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Analysis and characterization of arsenosugars in edible algae

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Vull expressar el meu sincer agraïment al meu tutor, Jose Fermín López, per la seva orientació i suport al llarg del desenvolupament d'aquest treball. Les seves recomanacions i comentaris han estat molt profitosos per millorar la qualitat del projecte i per ampliar la meva visió sobre el tema tractat. També voldria donar les gràcies a la meva família i amics per la seva comprensió i suport incondicional durant aquesta etapa. El seu recolzament m'ha donat la força necessària per superar els moments més complicats del procés, així com l'energia per continuar endavant amb il·lusió i determinació. A més, voldria expressar el meu agraïment als companys i professors que, amb les seves paraules d'encoratjament o amb petits gestos, han contribuït d'alguna manera a fer aquest camí més lleuger i enriquidor.

REPORT

IDENTIFICATION AND REFLECTION ON THE SUSTAINABLE DEVELOPMENT GOALS (SDG)

The study conducted on the analysis and characterization of arsenosugars in edible algae addresses critical intersections between environmental chemistry, public health, and food safety. By advancing knowledge in these areas, the project contributes significantly to several Sustainable Development Goals (SDGs), aligning particularly with the three of the five overarching areas defined as the "5 Ps": People, Planet, Prosperity, Peace, and Partnership.

This research holds profound implications for the "People" dimension, as it directly addresses the safety of food products consumed globally. The findings, which emphasize the need for improved understanding of arsenosugars and their potential health risks, align with SDG 3 (Good Health and Well-being). Specifically, the work supports target 3.9, which seeks to reduce mortality and illnesses resulting from hazardous chemical exposures. By enhancing the understanding of how arsenosugars are metabolized and detected, the study strengthens the foundation for mitigating public health risks associated with arsenic in marine-derived food products.

The project also contributes to the "Planet" dimension by shedding light on the biogeochemical cycling of arsenic in marine ecosystems and its bioaccumulation in algae. This focus is closely linked to SDG 14 (Life Below Water), particularly target 14.1, which prioritizes the prevention and reduction of marine pollution, including chemical contaminants. The study underscores the necessity of monitoring and managing arsenic contamination to safeguard marine biodiversity, reinforcing the interconnectedness between ecosystem health and food safety.

From a "Prosperity" perspective, this work has significant implications for sustainable food systems and aquaculture. By providing robust data on arsenosugars and their distribution across various algae species, the study supports the development of safe consumption guidelines for algae-based products. This aligns with SDG 12 (Responsible Consumption and Production), notably target 12.4, which emphasizes the environmentally sound management of chemicals throughout their lifecycle. The refinement of detection methods for arsenosugars also facilitates the creation of regulatory frameworks, thereby fostering trust in the global market for algae products.

The study's alignment with specific SDG targets further underscores its relevance. For instance, under SDG 3, the research directly contributes to target 3.9 by addressing chemical safety. Similarly, under SDG 12, it supports target 12.4 by advancing methods for managing chemicals in a sustainable manner. Additionally, the work aligns with SDG 14 by contributing to target 14.1, which focuses on reducing marine pollution. Notably, the research bridges SDGs 3 and 14, highlighting the interdependence of human health and marine ecosystem health. This overlap underscores the need for integrated approaches to policy and practice. Proposing a unified indicator that measures arsenic levels in marine-derived food products could enhance coordination between these goals, ensuring more holistic progress.

The potential future expansion of this research could have profound societal impacts. Establishing a comprehensive global database for arsenosugar concentrations across different algae species could guide policy development and ensure the safe consumption of algae products. Moreover, the development of standardized certified reference materials would enhance the reliability of arsenic analyses, fostering international cooperation and harmonization of safety standards. These advancements could contribute to more effective regulatory measures, ultimately ensuring consumer safety and promoting sustainable marine resource management.

CONTENTS

1. SUMMARY

This review critically examines the analytical techniques employed for the characterization and speciation of arsenosugars (AsSug) in edible algae, emphasizing their environmental, biological, and regulatory implications. Techniques for processing, extraction, clean-up, separation, and detection are analysed, showcasing their roles in overcoming the complex challenges of analysing arsenosugars in diverse algal matrices. Arsenosugars exhibit significant variability in their functional groups (OH, SO_3, SO_4, and PO_4) and total arsenic (tAs) distribution, influenced by species type and environmental factors. Brown algae show the highest arsenic concentrations (15.3–150 mg/kg), followed by red (2.2–48 mg/kg) and green algae (0.32–28.4 mg/kg), with arsenosugars constituting a substantial fraction of total arsenic across all groups.

