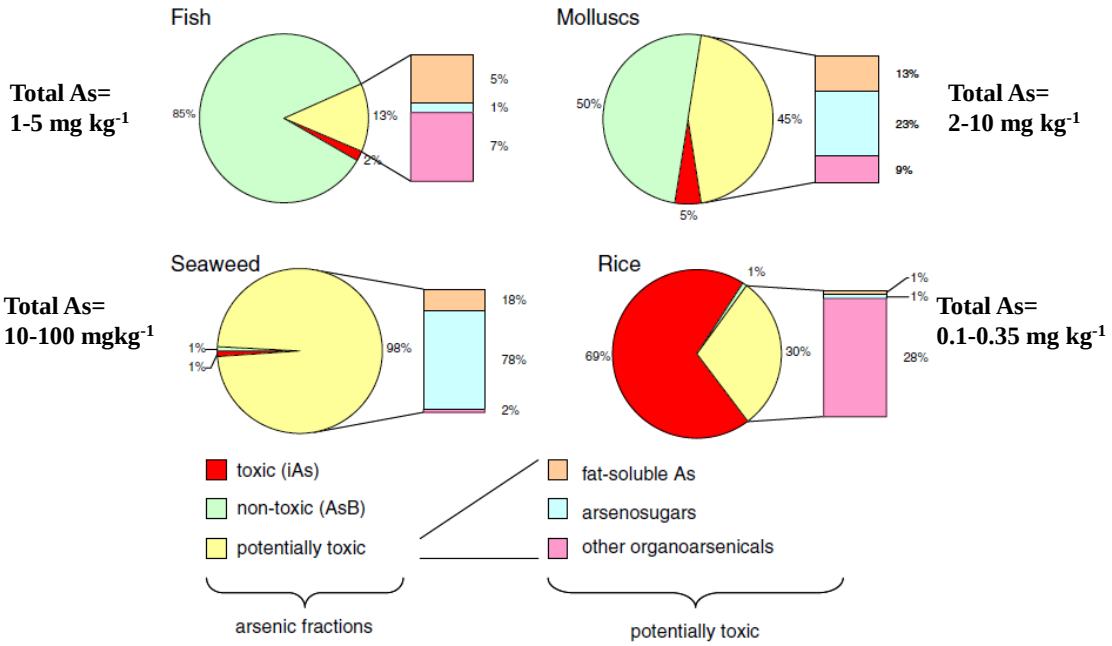

Figura 7. Proporciones esperadas de las tres fracciones de arsénico en pescado, moluscos, algas y arroz.

El grupo CONTAM de la EFSA concluyó que la exposición alimentaria al arsénico inorgánico para niños menores de tres años de edad es, en general, estima ser de 2 a 3 veces la de los adultos. Se concluyó que la ingesta semanal tolerable provisional (PTWI) establecida por el JECFA ya no es apropiado era como datos habían demostrado que el arsénico inorgánico provoca cáncer de pulmón y vejiga urinaria, además de la piel, y que una serie de efectos adversos se había informado al exposiciones inferiores a los revisados por el JECFA. El grupo CONTAM modela los datos de dosis-respuesta de los estudios epidemiológicos clave y selecciona una respuesta de referencia de riesgo adicional del 1%. Un rango de valores como punto de referencia de dosis límite inferior de confianza ($BMDL_{01}$) entre 0,3 y 8 mg kg^{-1} de peso corporal por día fue identificado para los cánceres de pulmón, la piel y la vejiga, así como lesiones en la piel. Además, las exposiciones alimentarias estimadas a arsénico inorgánico para el consumidor medio y de alto nivel en Europa estuvieron dentro del rango de los valores $BMDL_{01}$ identificados, y por lo tanto la conclusión de que hay poco o ningún margen de exposición y la posibilidad de un riesgo para algunos consumidores no pueden ser excluidos.

Entre las ocho propuestas por la EFSA, cuatro de ellas están relacionadas con la metodología analítica:

- La exposición alimentaria al arsénico inorgánico debe reducirse.

- Con el fin de perfeccionar la evaluación de riesgos de arsénico inorgánico, hay una necesidad de producir datos de especiación para diferentes productos alimenticios para apoyar los datos dietéticos de evaluación de la exposición y la dosis-respuesta de los posibles efectos sobre la salud.

• Aunque varios métodos de especiación de arsénico se han reportado, es necesario establecer su idoneidad para una serie de muestras de alimentos y / o especies de arsénico.

• Hay una necesidad de métodos analíticos validados robustos para la determinación de arsénico inorgánico en una gama de productos alimenticios.

• Se requieren materiales de referencia certificados especialmente para el arsénico inorgánico en productos tales como el agua, el arroz y el marisco. La producción de un material de este tipo debe ser una prioridad para facilitar futuros estudios sobre el contenido de arsénico inorgánico de los alimentos.

• Estudios epidemiológicos futuros deben incorporar mejor caracterización de la exposición al arsénico inorgánico incluyendo las fuentes de alimentos.

• Hay una necesidad de más información sobre los períodos de la edad crítica de la exposición al arsénico, en particular en la vida temprana. Los estudios deben incluir efectos en el futuro de la exposición a arsénico así como la exposición temprana.

• Existe una necesidad de mejorar la comprensión del metabolismo humano de organoarsenicals en los alimentos (arsenoazúcares, arsenolipids etc.) y las implicaciones para la salud humana.

Asimismo, el Comité Mixto FAO/OMS de Expertos en Aditivos Alimentarios (JECFA) ha evaluado la exposición alimentaria al iAs. Conclusiones similares al informe de la EFSA se publicaron, destacando que existe una necesidad de métodos validados para la extracción selectiva y la determinación del iAs en matrices alimentarias y de CRMs para iAs. Además, se destacó que hay una necesidad de mejora de los datos sobre la presencia de diferentes especies de arsénico y su biodisponibilidad en diferentes alimentos que se consumen con el fin de mejorar las estimaciones de la exposición dietética y sistémica. También publicó que se necesita más información sobre la toxicidad de especies de arsénico en los alimentos. Se recomendó que los futuros estudios epidemiológicos de los impactos en la salud de arsénico deben incorporar medidas adecuadas de la exposición total al iAs, incluyendo los alimentos y el agua de cocción así como el procesamiento de los alimentos. Por último, el Comité recomendó que los futuros estudios epidemiológicos no sólo se centren en los riesgos relativos, sino también analizar y reportar los datos de tal manera que son adecuados para la estimación de los niveles de exposición asociados con riesgos adicionales, a fin de que sus resultados sean utilizables para una cuantitativa evaluación del riesgo. Además, el límite de arsénico inorgánico más bajo en la dosis de referencia para un 0,5% de aumento en la incidencia de cáncer de pulmón ($BMDL_{0.5}$) se determinó a partir de estudios epidemiológicos que ser basada 3,0 μg As kg^{-1} de p.c. por día (2-7 μg As kg^{-1} de p.c. por día en la gama de la exposición dietética total estimada) utilizando

una serie de supuestos para estimar la exposición dietética total al arsénico inorgánico a partir del agua de bebida y comida. El Comité observó que la ingesta semanal tolerable provisional (PTWI) de 15 µg As kg^{-1} de p.c se encuentra en la región de la $BMDL_{0.5}$ y por lo tanto ya no era apropiado y el Comité retiró el PTWI anterior.

Recientemente, en 2014 la EFSA evaluó la exposición alimentaria al arsénico inorgánico en la población europea y proporcionó información sobre los niveles de arsénico, total e inorgánico, que se encuentran en una variedad de alimentos en el mercado europeo. Un conjunto de datos compuesta de 103,773 muestras de alimentos (incluida el agua potable) fueron recogidos en 21 países europeos y se utilizaron para calcular la exposición alimentaria a iAs. De ellos, 101.020 se basaban en tAs y 2753 a iAs. Entre los resultados presentados de tAs, el 66,1% estaba por debajo del límite de detección o cuantificación; por su parte de los datos reportados sobre iAs el porcentaje de datos fue de 41.9%. La mayor parte de los datos publicados (92,5%) de As fueron convertidos a iAs utilizando diferentes enfoques (en general un factor de conversión del 70% se utilizó) antes de calcular la exposición alimentaria a iAs. La exposición alimentaria estimada entre la población adulta fue considerablemente menor en comparación con el anterior dictamen de la EFSA de 2009, y osciló 0,09-0,38 µg As kg^{-1} de p.c por día, y las estimaciones de la exposición dietética percentil 95 variaron desde 0,14 hasta 0,64 µg As kg^{-1} de p.c por dia. Se concluyó que el principal contribuyente a la exposición alimentaria al arsénico inorgánico fue el grupo de los grupos de alimentos "productos elaborados a base de cereales (no basados en arroz) (**Figura 8**), en particular, el pan de trigo, para todas las clases de edad, excepto los bebés y niños pequeños. Otros grupos de alimentos que fueron importantes contribuyentes a la exposición del iAs fueron el arroz, la leche y los productos lácteos (principal contribuyente en los lactantes y niños pequeños), y el agua potable.

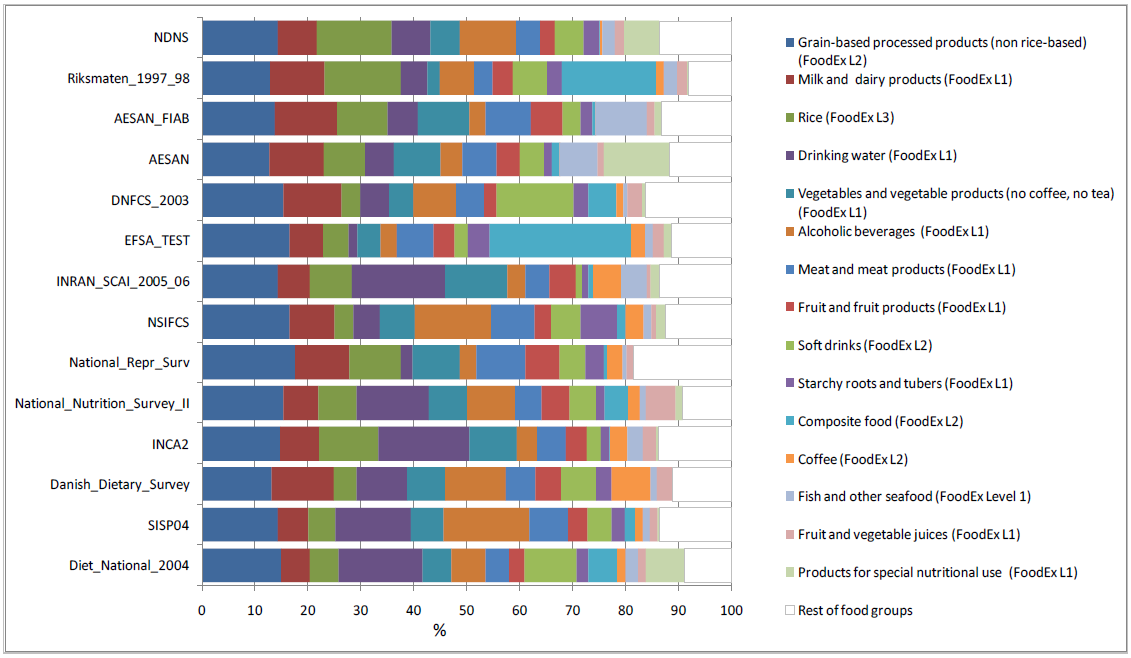

Figura 8. Principales grupos de alimentos que contribuyen (%) a la exposición alimentaria crónica media de iAs para la clase de edad '18 a 65 años de edad "(Adaptado de la EFSA, 2014).