Processing methods, such as freeze-drying and grinding, are essential for sample homogenization and enhancing extraction efficiency, particularly for cell-wall-bound arsenosugars. Extraction techniques like microwave-assisted and ultrasound-assisted methods are widely used, employing solvents such as MeOH-H_2O mixtures and nitric acid to achieve high recoveries. Clean-up processes, including filtration and solid-phase extraction (SPE), ensure the removal of matrix interferences and improve analyte isolation. High-performance liquid chromatography (HPLC) emerges as key separation technique, while inductively coupled plasma mass spectrometry (ICP-MS) remains the preferred detection method for its precision and sensitivity.

Despite these advancements, challenges persist due to matrix effects, the lack of certified reference materials (CRM), and limited analytical quality control frameworks. The review underscores the importance of refining these techniques and integrating analytical quality control measures to ensure reproducible results. Public health and regulatory implications are crucial, with certain algal species exceeding recommended inorganic arsenic thresholds. So harmonized standards and improved methodologies are essential to ensure the safe consumption of edible algae globally.

Keywords: Arsenosugars, arsenic, algae, seaweed, food safety.

2. RESUM

Aquest TFG examina les tècniques analítiques emprades per a la caracterització i l'especiació dels arsenosucres (AsSug) en algues comestibles, destacant les seves implicacions ambientals, biològiques i normatives. S'analitzen les tècniques de processament, extracció, neteja, separació i detecció, posant de manifest el seu paper en la superació dels reptes que planteja l'anàlisi dels arsenosucres en diferents matrius. Els arsenosucres presenten una variabilitat significativa en els seus grups funcionals (OH, SO_3, SO_4 i PO_4) i en la distribució total d'arsènic, influenciada pel tipus d'espècie i els factors ambientals. Les algues marrons mostren les concentracions més elevades d'arsènic (15,3–150 mg/kg), seguides de les algues vermelles (2,2–48 mg/kg) i les algues verdes (0,32–28,4 mg/kg), amb els arsenosucres constituint una fracció substancial de l'arsènic total en tots els grups.

Els mètodes de processament, com la liofilització i la mòlta, són essencials per a l'homogeneïtzació de les mostres i per millorar l'eficiència d'extracció, especialment en el cas d'arsenosucres units a les parets cel·lulars. Les tècniques d'extracció, com les assistides per microones i ultrasons, són àmpliament utilitzades i fan servir dissolvents com les mescles de metanol-aigua i l'àcid nítric per aconseguir recuperacions elevades. Els processos de neteja, incloent-hi la filtració i l'extracció en fase sòlida (SPE), garanteixen l'eliminació de les interferències de la matriu i milloren l'aïllament dels analits. La cromatografia líquida d'alt rendiment (HPLC) emergeix com una tècnica clau de separació, mentre que l'espectrometria de masses amb plasma acoblat inductivament (ICP-MS) es manté com el mètode de detecció preferit per la seva precisió i sensibilitat.

Malgrat aquests avenços, persisteixen reptes deguts als efectes de la matriu, la manca de materials de referència certificats (CRM) i la limitada implementació de marcs de control de qualitat analítica. El TFG subratlla la importància de perfeccionar aquestes tècniques i d'integrar mesures de control de qualitat analítica per garantir resultats reproduïbles. Les implicacions per a la salut pública i les normatives són crucials, ja que certes espècies d'algues superen els límits recomanats d'arsènic inorgànic. Per tant, és essencial establir estàndards harmonitzats i millorar les metodologies per garantir el consum segur d'algues comestibles a nivell global.

Paraules clau: Arsenosucres, arsènic, algues, seguretat alimentària.

3. INTRODUCTION

3.1. ALGAE PROPERTIES

The consumption of algae is growing not only in the food industry but also in the pharmaceutical and cosmetic industries. According to data from the Food and Agriculture Organization of the United Nations (FAO) [1, 2], algae production in 2014 was 28.4 million tons, and by 2022, it reached 37.8 million tons, representing a 33% increase in just eight years. It is more common in Asian countries, but over the years, it has spread among the population worldwide due to its numerous benefits. The consumption of algae provides essential nutrients (such as high levels of iodine, minerals, and vitamins) [3] that not only benefit human health but also animals, as it reduces methane production in livestock and has antibiotic uses [4]. Some studies suggest that the long-term consumption of kelp lowers blood pressure and helps prevent infections from certain viruses and even cancer [5]. On the other hand, it is known that algae also contain various species of arsenic (As), which in its inorganic forms are toxic and even carcinogenic, such as *Hizikia fusiforme* from Korea which contains a great amount of total arsenic (162 mg/kg) [6], significantly exceeding the arsenic derived from daily water consumption, estimated at around 20 µg (considering that the established limit of As in drinking water is 10 µg/kg).