Además, la mayor exposición alimentaria se estimó en los más jóvenes de la población (bebés y niños pequeños) y la exposición dietética media varió 0,20 a 1,37 µg As kg^{-1} de p.c por día, mientras que las estimaciones de la exposición dietética percentil 95 variaron desde 0,36 hasta 2,09 µg As kg^{-1} de p.c por dia. Los principales contribuyentes fueron “Leche y productos lácteos”, seguido de “agua potable", "productos elaborados a base de cereales (no basados en arroz)" y "Alimentos para lactantes y niños pequeños "(**Figura 9**). Además se destacó que el consumo de tres porciones (90 gramos/día) de base de arroz alimentación infantil podría representar una fuente importante de la iAs (1.59- 1.96 µg As kg^{-1} de p.c por día). Finalmente se concluyó que las fuentes de incertidumbre más importantes en la presente evaluación se relacionan con la heterogeneidad de los datos de consumo de alimentos, la conversión de tAs en iAs y al tratamiento de los datos censurados. Además, como recomendación se destacó que se necesitarían más datos analíticos sobre las iAs, en particular, en pescados y mariscos, y en los grupos de alimentos que proporcionan una contribución significativa a la exposición alimentaria al iAs (por ejemplo, productos de arroz y base de trigo) con el fin de reducir la incertidumbre de la evaluación a la exposición de arsénico inorgánico.

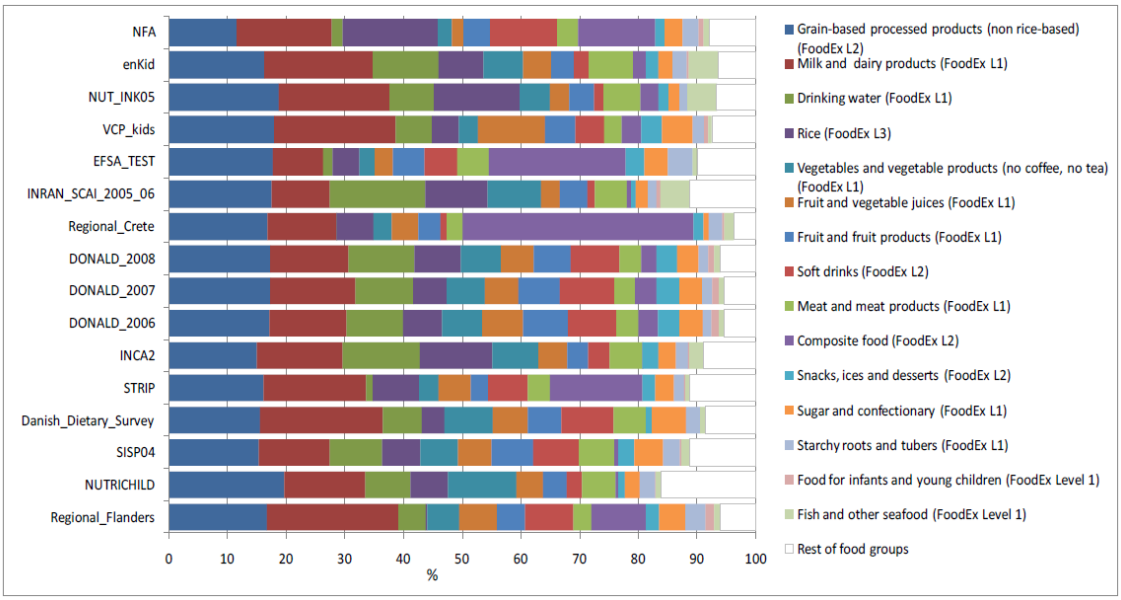

Figura 9. Grupos de alimentos principales que contribuyen (%) a la exposición alimentaria crónica media de iAs para la clase de edad '3 a 10 años de edad "(Adaptado de la EFSA, 2014).

1.6. Legislación Europea

La UE estableció un límite máximo de 10 µg As L-1para el arsénico en las aguas destinadas al consumo humano en base a la recomendación de la OMS. La Directiva 2002/32/ CE sobre sustancias indeseables en la alimentación animal establece contenidos máximos de arsénico total en un número de productos de alimentación (referido a un pienso con un contenido de humedad del 12%). La directiva establece que los contenidos de iAs por debajo de 2 mg kg^{-1} se recomiendan en las materias primas para piensos, especialmente los basados en las especies de algas Hiziki fusiforme. Muy recientemente, la Unión Europea publicó el

Reglamento (UE) 2015/1006 (Comisión 2015) que modifica el anexo del Reglamento (CE) no 1881/2006 (Comisión de 2006) con respecto a los niveles máximos de iAs en el arroz y los productos a base de arroz. Los nuevos niveles máximos de iAs van de 0,10-0,3 mg As kg^{-1} en función del producto de arroz. Además, recientemente la Unión Europea ha publicado una nueva recomendación por la cual los Estados miembros deberán realizar el monitoreo de la presencia de arsénico en los alimentos durante los años 2016, 2017 y 2018. El monitoreo debe incluir una amplia variedad de productos alimenticios que reflejan los hábitos de consumo, incluyendo alimentos como los granos de cereales, productos a base de cereales (incluido el salvado y el germen) , jugos de frutas y vegetales, el agua potable (incluyendo el agua embotellada), café, hojas de té secas, cerveza, pescado y mariscos, verduras, productos de algas (incluyendo hijiki), la leche, los productos lácteos, los alimentos destinados a los lactantes y los niños pequeños, los alimentos para usos médicos especiales y suplementos alimenticios a fin de permitir una estimación precisa de la exposición a iAs. Los Estados miembros deben llevar a cabo el análisis del arsénico, preferentemente mediante la determinación del contenido de las iAs y tAs y, si es posible, otras especies de arsénico pertinentes, haciendo uso de un método de análisis que se ha demostrado para generar resultados confiables.

1.7 Las técnicas analíticas para la determinación de arsénico total, las especies de arsénico y arsénico bioaccessible

Una revisión exhaustiva de los métodos de análisis y técnicas de medición para la determinación de arsénico inorgánico se revisan y se muestra en el **Artículo** I. Por lo tanto, en esta sección se presenta una visión general de las técnicas analíticas.

1.7.1 Determinación de arsénico total

Revisiones recientes de la bibliografía indican que las principales técnicas utilizadas para la determinación de arsénico en muestras biológicas son la espectrometría de absorción atómica con horno de grafito (GFAAS), espectrometría de emisión óptica con plasma acoplado inductivamente (ICPOES), espectrometría de masas con plasma acoplado inductivamente (ICP-MS) y la espectrometría de absorción atómica con generación de hidruros (HG-AAS). La selección apropiada del procedimiento de preparación de muestras en el análisis de trazas es esencial debido a la integridad de la información química que depende en gran medida de los pasos iniciales. Los métodos más utilizados en la preparación de muestras de alimentos son incineración en seco y digestión ácida asistida por microondas. La presencia de arsenobetaína (AB) en peces y especies marinas puede ser un problema en la determinación del tAs por GFAAS, HG-AFS o HG-AFS. Esta especie es considerada una especie metabólicamente estable y su descomposición química es muy difícil. La conversión de todas las especies de arsénico orgánico en iAs normalmente se requiere o la determinación de tAs por espectrometría atómica. En consecuencia, la alta estabilidad de AB se vuelve desfavorable para la determinación del contenido de tAs. Digestiones húmedas utilizando agentes oxidantes fuertes combinados con ácidos fuertes y altas temperaturas (280ºC), son necesarios para la degradación completa de AB. En algunos casos, incluso con el uso de estos reactivos a temperaturas más altas, AB no se

degrada completamente y el contenido tAs puede ser subestimado fácilmente. Por lo tanto, es necesario ser consciente de este hecho con el fin de seleccionar el procedimiento de digestión más adecuado para superar este problema.

1.7.2 Especiación de arsénico

Consideraciones generales de especiación de arsénico

En especiación analítica de un elemento el parámetro clave es asegurarse de que no hay alteraciones de las especies de arsénico en todo el proceso de análisis, incluyendo el muestreo. La especiación de arsénico todavía es un reto, sobre todo en el análisis de muestras de alimentos con una matriz orgánica compleja. El objetivo de la especiación es la extracción cuantitativa de todas las especies de arsénico sin cambiar sus características originales. Los pasos principales en el análisis de especiación son: (1) extracción, (2) separación y (3) medición y cuantificación de las especies. Como ejemplo, los pasos de análisis de especiación de arsénico por HPLC-ICP-MS se muestran en la **Tabla 5.** Los pasos necesitan optimización y evaluación adecuada para obtener una extracción cuantitativa y garantizar cambios mínimos a las especies originales, especialmente en matrices complejas, tales como diferentes productos alimenticios. Por ejemplo, la selección de los extractantes y el aparato utilizado son cruciales en el paso 1; fases móviles se consideran cuidadosamente para lograr una separación cromatográfica adecuada (2); y, finalmente, la selección de las condiciones ICPMS más adecuada es de importancia primordial en el paso 3.

Tabla 5. Pasos en la especiación de arsénico por HPLC-ICP-MS

Step	Evaluation
Extraction	Selection of extractants
	Maintaining integrity of species
	Quantitative extraction
Separation (HPLC)	Selection of mobile phases
	Interaction of species with column
	Availability of standards
	Elution mode
Measurement and quantification (ICPMS)	Nebulization
	Monitored masses
	Interferences

Extracción de especies de arsénico

Según Maher y colaboradores, las especies metaloides podrían ser clasificadas como: "especies fáciles de extraer" especies estables existentes como moléculas discretas o relativamente débilmente ligados a constituyentes celulares y " especies difíciesl de extraer " especies inestables que se disocian en la extracción y especies incorporadas dentro de

constituyentes celulares como las proteínas. En el caso del arsénico, las especies de arsénico solubles en agua y las especies de arsénico solubles en lípidos fueron clasificadas en el primer grupo, mientras que las fitoquelatinas de arsénico en las plantas en el segundo. Por lo tanto, la selección de un disolvente de extracción adecuado es la importancia primordial en el análisis de especiación de arsénico. En general, las condiciones de extracción pueden variar mucho dependiendo del extractante: muestra, el enfoque de extracción utilizado y de los intervalos de tiempo y temperatura. Las condiciones de extracción influyen no sólo la eficacia de la extracción, sino también la integridad de las especies de arsénico nativas durante la extracción. El protocolo de extracción debe ser optimizado para obtener resultados fiables sobre la base de la eficacia de la extracción. Por último, es fundamental prestar especial atención a la estabilidad de las especies de arsénico en los extractos. Otro punto fundamental es asegurar la estabilidad de las especies de arsénico en las etapas de almacenamiento de muestras y pre-tratamiento mediante el uso de la conservación de la muestra seleccionada adecuadamente, ya que varios factores pueden promover entre la conversión de As especies (por ejemplo, la actividad microbiana, temperatura y luz).

Una amplia variedad de disolventes de extracción han sido utilizados en los productos alimenticios: mezclas de MeOH:agua, agua, ácidos, bases, extracciones secuenciales y enzimáticas son los más utilizados con el apoyo de la extracción mecánica, extracción de placa caliente, extracción ultrasónico, baño de agua, extracción presurizada o extracción asistida por microondas. Los parámetros metodológicos cruciales que podrían afectar a la eficacia de extracción y la estabilidad especies son: el tipo de disolvente, el tamaño de partícula de la muestra, la relación sólido / líquido, el tiempo de extracción y la temperatura; y, la técnica de extracción. Mezclas de metanol, agua y agua-metanol se utilizan comúnmente para extraer las especies de arsénico solubles en agua de las algas y animales marinos, pero son ineficientes en la extracción de las especies inorgánicas de arsénico en los animales terrestres. Para estas muestras se recomienda el uso de un ácido diluido. Los procedimientos de extracción secuencial, por ejemplo: extracción de metanol-agua, seguido de extracción con ácido diluido, incrementan la eficiencia de extracción de las especies de arsénico "difíciles de extraer". Extracción asistida con microondas es ampliamente utilizado y se ha demostrado dar mejores recuperaciones en relación con la agitación mecánica o sonicación. Algunos especies de As como los arsenoazúcares y AB son relativamente estables, pero As(III), As(V), MA(III), DMA(III), As-GSH y As-PC especies no lo son. Se debe tomar una atención especial para asegurar que las especies determinadas no se tratan de artefactos de los procedimientos de preservación o de extracción. La especiación de As en muestras de alimentos requiere la extracción en condiciones suaves con el fin de mantener la integridad de todas las especies de arsénico. Los problemas asociados con la baja recuperación de las especies y con la oxidación o reducción entre las especies inorgánicas, As III) y As(V), y la conversión de las especies orgánicas a las especies NIC han sido ampliamente reportados.

Técnicas

El análisis de especiación por lo general implica muchos pasos, incluida la extracción, separación y detección. Varios métodos han sido empleados para llevar a cabo análisis de especiación de arsénico. Sin embargo, la aparición de técnicas acopladas ha permitido el desarrollo de métodos analíticos muy potentes para los propósitos de especiación. Las técnicas de separación más comunes utilizados para este propósito, son la cromatografía de gases (GC), cromatografía líquida de alto rendimiento (HPLC), incluyendo la cromatografía iónica (IC), electroforesis capilar (CE) y el fraccionamiento campo-flujo (FFF), entre otros. Entre los detectores acoplados a una técnica de separación, HG-AAS, HG-AFS, ICPOES y especialmente ICPMS, con HG y sin HG, son los más utilizados entre los detectores específicos de elementos. La selección de la técnica de separación será determinada por las propiedades de las especies de arsénico de interés, tales como volatilidad, carga y polaridad, mientras que la técnica de detección se determina por el nivel de concentración esperada en la muestra ensayada. El análisis más común que se utiliza para la especiación de arsénico es HPLC-ICPMS (**Figura 5**). Además, hay métodos analíticos adicionales adecuados para la obtención de datos para complementar la información sobre la especiación de arsénico obtenido cuando se aplica el método antes mencionado, por ejemplo: espectroscopia de absorción de rayos X (XAS) y espectrometría de masas por electrospray (ESI-MS). Métodos y técnicas de medición para la determinación de arsénico inorgánico analítica se revisan y se muestra en el **Artículo I**.

1.7.3 Determinación del contenido bioaccesible de arsénico

Los alimentos proporcionan nutrientes, y además componentes no nutricionales y contaminantes. Se necesitan las frecuencias de consumo para evaluar los riesgos y beneficios asociados a la ingesta de un alimento determinado. Por otra parte, dicha evaluación debe tener en cuenta que los alimentos suelen ser sometidos a más tratamiento culinario antes de la ingestión. Cocinar afecta contenido de arsénico total y la distribución de las especies también arsénico. Para un mejor conocimiento de los riesgos y beneficios asociados al consumo de alimentos, la evaluación de la biodisponibilidad de arsénico, el contenido total y de especies de arsénico, es fundamental para la evaluación completa seguridad de los alimentos. La biodisponibilidad se refiere a la fracción de la sustancia que alcanza la circulación sistémica (sangre) desde el tracto gastrointestinal (GI) (fracción biodisponible) y que está disponible para promover su acción en el organismo expuesto. Un primer paso en la evaluación de la biodisponibilidad es el estudio de bioaccesibilidad, que indica la fracción máxima de un elemento traza u otra sustancia en los alimentos que se libera teóricamente a partir de su matriz en el tracto GI (fracción bioaccesibles), y por lo tanto se convierte en disponible para la absorción intestinal (es decir, entra en el torrente sanguíneo).

Se han propuesto tanto *in vitro* como *in vivo* estudios para la evaluación de los métodos de biodisponibilidad de arsénico en los alimentos. Cada método tiene sus propias fortalezas y debilidades para la evaluación de bioaccesibilidad. Los métodos *in vivo* son principalmente ventajosos debido a su cercanía a la realidad, sobre todo por lo que, si los individuos elegidos como sujetos de experimentación pertenecen a la población objetivo, pero exigen mucho tiempo

experimental, requieren una planificación cuidadosa y recursos específicos para un control experimental adecuado, y demás tienen algunas limitaciones éticas . Por otro lado, los métodos *in vitro* proporcionan una aproximación eficaz para las situaciones *in vivo* y ofrecen las ventajas de una buena reproducibilidad, la simplicidad, rapidez, facilidad de control, bajo coste y alta precisión, ya que es posible controlar las condiciones mejor que con en pruebas *in vivo*. Las condiciones experimentales se controlan con un grado mucho mayor y la validación y estandarización con materiales de referencia es posible, lo que permite la reproducibilidad y reduce la incertidumbre. Adecuada temperatura, agitación, pH, tipo de enzima y la composición química deben ser seleccionados para simular las condiciones gástricas y/o gastrointestinales. Para los enfoques metodológicos *in vitro*, la mayoría de los estudios sólo se refieren a la disponibilidad para la absorción intestinal. Para este propósito, hay una división entre el modelado digestivo estática y dinámica. En las metodologías estáticas, la reactividad bioquímica encontrado en el tracto GI humano (cavidad oral, entorno gástrico, y lumen intestinal) se simula de forma secuencial. Metodologías dinámicas están destinadas a ser más realista, que abarca varios fenómenos que se producen in vivo, tales como, cizalladura, mezclado, hidratación, o la peristalsis. Por otra parte, estas metodologías intentan simular las condiciones y cómo cambian con el tiempo durante cada etapa principal digestivo (boca, el estómago y el intestino).

Diversos enfoques *in vitro* para evaluar la bioaccesibilidad se han reportado en los últimos años. Los enfoques *in vitro* más utilizados para estimar la bioaccesibilidad son: (1) la concentración máxima soluble del compuesto diana en la solución GI simulado (fracción bioaccesible); (2) la fracción soluble del compuesto (fracción BA) logrado mediante el uso microbiota GI humano (Simulador del Ecosistema Intestinal Microbiana Humano, SHIME); (3) la fracción dializable del compuesto, que puede dializar través de una membrana semi-permeable con un tamaño de poro especificado (dializado o fracción biodisponible) en equilibrio o de no equilibrio condiciones; y, (4) la fracción del compuesto capaz de ser retenido o transportado a través de un sólido o micro soportes porosos (fracción biodisponible) en los que se incorporan células Caco-2 humanas cultivadas (modelo epitelial intestinal).

Un número limitado de estudios bioaccesibilidad de arsénico se ha llevado a cabo en alimentos convencionales tales como: pescados y mariscos, algas comestibles, arroz o verduras. Hay así una falta de datos de bioaccesibilidad de especies de arsénico en los alimentos teniendo en cuenta los efectos de cocción. Con el fin de mejorar el proceso de evaluación de riesgos, la necesidad de realizar tales estudios parece ser evidente.