Arsenic exists in four oxidation states: –3, as in arsine gas or arsenic hydride (AsH_3); 0, as in crystalline arsenic; +3, as in arsenite (AsO_3^{3-}); and +5, as in arsenate (AsO_4^{3-}), covering a wide range of organic and inorganic arsenic compounds. It should be noted that the As species considered toxic and carcinogenic (As(III) and As(V)) are not predominant in algae. Other organic species also exist, such as monomethylarsonate (MMA) and dimethylarsinate (DMA), but the most abundant are arsenosugars [7].

3.2. ARSENOSUGARS: WHAT ARE THEY AND WHAT EFFECTS DO THEY HAVE?

Arsenosugars were first identified in 1981 after being isolated from the brown algae *Ecklonia radiata*. Since then, more than 20 types have been discovered, including dimethylated arsenosugars, dimethylated thioarsenosugars (thio-Sug), and trimethylated arsenosugars. While they are most found in marine organisms, they have also been detected in certain terrestrial species [8].

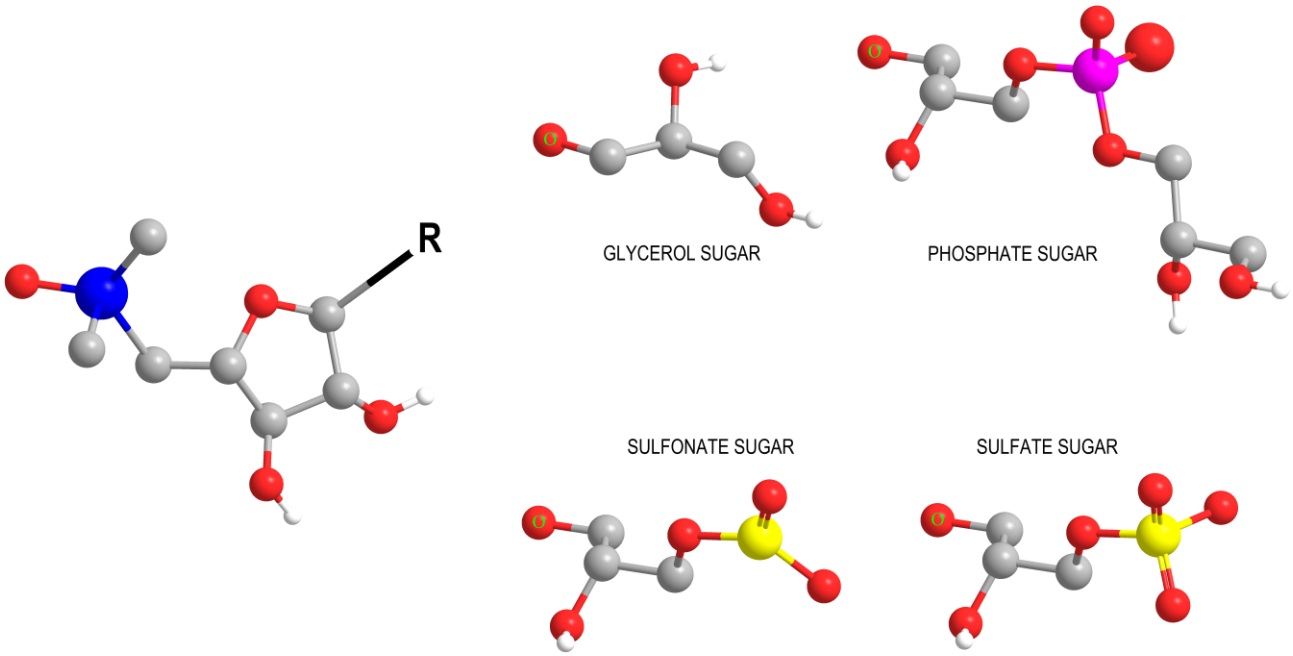
There are contradictory studies regarding the toxicity of arsenosugars, some of which report neurotoxic and cytotoxic effects [9], and there is also some controversy about their chronic effects on our health. For instance, there is debate on whether cooking these compounds or the digestion processes in our body could alter their toxicity. Additionally, it is believed that their toxicity depends on their structure, which in turn depends on the type of algae: brown, red, or green algae [10]. Brown algae have the highest arsenic content, followed by red and green algae. These differences could be attributed to variations in their physiological and biochemical mechanisms for arsenic uptake and storage.