1.8 Aseguramiento de la calidad en el análisis de la especiación

La determinación de especies de arsénico todavía no es un procedimiento de rutina, y por lo tanto criterios claros de calidad aún no se han establecido. La fiabilidad de los datos especiación depende de la exactitud del métodos de especiación. Para ello, la necesidad de minimizar los errores que pueden ocurrir durante el muestreo, preparación de muestras, separación y detección es de suma importancia. Una forma común para verificar procedimiento analítico y evaluar la exactitud del método es analizando materiales de referencia certificados

(CRMs). Estos deben ser lo más similar posible a la muestra real y deben ser tratados de la misma manera que las otras muestras. Los CRMs ofrecen una excelente manera de asegurarse de que el método empleado proporciona resultados aceptables. Varias CRMs están disponibles para el contenido de tAs en varias matrices, pero sólo unas pocos CRMs existen para la especiación de arsénico. Además, como control de calidad externo, pruebas de aptitud (PT) o comparaciones entre laboratorios son una valiosa herramienta para poner a prueba la fiabilidad de un método mediante la comparación de los resultados con un valor de referencia asignado. Un resumen de los CRMs disponibles con contenidos certificados para especies, así como los PTs para el análisis de especiación de arsénico inorgánico se describen en el **Artículo I**.

PARTE II: OBJETIVOS

Capítulo 2
Objetivos

El interés en la determinación de arsénico inorgánico (IAS) en los alimentos se debe al amplio reconocimiento de sus efectos tóxicos en los humanos, incluso a bajas concentraciones. De la literatura revisada, varios métodos de especiación de arsénico se han publicado, sin embargo es necesario establecer su idoneidad para una serie de muestras de alimentos y /o especies de arsénico Además, existe una necesidad urgente de métodos analíticos robustos y validados y adecuados para la determinación de arsénico inorgánico en una gama de productos alimenticios. Esta necesidad se destacó por varias agencias internacionales de seguridad y salud, y por las organizaciones encargadas de establecer los niveles máximos de la iAs en los productos alimenticios.

En vista de todo esto, el objetivo principal de esta tesis es desarrollar metodología analítica robusta para la determinación de arsénico inorgánico, así como otras especies de arsénico en alimentos. La metodología propuesta es aplicada a varios productos alimenticios proporcionando resultados fiables como una respuesta a la demanda de las agencias internacionales de seguridad.

Este objetivo general se puede dividir en objetivos específicos que se detallan a continuación:

• Establecimiento y validación de métodos para la determinación de arsénico total y de las especies de arsénico en los productos alimenticios.

• Como control de calidad externo de los métodos validados, la participación en estudios de viabilidad para la preparación de materiales de referencia certificados con especies de arsénico certificadas y en pruebas de aptitud para la determinación de arsénico inorgánico.

• Aplicación de la metodología propuesta en varios alimentos de origen tanto terrestres como marinos: arroz, alimentos a base de cereales, productos infantiles, setas, algas, peces, crustáceos y bivalvos.

• Estimación de la exposición dietética diaria de ingesta de arsénico total e inorgánico para evaluar las implicaciones toxicológicas de la ingesta de los alimentos seleccionados.

Publicaciones

- **Article I**

Recent developments and quality assessment of inorganic arsenic determination in food: a review

Llorente-Mirandes, T., Rubio, R., López-Sánchez, J. F.

Artículo en revisión

- **Article II**

A fully validated method for the determination of arsenic species in rice and infant cereal products.

Llorente-Mirandes, T., Calderón, J., López-Sánchez, J. F., Centrich, F., Rubio, R

Pure Applied Chemistry, 2012, 84(2), 169–333.

- **Article III**

A need for determination of arsenic species at low levels in cereal-based food and infant cereals. Validation of a method by IC-ICPMS.

Llorente-Mirandes, T., Calderón, J., Centrich, F., Rubio, R., López-Sánchez, J.F.

Food Chemistry, 2014, 147, 377–385.

- **Article IV**

Occurrence of inorganic arsenic in edible Shiitake (Lentinula edodes) products.

Llorente-Mirandes, T., Barbero, M., Rubio, R., López-Sánchez, J.F.

Food Chemistry, 2014, 158, 207–215.

- **Article V**

Arsenic speciation in commercial edible mushrooms and mushroom supplements by HPLC-ICPMS. Assessment of inorganic arsenic exposure.

Llorente-Mirandes, T., Barbero, M., Rubio, R., López-Sánchez, J.F.

Artículo enviado para su publicación

- **Article VI**

Measurement of arsenic compounds in littoral zone algae from the Western Mediterranean Sea. Occurrence of arsenobetaine.

Llorente-Mirandes, T., Ruiz-Chancho, M.J., Barbero, M., Rubio, R., López-Sánchez, J. F.

Chemosphere, 2010, 81, 867–875.

- **Article VII**

Determination of Water-Soluble Arsenic Compounds in Commercial Edible Seaweed by LC-ICPMS.

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Journal of Agricultural and Food Chemistry, 2011, 59, 12963–12968.

- **Article VIII**

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Food Chemistry, 2015, 173, 1073–1082.

- **Article IX**

Assessment of arsenic bioaccessibility in raw and cooked edible mushrooms by a PBET method.
Llorente-Mirandes, T., Llorens-Muñoz, M., Funes-Collado, V., Sahuquillo, A., López-Sánchez, J. F.
Food Chemistry, 2016, 194, 849–856

- **Article X**

Performance of laboratories in speciation analysis in seafood. Case of methylmercury and inorganic arsenic.
Baer, I., Baxter, M., Devesa, V., Vélez, D., Raber, G., Rubio, R., **Llorente-Mirandes, T**., Sloth, J.J., Robouch, P., de la Calle, B.
Food Control, 2011, 22, 1928-1934.

- **Article XI**

Is it possible to agree on a value for inorganic arsenic in food? The outcome of IMEP-112.
de la Calle, M. B., Baer, I., Robouch, P., Cordeiro, F., Emteborg, H., Baxter, M. J., Brereton, N., Raber, G., Velez, D., Devesa, V., Rubio, R., **Llorente-Mirandes, T**., Raab, A., Feldmann, J., Sloth, J. J., Rasmussen, R. R., D'Amato, M., Cubadda, F.
Analytical and Bioanalytical Chemistry, 2012, 404, 2475–2488.

- **Article XII**

Determination of total cadmium, lead, arsenic, mercury, and inorganic arsenic in mushrooms: the outcome of IMEP-116 and IMEP-39.
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- **Article XIII**

Accuracy of a method based on atomic absorption spectrometry to determine inorganic arsenic in food: Outcome of the collaborative trial IMEP-41.
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Artículo enviado para su publicación

PARTE III: RESULTADOS Y DISCUSIÓN

CAPÍTULO 3

Desarrollo y validación de métodos para la determinación de especies de arsénico en los productos alimenticios

En 2009, la Comisión Técnica de Contaminantes de la Cadena Alimentaria (CONTAM) de la EFSA evaluó los riesgos para la salud humana relacionados con la presencia de arsénico en los alimentos de la población europea. Como recomendación general se reportó que la exposición alimentaria al arsénico inorgánico se debe reducir. Entre las conclusiones de este informe, el arroz, los cereales y los productos a base de cereales fueron identificados como los contribuyentes principales a la exposición diaria de iAs en la población general europea. Además se informó que los niños menores de tres años son los más expuestos al iAs, que se relacionan directamente con el consumo de productos a base de arroz. Por otra parte, el informe de la EFSA hizo hincapié en que hay una necesidad de métodos analíticos validados robustos para determinar el iAs en una gama de productos alimenticios. Recientemente, la Unión Europea publicó el Reglamento (UE) 2015/1006 (Comisión Europea 2015) que modifica el anexo del Reglamento (CE) no 1881/2006 (Comisión Europea 2006) con respecto a los niveles máximos de la NIC en el arroz y los productos a base de arroz. Los nuevos niveles máximos de de iAs van de 0,10-0,3 mg As kg^{-1} en función del producto de arroz. Además, la Comisión Europea ha publicado recientemente una recomendación sobre el seguimiento de arsénico en los alimentos por los Estados miembros durante los años 2016, 2017 y 2018. El monitoreo debe incluir una amplia variedad de productos alimenticios y los Estados miembros deben llevar a cabo el análisis del arsénico, preferentemente mediante la determinación del contenido de iAs y tAs, si es posible, otras especies de arsénico pertinentes.

Debido a todo esto, los laboratorios de análisis de control de los alimentos tienen que estar preparados para determinar iAs en arroz y productos de arroz y ahora también deben estar preparados para analizar iAs y tAs en otros productos alimenticios, por lo que necesitarán métodos sensibles y robustos y que estén validados como requisito de la norma ISO-UNE-EN 17025, que es obligatoria para los laboratorios de análisis que trabajan en el control de alimentos. Un esquema integral de aseguramiento de la calidad en los laboratorios de química analítica incluiría los siguientes elementos: validación de los métodos analíticos; participación en ensayos de aptitud (PT); uso de CRMs y la aplicación en rutina del control de calidad (QC) interno. Los ensayos de aptitud o ejercicios de interlaboratorio es un medio para asegurar que la

validación del método y los procedimientos de control de calidad internos son satisfactoriamente. La participación en PT puede ser útil para detectar interferencias del método validado e iniciar la solución de problemas relacionados con las fuentes no reconocidas de error. Su principal virtud es que proporciona un medio por el que los participantes pueden obtener una evaluación externa e independiente de la exactitud de sus resultados. Por lo tanto, la participación en PT es una herramienta valiosa para poner a prueba la fiabilidad de un método mediante la comparación de los resultados obtenidos con un valor de referencia asignado.

En vista de todo esto, nos propusimos como objetivo desarrollar y validar métodos analíticos para la determinación de especies de arsénico en alimentos, con especial énfasis en el arroz y los alimentos a base de cereales que puedan ser aplicados en el análisis de rutina de los laboratorios de control de alimentos. El proceso de análisis para el desarrollo y validación de un método de especiación en estas matrices es compleja. Diferentes aspectos tienen que ser considerados, incluyendo, tratamiento de la muestra, los procedimientos de preparación, método de detección, la estrategia de calibración así como la evaluación del control de calidad que incluye la evaluación de control de calidad interno, el establecimiento y validación de los parámetros del método de evaluación y el control de calidad externo. Todos los trabajos de investigación relacionados con el desarrollo y validación de métodos de especiación se presentan en el **Capítulo 3**.

A modo de resumen, las publicaciones incluidas en el este capítulo se presentan a continuación: **Article II, Article III, Article X, Article XI, Article XII** and **Article XIII**

Todos los métodos analíticos desarrollados, establecidos y aplicados en la tesis se resumen en la **Tabla 6**. Los métodos llevan nombres de A a la F. De este modo, el método para la determinación de arsénico total se denomina el método A, los métodos para análisis de especiación de arsénico se llaman B, C, D y E; y el método para la estimación de la bioaccesibilidad de arsénico es el método F (**Tabla 6**).

Un resumen de los parámetros de validación evaluados en los métodos desarrollados para la determinación de especies de arsénico se muestra en la **Tabla 7**.

Tabla 6. Métodos evaluados para la determinación de especies de arsénico, arsénico total y arsénico bioaccesibles

Method	Sample weight (dry mass)	Reagents	Apparatus	Time and temperature conditions	Sample treatment	Measurement of As
Total arsenic						
A	0.10-0.50g	8 mL HNO_3 + 2 mL H_2O_2	Microwave digestion	60 min, max. T= 190ºC	Dilution in water up to 20/25 mL.	ICPMS [b]
Arsenic species						
B	0.10-0.30g	10 mL of 0.2% HNO_3/ 1% H_2O_2 solution (w/v)	Microwave-assisted extraction	45 min, max. T= 90ºC		
C	0.10-0.30g	10 mL of 0.2% HNO_3 solution (w/v)	Microwave-assisted extraction	45 min, max. T= 90ºC	Centrifugation at 3500 rpm (10 min) and filtration (PET filters, pore size 0.45 µm).	
D	0.10-0.30g	10 mL of H_2O solution	End-over-end shaker	30 rpm for 16 h at room temperature		Anion and cation exchange chromatography coupled to ICPMS [a]
E	0.2g	20 mL of a MeOH/H_2O solution (1:1, v/v)	Microwave-assisted extraction	10 min at 40W	Centrifugation at 2500 rpm (10 min). Evaporation of MeOH under an IR lamp (T<40 °C, 4h). Dilution of extract in water up to 20 mL and filtration through a nylon membrane (0.2µm). Clean-up with a C18 cartridge.	
Arsenic bioaccessible						
F	0.50g	Gastric and Gastrointestinal solutions [b]	Thermo-agitator with orbital–horizontal shaker	1h at 37ºC + 3h at 37ºC	Centrifugation at 3500 rpm (10 min) and filtration (PET filters, pore size 0.45 µm)	ICPMS [b]

.**Table 7**. Resumen de los parámetros de validación evaluados en los métodos desarrollados para la determinación de especies de arsénico.

Publication	Food commodity	Analyte	Method [a]	Validation parameters									
				LOD	LOQ	Linearity	Repeatability	Intermediate Precision	Trueness	Accuracy	Selectivity	Uncertainty	External QC
Article II	Rice	DMA, MA and iAs	B and C	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Article III	Cereal-based foods	DMA MA and iAs	B	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Article IV	*L. edodes* products	DMA, MA, iAs, AB, AC, TMAO, unknowns	B	✓	✓	**n.e**	**n.e**	**n.e**	✓	✓	**n.e**	**n.e**	✓
Article V	Edible mushrooms	DMA, MA, iAs, AB, AC, TMAO, unknowns	B	✓	✓	**n.e**	**n.e**	**n.e**	✓	✓	**n.e**	**n.e**	**n.e**
Article VI	Marine seaweeds	DMA, MA, iAs, AB, AC, TMAO, arsenosugars and unknowns	D	✓	✓	**n.e**	**n.e**	**n.e**	**n.e**	✓	**n.e**	**n.e**	**n.e**
Article VII	Edible seaweeds	DMA, MA, iAs, AB, AC, TMAO, arsenosugars and unknowns	D	✓	✓	**n.e**	**n.e**	**n.e**	**n.e**	✓	**n.e**	**n.e**	**n.e**
Article VIII	Fish and shellfish	DMA, MA, iAs, AB, AC, TMAO and unknowns	B	✓	✓	**n.e**	**n.e**	**n.e**	✓	✓	✓	**n.e**	**n.e**

[a] Details of method A are shown in **Table 6.**

n.e means not evaluated

CAPÍTULO 4

Ocurrencia de las especies de arsénico en alimentos

Los alimentos proporcionan nutrientes y también componentes no nutricionales y contaminantes. Para evaluar los riesgos y beneficios asociados a la ingesta de un alimento determinado, los niveles de las frecuencias de consumo deben tomarse en cuenta. La comida se consume generalmente en forma elaborada y típicamente se somete a un tratamiento culinario adicional antes de la ingestión que puede alterar las formas de concentración y químicas de un elemento. Los seres humanos están expuestos al arsénico en el medio ambiente principalmente a través de la ingestión de alimentos y agua. El procesamiento puede causar un aumento considerable o disminución en la ingesta de arsénico real a partir de los alimentos. Además, para un mejor conocimiento de los riesgos y beneficios asociados al consumo de alimentos, se requiere la estimación de la biodisponibilidad de arsénico. El término biodisponibilidad incluye bioaccesibilidad y, en consecuencia los estudios de bioaccesibilidad pueden ser un enfoque alternativo para medir la potencial biodisponibilidad de arsénico. Por lo tanto, futuros estudios de evaluación de riesgos deberían considerar el efecto de la cocina, así como la biodisponibilidad (o bioaccesibilidad) de las especies de arsénico en los alimentos.

Algunos productos alimenticios son capaces de acumular altos niveles de arsénico que pueden representar un riesgo grave para la salud de los consumidores. El arsénico se bioacumula en la cadena alimentaria marina y concentraciones de tAs en el rango mg As kg^{-1} por lo general se encuentra en los organismos marinos. Niveles de arsénico típicos en algas son por lo general en el rango de 1-100 mg As kg-1 en función de las especies de algas, mientras tanto, las concentraciones de arsénico en el rango de 1-30 mg As kg^{-1} se ha informado en los pescados y mariscos. Por otro lado, en muestras de origen terrestre la concentración tAs es típicamente <0,3 mg As kg^{-1}. Sin embargo, en ciertos casos concentraciones de hasta de 1 mg kg^{-1} han sido reportadas. El arsénico tiene una química bastante compleja, sobre todo en el medio marino, donde más de 50 diferentes compuestos naturales de As se han identificado , que comprenden formas tanto orgánicos como inorgánicos. Entre ellos y simplificando, AB no tóxico es el compuesto principal en pescado y los mariscos y los arsenoazúcares potencialmente tóxicos son predominantes en las algas marinas. El análisis de especiación en los alimentos terrestres es menos complejo que en los alimentos marinos, se han reportado algunas especies de arsénico, pero el arsénico inorgánico generalmente predomina antes de que otras especies de arsénico, es decir, especies metiladas. Así, el arsénico inorgánico es generalmente predominante en arroz, cereales infantiles y productos de cereales que son los que más contribuyen a la exposición alimentaria al arsénico inorgánico.

En cuanto a los niveles máximos de la UE de en iAs en los productos alimenticios, un valor máximo de 10 µg As L^{-1} se establece para las aguas destinadas al consumo humano, sin distinguir las formas de arsénico, mientras que para las aguas minerales naturales, una dosis máxima de 10 µg As L^{-1} está previsto para el total de arsénico. Muy recientemente, la Unión Europea ha establecido niveles máximos de la iAs en arroz y productos de arroz. Actualmente, no hay niveles máximos establecidos para el arsénico otros productos alimenticios distintos del arroz a nivel de la UE, aunque algunos ML se establecen en la legislación nacional en algunos Estados miembros. A pesar de que la toxicología del arsénico depende sobre todo de su forma química, la mayoría Regulaciones y Directivas de alimentos no definen una concentración máxima permitida en función de una/as especie/s de arsénico determinada/s, sino en términos de contenido total de arsénico.

Con estas consideraciones en mente, y debido a la toxicidad del arsénico depende de sus especies químicas, nos propusimos como objetivo proporcionar datos de especiación de arsénico fiables en varios productos alimenticios. Estos estudio de especiación pueden ser útiles para nuevos estudios sobre la evaluación de riesgos y también en los debates en curso en la Comisión Europea y el CODEX Alimentarius para el establecimiento y la aplicación de los futuros niveles máximos de arsénico inorgánico en los productos alimenticios. Todos estos estudios de especiación se presentan en el **Capítulo 4**.

A modo de resumen, las publicaciones incluidas en este capítulo se presentan a continuación: **Article VI; Article VII; Article VIII**; **Article IV; Article V** and **Article IX.**

A continuación se muestran un resumen de los principales resultados de la ocurrencia de las especies de arsénico en alimentos.

Contenido de arsénico en todos los grupos de alimentos estudiados

Con el fin de comparar la presencia y distribución de arsénico en todos los alimentos estudiados en esta tesis, un resumen se presenta a continuación.

Arsénico total

El contenido total de arsénico en los alimentos analizados se muestra en la **Tabla 8** y varió 0,004-121,7 mg As kg^{-1} (**Figura 10**). La concentración media de arsénico de todos los productos alimenticios ensayados fue de 7,7 mg As kg^{-1} (mediana = 0,20, n = 137) con una alta variabilidad del contenido de arsénico en función del grupo de alimento. Se encontraron bajos niveles de arsénico en los alimentos terrestres analizados (es decir, el arroz, los productos a base de cereales, cereales infantiles y setas) y el valor promedio fue de 0,26 mg As kg^{-1} (mediana = 0,11, n = 91) y variaron desde 0,004 hasta 2,83 mg As kg^{-1}. El contenido de arsénico fue inferior a 0,32 83 mg As kg^{-1} en el arroz, en cereales y productos derivados y en alimentos infantiles, y por debajo de 2,8 83 mg As kg^{-1} en las setas. Nuestros resultados están de acuerdo con los reportados en la literatura, los alimentos terrestres suelen tener niveles por debajo de 0,3 83 mg As kg^{-1}, con la excepción de algunas especies de hongos que son organismos bioacumuldores de arsénico y se han reportado concentraciones de hasta 146,9 mg As kg^{-1}.

Por otro lado, altos contenidos de arsénico total se encontraron en los alimentos marinos analizados (es decir, peces, crustáceos y algas marinas). La concentración promedio de todas las muestras de marinas fue de 22,6 mg As kg^{-1} (mediana = 9,1, n = 46) y varió desde 1,2 hasta 121,7 mg As kg^{-1}1. Entre ellos, las algas marinas tenían mayores niveles de tAs que los peces o mariscos: El contenido total de arsénico varió desde 2,0 hasta 121,7 y de 1,2 a 35,2 mg As kg^{-1} para las algas y, pescados y mariscos, respectivamente. Estos resultados son concordantes con la literatura ya que los alimentos marinos son capaces de bioacumular altos niveles de arsénico del agua de mar, y, contenidos de arsénico hasta 150 y 75 mg As kg^{-1} han sido reportados en algas y muestras de peces, respectivamente.