Arsenosugars are part of the arsenic transformation cycle in marine plant organisms. Phytoplankton and other lower trophic level organisms are responsible for assimilating inorganic arsenic species (iAs) [9], which are transferred to organisms that feed on them, becoming organic arsenic species (oAs). This means that of the total arsenic identified in seafood (8–22 mg/kg), only a small percentage exists as iAs (<1% of the total As), while more than 80% are arsenosugars [11]. However, there are some exceptions, such as the brown algae family hijiki, which contains concentrations of up to 107 mg/kg of As(V).

This high bioaccumulation of As in marine organisms occurs, among other factors, due to competition between PO_4^{3-} and AsO_4^{3-} for uptake by the algae cells, as these two ions are considered chemically analogous because of their similar size and geometry. Nevertheless, seawater contains between 1 and 5 µg/L of As, and according to various reports, the level of As accumulated in these marine organisms is several times higher. This is also attributed to biological, chemical, and environmental factors that significantly contribute to the increased storage capacity of As in macroalgae.

In Figure 1, the most typical arsenosugars found in macroalgae can be observed. The structure on the left represents the common chain and the structures on the right are the different arsenosugars depending on their functional group, which are attached via the oxygen atom that is marked: glycerol (OH), phosphate (PO_4), sulfonate (SO_3), and sulfate (SO_4).

Figure 1. Structures of the most common arsenosugars in macroalgae. The 4 structures on the right attach with the left one via the oxygen atom that is marked forming the arsenosugars indicated in the figure. Blue atoms represent As, red ones represent O, grey ones represent C and white ones represent H. Methyl and methylene H have been omitted.



3.3. ARSENOSUGARS SPECIATION AND QUANTIFICATION: CERTIFIED REFERENCE MATERIALS, SEPARATION AND DETECTION TECHNIQUES

The controversy surrounding the toxicity of arsenosugars arises from the lack of CRM and the difficulty in synthesizing them [7]. Moreover, the few available CRM are not suitable for all algae types, as one of the most well-known, CRM 7405-b (hijiki algae), would not be a good reference material for red or green algae. The inability to determine toxicity infallibly leads to the lack of legislation regarding these compounds. This highlights the importance of developing appropriate methods and techniques to aid in the creation of new CRM and the precise analysis of the toxicity of these compounds. Several attempts to develop quantification methods have been documented in various scientific articles, some of which suggest the possibility of using the calibration curve of other well-known As species, such as As(V) and DMA, for arsenosugars. Methods used for the speciation of chemical compounds are a combination of separation and detection techniques [11], and in the specific case of As speciation, there have been no significant advances in recent decades.

The first articles on this subject were published by Braman and Foreback [12] in 1973, based on the differences in the boiling points of various As species to form arsine. Starting in 1975, chromatographic techniques such as gas chromatography (GC) [13] and high-performance liquid chromatography (HPLC) [14] began to be used, and more recently, techniques like capillary zone electrophoresis (CZE) [15] have been developed. Other studies have used instrumental neutron activation analysis (INAA) to measure and assign values to different arsenosugars without needing calibration standards [5]. Despite all the advancements in analytical techniques, HPLC has proven to be one of the preferred methods for separating As species in algae. For detection techniques, the most used are atomic spectrometry and mass spectrometry. In atomic spectrometry, the most reported techniques in the scientific literature are hydride generation coupled to atomic fluorescence spectrometry (HG-AFS) [16], hydride generation coupled to atomic absorption spectrometry (HG-AAS) [17] and inductively coupled plasma atomic emission spectroscopy (ICP-AES) [18]. In mass spectrometry, both inductively coupled plasma mass spectrometry (ICP-MS) [19] and electrospray mass spectrometry (ESI-MS) [20] play a significant role. Regarding detection techniques, mass spectrometry are the preferred ones according to all scientific reports, and in most cases, it is coupled with HPLC.

4. OBJECTIVES

This review seeks to offer a summary of the primary analytical techniques used in arsenic research within the environment over the past six years. In terms of its scope, five distinct objectives were considered:

- Study the toxicity of algae based on more detailed studies and refer to the relevant legislation.
- Emphasize the existing challenges in the development of analytical methods for arsenic speciation.
- Conduct a study of the analytical methods for As speciation (from the processing of macroalgae to their detection and quantification), detailing the scope of each technique.
- Determine the use of CRM in peer-reviewed publications and classify them.
- Develop a quality analysis approach.