Clasificando por grupos de alimentos, el contenido de arsénico aumenta en el siguiente orden: Productos a base de cereales <alimento infantil <arroz y arroz productos <Setas <Marisco<Pescado <Algas. Este hecho se puede observar claramente en la **Figura 10**.

Tabla 8. Media y mediana de las concentraciones totales de arsénico (mg As kg^{-1}) en los alimentos ensayados. Se indica el número de muestras analizadas (n) y el rango de valores.

Food group	Number of samples (n)	Total Arsenic		
		Mean value	Median value	Range
Cereal-based products	21	0.007	0.007	0.004 to 0.023
Infant food	18	0.073	0.038	0.008 to 0.31
Rice and rice products	20	0.170	0.172	0.08 to 0.32
Mushrooms	32	0.588	0.280	0.05 to 2.8
Shellfish	8	10.2	10.3	1.2 to 24.6
Fish	14	10.2	4.3	1.4 to 35.2
Seaweeds	24	34.4	23.7	2.0 to 121.7

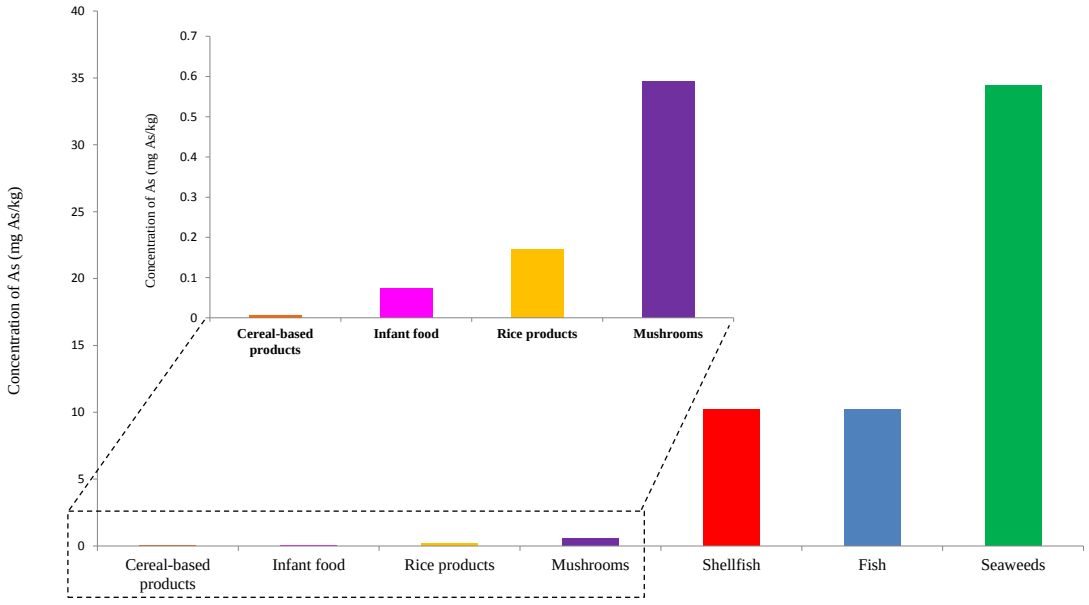

Figura 10. Concentración de arsénico total en los grupos de alimentos estudiados.

7.5.2 Especies de arsénico

La concentración de las especies de arsénico y su distribución (%) para todos los grupos de alimentos estudiados se muestran en la **Tabla 9** y la **Figura 11**, respectivamente. La presencia de especies de arsénico en los alimentos es diferente según el tipo de grupo de alimentos, que está de acuerdo con la literatura. Las diferencias entre los patrones de especiación se muestran claramente en la **Figura 11**, que muestra las proporciones de los compuestos de arsénico encontrados para cada uno de los grupos de alimentos analizados. El tóxico arsénico inorgánico se determinó en todos los grupos de alimentos, excepto en muestras de peces que fue por debajo de los límites de detección, el iAs varió entre 2,6 y 100% de la suma de las especies de arsénico en función del tipo de alimento y es el compuesto de arsénico predominante en cereales y productos derivados, cereales infantiles, arroz y setas y que representa entre un 69,5% a 100% con una media del porcentaje de 79,8% en estos grupos de alimentos. Arsenobetaína no tóxica fue encontrada en hongos, algas, crustáceos y peces; siendo un compuesto de menor importancia en los dos primeros grupos y el compuesto predominante en los dos últimos representa el 84,6% y 91,3% de la suma de las especies de arsénico, respectivamente. AB no se detectó en los productos a base de cereales, cereales infantiles y muestras de arroz. Nuestros datos muestran claramente que los arsenoazúcares son los más compuestos más abundantes de arsénico en algas marinas, 63,8% de la suma de las especies. Además, éstos fueron cuantificados en algunas especies de hongos, pero no se han detectado en los productos a base de cereales, cereales infantiles, arroz, mariscos y muestras de peces. Otros organoarsenicales, que podrían ser considerados como potencialmente tóxicos, como DMA, MA, TMAO, AC, TMAP y TETRA se encontraron en menor proporción en función del grupo de alimentos. DMA se detectó en todos los grupos de alimentos estudiados, excepto en los productos a base de cereales y varió desde 1.0 a 28.6% con un valor medio de 11,5%. MA, AC y TMAO se detectaron en algunos grupos de alimentos y representan un pequeño porcentaje de las especies extraídas; MA representó el 0,1 hasta el 5,4%, AC representó el 0,1 a 0,6% y TMAO representó 0,6 a 1,8% de la suma de las especies de arsénico. Debido a la falta de patrones apropiados, TMAP y TETRA fueron identificados por comparación con la literatura debido a la comparación del tiempo de retención cuando se utilizan las mismas condiciones cromatográficas. Sin embargo, estas identificaciones deben verificarse con experimentos de fortificación y experimentos de espectrometría de masas moleculares con unos patrones apropiados. TMAP fue encontrado en setas, crustáceos y bivalvos y peces y representó el 0,2 a 5,4%. Además, TETRA se cuantificó en las setas y los crustáceos y bivalvos y representó el 0,2 y 1,7%, respectivamente. Dos compuestos aniónicos desconocidos, UA-A y UA-B, se encontraron en algunas muestras de mariscos. Estas especies desconocidas fueron bien separado de otros compuestos de arsénico y se encuentran como especies minoritarias. Estos picos desconocidos representaron el 0,2 y 5,4% para UA-A y UA-B, respectivamente. Además, otros compuestos desconocidos, tanto aniónicos como catiónicos, se encontraron en varias algas marinas. Hemos decidido agrupar estas especies de arsénico desconocidas en algas como la suma de estas especies aniónicos y catiónicos, UA y la UC, respectivamente (**Tabla 9 y Figura 11**).

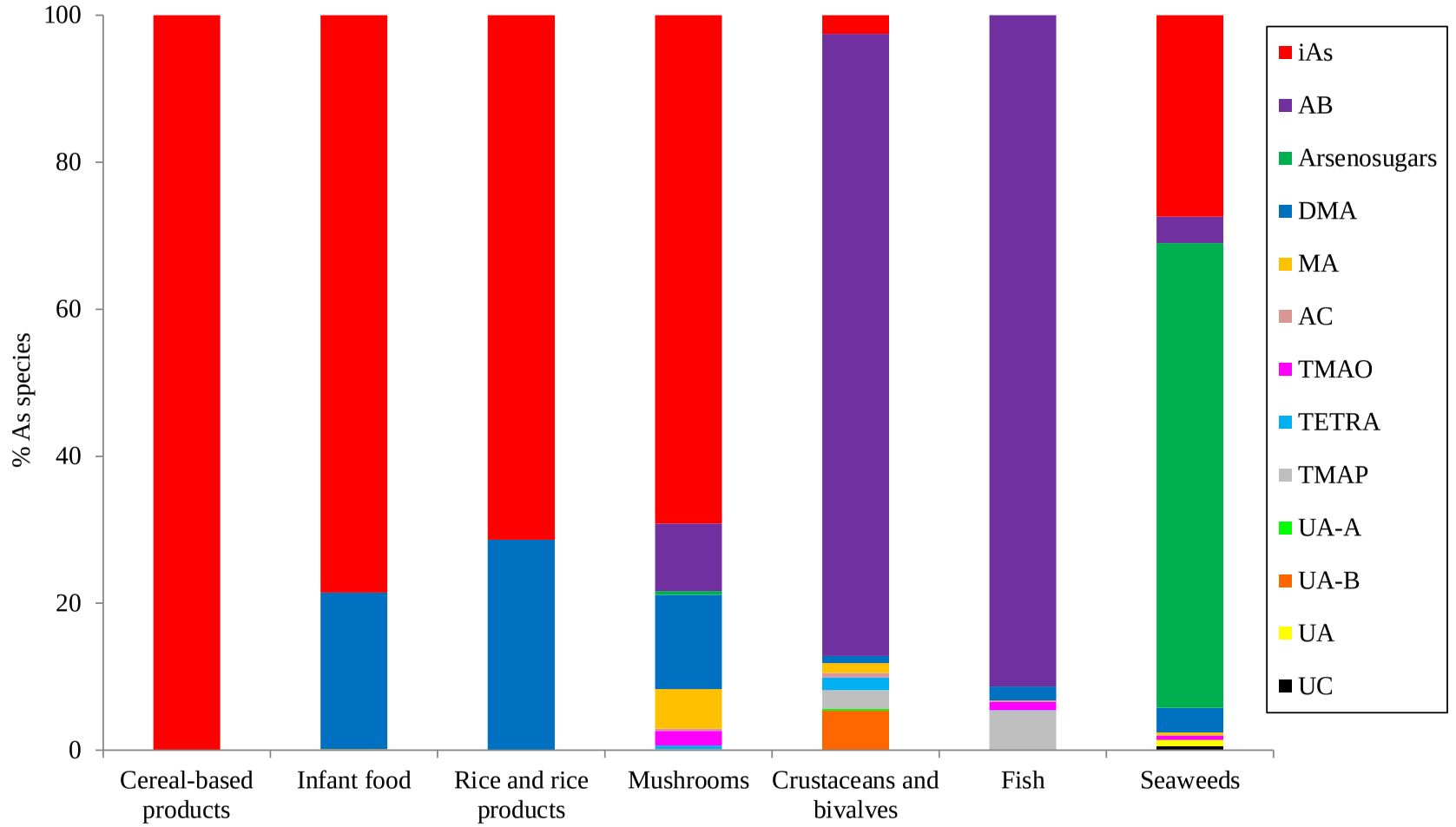

Figure 11. Proporciones de especies de arsénico en extractos de productos alimenticios estudiados con respecto a la suma de las especies de arsénico,. UA-A y UA-B son especies de arsénico aniónicas desconocidas que se encuentran en algunas muestras de moluscos que no responden a ningún patrón disponibles. UA y UB representan la suma de todas las especies de arsénico aniónicas y catiónicas encontrados en algunas muestras de algas, respectivamente.