5. METHODS

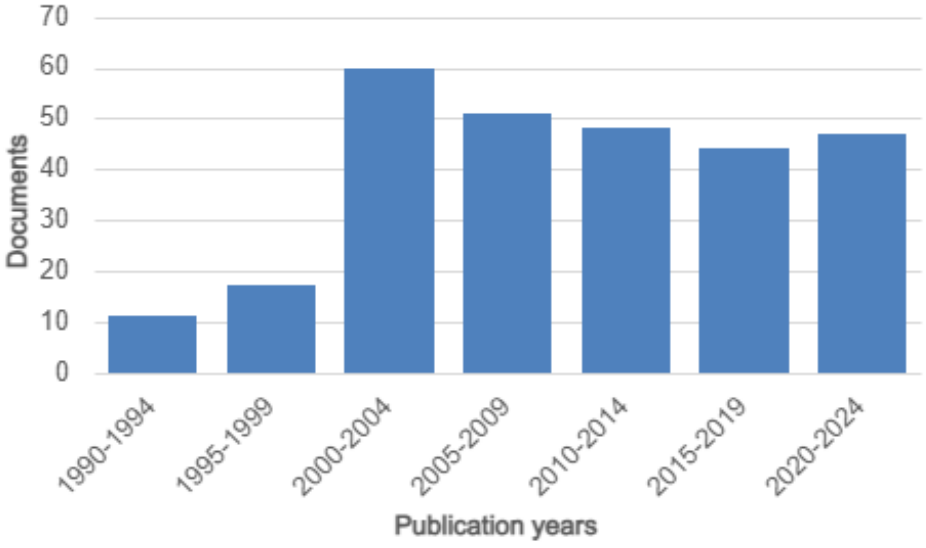
Bibliographic research was done using Web of Science. In the first attempt, the following keywords were used: “arsenic” and “algae”. The research gave over two thousand documents so “CRM” was introduced to limit the results. Some of the documents obtained were discarded from deviation of the main topic and the ones reviewed date from 2002 to 2022.

To focus on the topic, a second research was done using the first two keywords and “arsenosugars” as the third one. Nearly three hundred documents were found but one interesting review was selected *Analytical methodologies for arsenic speciation in macroalgae: A critical review* which includes articles since 1973, although mainly from 1998 to 2017. Then, all the articles from 2018 until 2023 were studied, resulting in a total of sixty-five documents. The first part of the research was carried out in mid-October and the second part at the end of the month.

The SciFinder database was also used for document searches. Most of the articles matched and those that deviated from the main research topic, were therefore discarded.

Figure 2 shows the evolution over time of the documents published on the topic discussed. It can be determined that it was not a subject of relevance until the early 2000s, and since then, the level of interest has remained constant.

Figure 2. Evolution over time on arsenosugars documents.



6. RESULTS AND DISCUSSION

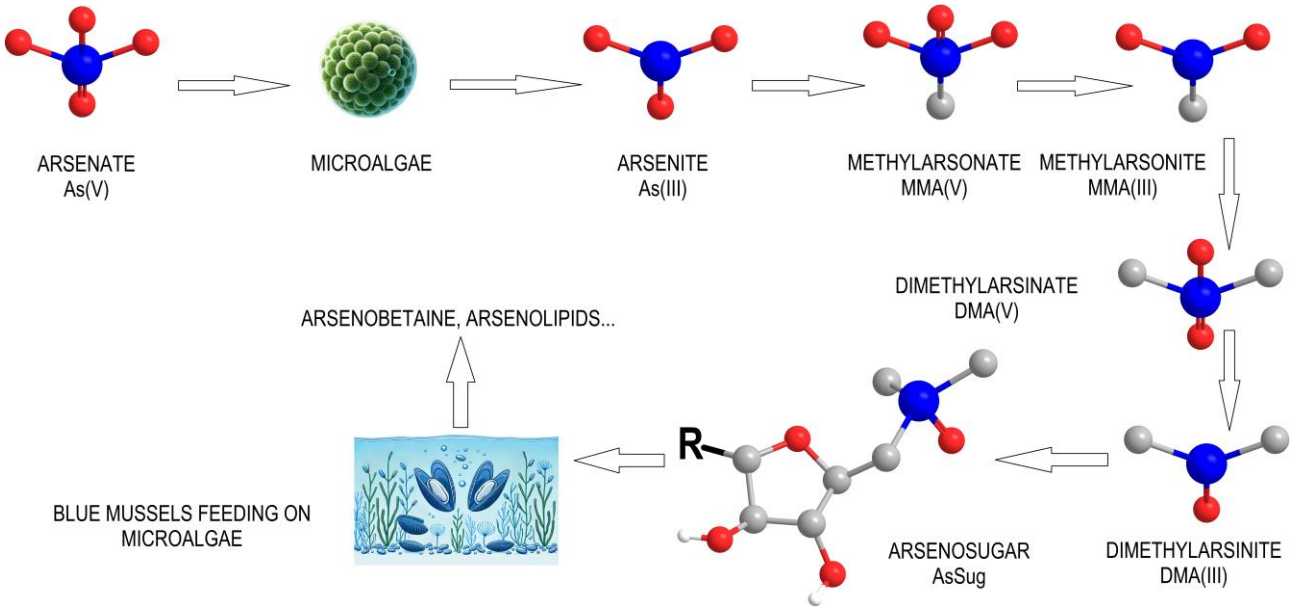
6.1. ARSENIC BIOTRANSFORMATION AND TOXICITY PARAMETERS

6.1.1. Arsenic biotransformation in algae

Microalgae play a central role in arsenic cycling within marine ecosystems, serving as the primary interface for arsenic transfer from seawater into the food chain. These organisms predominantly uptake arsenate (As(V)) from seawater and, through detoxification mechanisms, transform it into a range of oAs species, including AsSug and arsenolipids (AsLip). These processes were first described by Challenger in 1945 [19] and involve methylation and reduction steps to produce compounds such as DMA. The mechanism of arsenic biotransformation is shown in Figure 3. Experimental work with *Dunaliella tertiolecta* [21] shows that As(V) is rapidly methylated to DMA, which subsequently participates in the formation of AsSug and AsLip. Concentrations of these complex oAs increase with time, and cultures maintained in continuous systems exhibit 2–3 times higher concentrations compared to batch systems. Also, it has been suggested that AsSug are not merely detoxification products but might instead be normal by-products of biological activity. For instance, in *Laminaria digitata* [22], these compounds are likely bound to cell membranes within the algal cells, requiring lyophilization to achieve quantitative release.

Freshwater phytoplankton exhibit varying arsenic biotransformation efficiencies [23]. Species like *Botryococcus braunii* and *Staurastrum paradoxum* oxidize As(III) into As(V), while others such as *Achnanthidium minutissium* and *Closterium aciculare* excel at methylating iAs into complex oAs. There is a third group which is the one that cannot efficiently biotransform As and thus rather maintain As(V) inside their cells (*Pediastrum duplex*). These variations may explain the observed diversity in arsenic metabolism across aquatic ecosystems.

Figure 3. Pathway for the biotransformation of iAs to more complex oAs species in microalgae. Blue atoms represent As, red ones represent O, grey ones represent C and white ones represent H. Methyl and methylene H have been omitted.



6.1.2. Arsenic biotransformation in animals/humans

An in vitro artificial digestion system was used to examine the metabolism of an AsSug compound in the gastrointestinal tract [24] and the results showed that glycerol arsenosugars (Gly-Sug) are partially broken down into thio-Sug during digestion but retain their sugar component. Absorption occurs in the intestinal tract, with subsequent metabolism in the liver converting them to DMA. Gly-Sug are incubated with gastric juice for 4 hours, followed by bile-pancreatic juice for 0.5 hours, and finally with an enteric bacteria solution for 24 hours. The transformation of arsenic compounds after artificial digestion was analysed using HPLC-ICP-MS and HPLC coupled with ESI quadrupole time-of-flight mass spectrometry (HPLC-ESI-QTOF-MS) and the findings indicate that the artificial gastrointestinal digestion process converted Gly-Sug into thio-Gly-Sug, but no DMA(V) formation was observed. During the artificial digestion, the 5-

deoxyribofuranose structure of AsSug remained intact. Therefore, it is suggested that AsSug is likely absorbed in the intestinal tract after partial decomposition of its sugar component and may then be metabolized to DMA(V) in the liver and eventually excreted in the urine.

Another experiment showed thio-DMA and its excretion detected through urine after exposure to iAs and AsSug consumed by marine algae [25]. In this study, the production of thio-DMA has been linked to cellular disruptions, such as mitotic arrest caused by the phosphorylation of BubR1, a protein of the spindle assembly checkpoint (SAC) complex that accumulates in the cell in the mitotic phase. Though the biological significance or the mechanism by which thio-DMA induced mitotic phase accumulation occurs is yet to be understood.