Tabla 9. Las concentraciones medias de especies de arsénico en los alimentos (mg kg-1). Rango de valores, se muestra el número de muestras (n) y el número de veces detectados para cada especie de arsénico (N).

Food type		DMA	MA	iAs	AB	TMAO	AC	TMAP	TETRA	PO_4-sugar	SO_3-sugar	SO_4-sugar	Gly-sug	Unknown anions [a]	Unknown cations [b]	UA-A [c]	UA-B [c]
Cereal-based products	**mean**	<LOD	<LOD	0.007	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
n=21	**range**	…	…	0.003-0.023	…	…	…	…	…	…	…	…	…	…	…	…	…
	N	0	0	21	0	0	0	0	0	0	0	0	0	0	0	0	0
Infant food	**mean**	0.041	0.006	0.044	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
n=18	**range**	0.000-0.183	0.000-0.0063	0.000-0.200	…	…	…	…	…	…	…	…	…	…	…	…	…
	N	12	1	18	0	0	0	0	0	0	0	0	0	0	0	0	0
Rice and rice products	**mean**	0.041	<LOD	0.112	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
n=20	**range**	0.016-0.081	…	0.034-0.239	…	…	…	…	…	…	…	…	…	…	…	…	…
	N	20	0	20	0	0	0	0	0	0	0	0	0	0	0	0	0
Mushrooms	**mean**	0.076	0.057	0.284	0.331	0.030	0.022	0.029	0.030	0.051	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
n=32	**range**	0-0.867	0-0.760	0-1.380	0-1.493	0-0.173	0-0.045	0-0.049	0-0.077	0-0.067	…	…	…	…	…	…	…
	N	27	21	32	12	12	4	2	5	3	0	0	0	0	0	0	0
Shellfish	**mean**	0.124	0.064	0.141	6.57	0.06	0.08	0.19	0.12	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.110	0.840
n=8	**range**	0-0.250	0-0.130	0-0.350	0-15.90	0.06	0-0.290	0-0.460	0-0.500	…	…	…	…	…	…	0-0.180	0-2.07
	N	5	5	8	8	1	6	8	5	0	0	0	0	0	0	2	6
Fish	**mean**	0.086	0.012	<LOD	8.46	0.03	0.05	0.88	0.10	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
n=14	**range**	0-0.240	0-0.014	…	0-33.5	0-0.080	0-0.070	0-4.53	0-0.100	…	…	…	…	…	…	…	…
	N	11	3	0	14	10	2	8	1	0	0	0	0	0	0	0	0
Seaweeds	**mean**	1.037	0.290	12.111	0.188	0.190	0.147	<LOD	<LOD	4.34	10.77	5.29	4.20	0.463	0.163	<LOD	<LOD
n=24	**range**	0-4.70	0-0.730	0-71.2	0-0.540	0-0.500	0-0.200	…	…	0-27.6	0-39.4	0-7.3	0-16.0	0-1.17	0-0.510	**…**	**…**
	N	19	4	19	15	6	3	0	0	14	10	4	24	4	6	n= 0	n= 0

[a] Suma de especies de arsénico aniónicas desconocidas que se encuentran en algunas muestras de algas marinas. [b] Suma de especies de arsénico catiónicas desconocidas que se encuentran en algunas muestras de algas marinas. [c] Especies desconocidas de arsénico, aniónicas (UA-A) y catiónicas UA-B que se encuentran en algunas muestras de mariscos.

CAPÍTULO 5

Conclusiones

Las conclusiones derivadas del trabajo llevado a cabo en la presente tesis doctoral se dividen en cuatro partes: la primera concierne sobre el artículo de revisión centrado en los métodos desarrollados y en la evaluación de los parámetros de calidad para la determinación de arsénico inorgánico en los alimentos, en segundo lugar centrado en el desarrollo y validación de métodos para la determinación de arsénico especies, el tercero basado en la ocurrencia y distribución de las especies de arsénico en los productos alimenticios y el cuarto sobre la evaluación de la exposición al arsénico y los riesgos de salud asociados.

Estado actual del desarrollo de métodos y evaluación de la calidad en la determinación de arsénico inorgánico en los alimentos

- ✓ Varios métodos cromatográficos y no cromatográficos se han desarrollado para determinar iAs en los alimentos. Entre ellos, la extracción química de las especies de iAs y posterior determinación por HPLC-ICPMS es sin duda el método más popular utilizado en el análisis de iAs en los alimentos.

- ✓ Aunque varios métodos de especiación de arsénico se han reportado, hay una necesidad para el desarrollo de métodos analíticos robustos para la extracción, separación y determinación de arsénico inorgánico en matrices con una compleja distribución de especies de arsénico, es decir, los alimentos de origen marino.

- ✓ Se requieren materiales de referencia certificados especialmente para iAs alimentos de origen marino. La producción de tales CRMs, así como la participación en ejercicios de comparación entre laboratorios ayudarían en la validación de los métodos y en facilitar futuros estudios sobre el contenido de iAs de los alimentos.

- ✓ Hay una necesidad validación de métodos selectivos y sensibles para la determinación de iAs para permitir su futura implementación en el análisis de rutina en los laboratorios de control de alimentos de acuerdo con la norma ISO / IEC 17025.

- ✓ Hay una necesidad de producir datos de especiación fiables, especialmente iAs, para diferentes productos alimenticios con el fin de perfeccionar los estudios de evaluación de riesgos. Para ello, el establecimiento y validación de métodos para la determinación de iAs son de suma importancia, y permitirán conclusiones consistentes en estudios de exposición en la dieta.

Desarrollo y validación de métodos para la determinación de especies de arsénico en alimentos

- ✓ El uso de microondas con una digestión ácida en un recipiente cerrado para la preparación de la muestra y la detección por ICPMS proporcionan una determinación precisa y exacta del contenido de tAs en varios tipos de muestras de alimentos (matrices de origen marino y terrestre y de productos alimenticios infantiles).

- ✓ El método propuesto para la determinación tAs es evaluado con éxito mediante los parámetros de validación seleccionados (linealidad, LOD, LOQ, exactitud, precisión, control de calidad externo) que demuestran la idoneidad del método. Además, la evaluación interna y externa de los controles de calidad se llevó a cabo para lograr un nivel de calidad satisfactorio para análisis de rutina.

- ✓ A partir de los métodos de extracción ensayados para la especiación de arsénico, la extracción asistida por microondas con el extractante HNO_3/ H_2O_2 es el procedimiento más adecuado para una extracción cuantitativa de todas las especies de arsénico en los productos alimenticios, así como de las especies tóxicas iAs sin degradación de otros compuestos de arsénico. El uso de ambas cromatografía de intercambio catiónico y aniónico acoplado a ICPMS (HPLC-ICPMS) proporcionó una separación satisfactoria de las especies de arsénico, así como una determinación sensible y selectiva.

- ✓ Un método para la determinación de especies de arsénico en el arroz y productos de arroz por HPLC-ICPMS fue desarrollado y plenamente validado. Linealidad, LOD, LOQ, repetitividad, precisión intermedia, veracidad, exactitud, la selectividad, así como la incertidumbre expandida se establecieron para iAs, MA, y DMA. El método se utilizó para analizar especies de arsénico en varias muestras que incluyen varios tipos de arroz, productos de arroz y productos de arroz infantiles, que muestran la amplia aplicabilidad del método.

- ✓ Con el fin de utilizar el método de arroz para determinar el contenido de arsénico en productos a base de cereales (As<0.010 mg As kg^{-1}), los LOD se han mejorado mediante la modificación de las condiciones instrumentales y parámetros de HPLC-ICP-MS. Entonces, se establecieron los principales parámetros de validación para iAs, MA, y DMA. Para evaluar la aplicabilidad del método, se analizaron varias muestras a base de cereales: pan, galletas, cereales de desayuno, harina de trigo, aperitivos de maíz, pasta y cereales infantiles. Este método optimizado se puede utilizar como un sustituto del método anterior validado en muestras de arroz y expande la aplicabilidad del método a los productos a base de cereales.

- ✓ El método de especiación propuesto fue validado con éxito de acuerdo con la norma 17025 ISO/IEC: 2005, es sensible y selectivo y podría ser considerado apropiado para su finalidad, es decir, la determinación de las iAs en los alimentos. Este método es sencillo para análisis de rutina para la determinación del iAs tóxico y otras especies de arsénico en varios productos alimenticios, incluso en matrices difíciles tales como las de origen marino. Además, puesto que los límites máximos de iAs en arroz y productos de arroz recientemente han sido establecidos por la Unión Europea, el método podría ser útil en los laboratorios de control de los alimentos.

- ✓ En términos de control de calidad externo, el método fue probado satisfactoriamente en ensayos de aptitud (IMEPs y FAPAS), que mostraron la idoneidad del método desarrollado para proporcionar resultados fiables y precisos sobre el arsénico inorgánico en los productos alimenticios.

- ✓ La participación en estudios de certificación de CRMs (caracterización, estabilidad y estudios de confirmación) mostró que el método de especiación es adecuado para la determinación de la especiación de arsénico en matrices de alimentos.

- ✓ El ensayo colectivo en la determinación de iAs en los alimentos de origen vegetal marino por HPLC-ICPMS (CEN TC275/WG10) produjo resultados que fueron consistentes con los valores asignados. Un método para la determinación de la iAs en los alimentos de origen marino y vegetal se desarrolló y se evaluaron las principales parámetros del método. Este método es adecuado para el análisis cuantitativo de la iAs en los alimentos de origen vegetal y marino y el proceso de normalización está en curso.