In another report, mice were fed with Fujian laver in different concentrations in order to establish a correlation between the amount of laver consumed and the excretion of As(V) in mouse feces and its residues in mouse muscles and organs [26]. The experiment showed that most AsSug and DMA are excreted directly in feces, with minimal bioaccumulation in muscles or organs. Some of the As(V) ingested by the mice was methylated into DMA(V) and directly excreted through feces from the mouse's body unchanged, with only a small amount accumulating in the body, leading to DMA(V) residue in the muscles and organs only after high-dose laver digestion. The primary As species in laver, such as methoxy arsenosugar (meth-Sug) and phosphate arsenosugar (PO_4-Sug), did not accumulate in the mouse muscles and organs. Most meth-Sug was converted into other As species before excretion like PO_4-Sug and DMA(V) during digestion but PO_4-Sug was excreted unchanged. Notably, increasing laver intake from 69 µg to 914 µg resulted in a decline in residual arsenic levels in tissues, from 0.78% to 0.12%, and a rise in fecal excretion, from 65% to 77% so there is not a clear correlation of the amount of laver consumed and the excretion of As(V) in mouse feces and its residues in mouse muscles. As high-dose laver intake did not result in a significant increase in As(V) levels in the mouse these findings suggest very limited health risks from typical dietary intake of laver.

6.1.3. Phosphorous influence on arsenosugars

Picocystis strain ML was studied under different incubation conditions [27] and there were significant variations in the composition of oAs species extracted. Under low-phosphorus conditions, most of the extractable arsenic was in the form of AsLip which made up 82% and 88% of the recovered material from the As(III) and As(V) amended samples, respectively. For the As(III) sample, the majority of the arsenic was taken up after being externally oxidized to As(V), and only a small proportion of water-soluble oAs species was detected. In contrast, under phosphorus-replete conditions, especially with the As(III) sample, AsLip represented less than 1% of the extracted oAs compounds, with the rest being water-soluble As compounds. This trend was also observed in the As(V) sample, although AsLip constituted a larger fraction (~32%) of the extractable arsenic. It is concluded that the imported As(V) likely serves some of the roles of phosphate under phosphorus-limited conditions. However, the substantial presence of AsLip in the high-phosphorus As(V) sample suggests that they may also play a key role in basic cellular functions, such as incorporation into the cell membrane structure. In fact, under low phosphate conditions, *Picocystis* strain ML tends to produce more AsSug, such as Gly-Sug, which may act as energy storage molecules. When phosphorus is abundant, AsLip are likely incorporated into cell membranes and dominate the arsenic profile.

In *Porphyra haitanensis*, sufficient phosphorus supply prevents lipid peroxidation and oxidative stress in algal cells exposed to arsenic [28]. Under low-phosphate conditions, arsenate replaces phosphate in biomolecular processes, including DNA binding and membrane synthesis, leading to elevated iAs levels in the algae but these seaweeds uptake the arsenate and convert it into arseno-sugars to alleviate toxicity and Gly-Sug and PO_4-Sug are the abundant species. This dynamic is consistent across several species. For example, a study done with *Dunaliella tertiolecta* shows that arsenate lowers its concentration under P-starvation (even though arsenate was added to the cultures) and Gly-Sug is the major species [29]. This would imply that the alga could efficiently biosynthesize this arsenic at the tested exposure levels. In P-replete conditions, PO_4-Sug are more abundant showing higher amounts at the higher P conditions, contrary to Gly-Sug. So, under phosphorus-rich conditions, arsenate substitutes for phosphate in vital biomolecular processes, while phosphorus scarcity promotes Gly-Sug production. All these results underscore the interplay between environmental phosphorus levels and arsenic bioaccumulation.

6.1.4. Human exposure and regulatory concerns

In an analysis of 25 seaweed species, iAs concentrations ranged from 0.05 mg/kg in *Chlorella pyrenoidosa* to 67.5 mg/kg in *Hizikia fusiformis* [6]. Estimated daily intake (EDI) of As through algae consumption was calculated using the following equation:

$$EDI = \frac{Ci\ x\ Di}{BW}$$

where Ci refers to the concentration of As species in dry seaweed biomass (µg/g); Di is the daily algae intake (g/day), and BW represents body weight (70 kg). To calculate the EDI, the iAs concentrations for the 25 seaweed samples analysed were derived by adding the levels of As(III) and As(V), and these values were compared to the lower confidence limit of the benchmark dose set by the European Food Safety Authority (EFSA), which ranges from 0.3 to 8 µg/kg bw per day. The EDI for low seaweed consumption (2 g/day) was calculated to range from 0.001 to 1.93 µg/kg·day, while high consumption (8 g/day) ranged from 0.01 to 7.71 µg/kg·day. EFSA's reference value for iAs exposure is 0.3 µg/kg·day, and only *Hizikia fusiformis* from Japan and Korea exceeded this threshold under low-consumption scenarios. Under high consumption, two additional species, *Porphyra umbilicalis* and *Himanthalia elongata*, also exceeded this value [6]. Although information on seaweed consumption in Europe is scarce, it is anticipated to be less than half when compared to Asian nations, where adults consume an average of 4 to 8.5 grams of seaweed daily, with South Korea showing the highest intake and Japan the lowest.