- ✓ Se estableció un método *in vitro* PBET para estimar la bioaccesibilidad de arsénico y se evaluaron los principales parámetros de control de calidad. El método se aplicó a setas frescas y cocinadas y, a priori, podría aplicarse para evaluar bioaccesibilidad de arsénico en otras muestras de alimentos; sin embargo una mayor investigación en este aspecto debe llevarse a cabo.

Ocurrencia y distribución de especies de arsénico en los productos alimenticios

- ✓ Sólo especies de iAs fueron encontradas en los productos de cereales y el iAs fue el principal compuesto encontrado en los productos de arroz, poniendo de relieve la importancia de estos grupos de alimentos como una posible fuente de iAs en las dietas a base de arroz y cereales. Se halló una correlación positiva entre el contenido de tAs y iAs en arroz y productos de cereales, con el contenido de iAs dependiente de tAs en las muestras ensayadas.

- ✓ La concentración de tAs y iAs fue mayor en los productos a base de arroz que en productos preparados usando otros cereales (trigo, cebada, avena y maíz) o mezclas de

ellos. Las concentraciones de iAs en productos analizados a base de cereales, arroz, y productos infantiles estaban por debajo de los niveles máximos establecidos. Sólo un arroz integral (arroz integral de grano medio) y una muestra de alimento para bebé (arroz integral orgánico) superaron los niveles máximos establecidos por la China.

- ✓ Gran variabilidad en el contenido de las especies orgánicas como inorgánicas de arsénico se encontró en el estudio de especiación en las setas. Dado el pequeño número de especies de setas analizadas, no somos capaces de hacer una generalización sobre el patrón de especiación de arsénico en las setas. Además, para obtener datos fiables de especiación de arsénico, las setas deben muestrearse en cada origen específico y analizadas de forma individual.

- ✓ Altas proporciones de iAs fueron encontradas en los productos base *L. edodes* (Shiitake), incluyendo las setas cultivadas y compradas. Sin embargo, no es del todo claro si las setas Shiitake acumulan el iAs del sustrato, o lo producen a través de una biotransformación.

- ✓ Los potencialmente tóxicos arsenoazúcares fueron los compuestos de arsénico predominantes que se encontraron en la mayoría de las algas estudiadas, mientras que iAs se encontró en proporciones bajas, salvo en algunas excepciones, junto con otras especies de arsénico. Porcentajes significativos de AB, que no es un compuesto común en las algas marinas, se detectaron en *U. rigida* y *E. compressa*. Los resultados de las algas marinas comestibles mostraron que el contenido de iAs estaba por debajo de los límites máximos establecidos por Francia, los EE.UU., Australia y Nueva Zelanda, con la excepción de *S. fusiforme*.

- ✓ Un cuidado especial se debe tomar en el consumo de *S. fusiforme* (Hijiki), ya que encontramos altos niveles de arsénico total e inorgánico: tAs> 100 mg As kg^{-1} y iAs > 60 mg As kg^{-1} que superan los límites máximos establecidos por Francia, EE.UU., Australia y Nueva Zelanda.

- ✓ Diez especies de arsénico se determinaron en los extractos de pescado y marisco y arsenobetaína fue el principal compuesto identificado (aprox. 90% del contenido de tAs). El arsénico inorgánico no se detectó en muestras de peces y se encontró por debajo de 0,4 mg As kg^{-1} en los crustáceos y bivalvos. En base a los resultados en los alimentos marinos, una amplia variabilidad en las especies de arsénico se puede esperar cuando se trata de pescados y mariscos, como algas, moluscos y crustáceos, destacando la necesidad de llevar a cabo especiación analítica para discernir de especies tóxica de las que no lo son.

Evaluación de la exposición al arsénico y los riesgos para la salud asociados

- ✓ Todos los datos de especiación de arsénico obtenidos en esta tesis son útiles para evaluar la ingesta diaria de arsénico en la población Catalana. Además, estos resultados pueden contribuir a los debates en curso en relación con el establecimiento y aplicación de niveles máximos de arsénico inorgánico en los productos alimenticios, tal como se recomienda en la Unión Europea.

- ✓ Los principales contribuyentes a la ingesta dietética de tAs son el pescado y el marisco, que contienen altas proporciones de la no tóxica arsenobetaína. Por otro lado, los cereales son los principales contribuyentes de iAs en la población adulta Catalana.

- ✓ La ingesta diaria promedio de iAs estimada en la población adulta Catalana está por debajo de los rangos BMDL de la EFSA y JECFA, pero teniendo en cuenta la relación entre la ingesta de iAs y algunos BMDL para distintos puntos finales, la posibilidad de riesgo para los grandes consumidores de arroz y productos de arroz no pueden ser excluidos.

- ✓ La exposición diaria a iAs para los consumidores de suplementos de setas es relativamente baja en comparación con los rangos BMDL y el riesgo toxicológico podría considerarse insignificante en comparación con la contribución de otras fuentes de iAs en la dieta. Sin embargo, los altos consumidores de *S. fusiforme* (Hijiki) estarían expuestos a la ingesta iAs dentro del rango $BMDL_{01}$ identificado por la EFSA, por lo que la posibilidad de riesgo no puede ser excluida.

- ✓ El consumo de cereales para bebés conduciría a una ingesta diaria de iAs por debajo del límite de referencia recomendados por la EFSA. Una alta ingesta diaria de iAs se estima para los bebés que consumen altas cantidades de productos a base de arroz, es decir arroz para bebés y galletas de arroz. El consumo de dos o más raciones al día de estos productos alimenticios daría lugar a la exposición por encima del punto de referencia más bajo establecido por la EFSA, por lo que la posibilidad de riesgo no puede ser excluido para los lactantes que consumen estos productos a base de arroz.

- ✓ En vista de nuestra exposición estimada de los bebés al iAs, hay una necesidad fundamental para reducir el contenido de iAs en productos de arroz para niños con el fin de reducir su exposición al iAs, clasificado como clase uno carcinógeno humano.

- ✓ Se encontraron valores altos de bioaccesibilidad de arsénico las setas frescas y en las cocinadas e incluso cuando la cocción lleva una disminución en el contenido de tAs, la bioaccesibilidad de tAs se mantuvo alta (> 76%) para ambas fracciones gástrica y gastrointestinal.

Futuras tendencias

- ✓ Hay una necesidad de métodos robustos para la determinación de arsénico inorgánico en una amplia gama de productos alimenticios, en particular los de origen marino, que necesitan ser validados estrictamente y aplicados posteriormente en el análisis de rutina por los laboratorios de control de los alimentos.

- ✓ Con el fin de validar métodos para la determinación de iAs, un valor certificado de iAs en un CRM debe estar disponible ya que esto sería crucial para asegurar la exactitud del método. Además, puesto que hasta la fecha, los ensayos de aptitud para la determinación de iAs en matrices marinas han sido insatisfactorios, más ejercicios de intercomparación entre laboratorios para iAs en matrices de origen marino son necesarios urgentemente.

- ✓ Con el fin de perfeccionar la evaluación de riesgo, existe la necesidad de producir datos de arsénico inorgánico fiables para diferentes productos alimenticios que apoyarían evaluación de la exposición dietética e indicarían los posibles efectos sobre la salud.

- ✓ La inclusión de datos bioaccesibilidad de iAs en la evaluación del riesgo ayudaría aún más a refinar y mejorar la evaluación de la exposición alimentaria y los márgenes de exposición. Por lo tanto, deben llevarse a cabo nuevos estudios sobre la bioaccesibilidad de especies de arsénico, especialmente del iAs, en los productos alimenticios, que también consideren el efecto de la cocción.

- ✓ La producción de un CRM con un contenido de arsénico bioaccesible en matrices alimentarias sería útil para fines de control de calidad y permitir comparaciones entre los métodos bioaccesibilidad establecidos.

- ✓ Más estudios *in vivo* de biodisponibilidad de arsénico serían deseables para demostrar la idoneidad y validar los métodos de bioaccesibilidad *in vitro*.

- ✓ Debido a que los estudios existentes de toxicidad de los arsenoazucares no son concluyentes, hay una necesidad de mejorar la comprensión del metabolismo humano de los arsenoazúcares en alimentos y sus implicaciones para la salud humana. Por lo tanto, se deben realizar más estudios sobre la toxicidad arsenoazucares para refinar la evaluación del riesgo y la nueva legislación sobre el arsénico.

- ✓ Ya que los arsenolípidos son particularmente abundantes en los aceites y grasas marinas, se necesitan métodos de análisis para una mayor investigación de estos compuestos en los peces marinos, alimentos para peces y mariscos en general. Más investigación de la abundancia y la toxicidad de estos compuestos también será

importante para la evaluación de riesgos y la legislación sobre el arsénico en relación con la seguridad alimentaria.

- ✓ El establecimiento de métodos estandarizados para la determinación del arsénico inorgánico en los alimentos es de suma importancia y una herramienta necesaria para la aplicación de las directivas futuras respecto a los niveles máximos de iAs en los productos alimenticios.

ANNEX II

Other contributions

Other relevant scientific contributions that are not included in the present thesis e.g.: research articles, book chapter publication and certification studies of heavy metals in environmental samples are summarized in this section.

Publications

Article

Direct solid sample analysis with graphite furnace atomic absorption spectrometry – a fast and reliable screening procedure for the determination of inorganic arsenic in fish and seafood.
Zmozinski, A.V., **Llorente-Mirandes, T**., Damin, I.C.F., López-Sánchez, J.F., Vale, M.G.R., Welz, B., da Silva, M.M.
Talanta, 2015, 134, 224–231

Book Chapter

Chapter title: *Arsenic occurrence in marine biota: the analytical approach*.
Sahuquillo, A., López-Sánchez, J.F., **Llorente-Mirandes, T**, Pell, A., Rubio, R., Ruiz-Chancho M. J.
Environmental problems in marine biology: methodological aspects and applications.
Science publishers. Edited by Tamara García-Barrera and José Luis Gómez-Ariza

Certification studies of reference materials

· Stability monitoring of BCR-700. Extractable elements in organic-rich soil

· Stability test of BCR CRM 483 and BCR CRM 484 extract metal contents

· Characterisation Study of ERM CC-144 soil