EFSA has raised health concerns regarding current iAs exposure levels, particularly from food sources and countries such as France, Australia, and New Zealand have set limits on iAs concentrations in seaweed [30]. France allows up to 3 mg/kg in seaweed condiments, while Australia and New Zealand enforce stricter limits of 1 mg/kg. China has a 0.3 mg/kg limit for iAs in complementary foods containing seaweed intended for infants [31]. In March 2022, EFSA issued a request for the ongoing collection of data on the presence of chemical contaminants in food and feed, including oAs compounds such as thiolated compounds and others (MA, DMA, AsLip and AsSug). Despite this, the European Union has not yet implemented maximum levels for iAs in seaweed. Instead, the decision-making on this matter has been delegated to its member states through Commission Directive 2002/32/EC, which states that upon request of the competent authorities, it is the responsible operator who must perform an analysis to demonstrate that the content of inorganic As is lower than 2 mg/kg [32]. While the EU has yet to establish specific limits for iAs in seaweed, regulations for other food categories, such as rice and seafood, are already in place. Drinking water standards have also become stricter over time. For example, the World Health Organization (WHO) guideline for tAs was reduced from 50 µg/L to 10 µg/L [31], and the Netherlands adopted an even lower voluntary limit of <1 µg/L through its national water associations. These measures underscore the increasing recognition of arsenic toxicity and the need for harmonized safety limits.

Also, the study of other compounds in edible algae, such as D-cysteinolic acid [33], is essential for advancing AsSug speciation techniques, understanding potential toxicological impacts, and guiding regulatory measures. D-cysteinolic acid, found in various algae, is hypothesized to serve as an osmolyte and may play a role in lipid digestion and gut microflora regulation, drawing parallels to taurine. Despite its significance in marine ecosystems, limited research exists on its effects on human health. Including such compounds in arsenic speciation research could deepen insights into the metabolic pathways of AsSug and their broader implications for both toxicity assessments and legislative standards for algae-based foods.

6.2. ANALYTICAL METHODS FOR ARSENIC SPECIATION: PROCESSING, EXTRACTION, CLEAN-UP, SEPARATION, DETECTION AND QUANTIFICATION

To guarantee the reliability of analytical results for practical applications, it is essential to address certain considerations across the various steps of speciation methodologies for As. From processing to quantification, all steps are explained in this review alongside different examples discussed in several scientific articles.

6.2.1. Processing

The preparation and processing of algae and seagrass samples are crucial steps to ensure reliable and reproducible analysis of arsenic species. Proper treatment not only aids in preserving sample integrity but also facilitates the extraction and quantification of arsenic compounds, including arsenosugars. Several sample processing techniques referenced in the provided studies are

summarized. The choice of methods such as washing, drying, grinding, and storage significantly influences the efficiency of AsSug extraction and the reliability of results, as highlighted in studies comparing fresh and freeze-dried materials.

Sample washing is usually performed with double-deionized water to remove contaminants and salts prior to subsequent treatments [14]. Similarly, for microalgae, the precipitates from experimental cultures are often washed using a diluted sterile seawater solution (20% ultrapure water), pooled, and centrifuged [19].

Drying methods vary across studies. For instance, in some studies, samples were dried in an oven at 40°C for 24 hours [3, 14] but also freeze-drying was used in multiple cases, especially for microalgae, to preserve samples and improve the efficiency of arsenic extraction [19, 22]. Freeze-drying has proved essential for efficient arsenic extraction, yielding an average extraction efficiency of 103% compared to 68% for fresh samples [22]. This suggests that freeze-drying facilitates cell wall and membrane disruption, enhancing the release of AsSug.

Grinding is consistently highlighted as a key step for homogenization and preparing fine powders suitable for analysis. In some cases, a mixer such as a Retsch MM 301 mixer mill with tungsten carbide jars and balls was used to grind the algae samples [3] and in other ones, tungsten carbide disc mills were utilized for grinding the edible algae to fine powders [14, 34]. Manual grinding in glass mortars is also a commonly used method for specific sample forms such as algae pellets [34]. Centrifugation also plays a pivotal role in processing microalgae cultures. In some studies, transferring the cultures to centrifuge tubes is done to remove the supernatant and then the precipitate can be re-suspended and further processed [19, 21].

Ground samples are typically stored in pre-cleaned polyethylene containers at room temperature [3, 34]. This minimizes contamination and preserves sample integrity before analysis. The findings indicate that the samples maintain their stability for a minimum of 12 months when stored in polystyrene containers at +20°C, with no evidence of degradation in the analytical signals.

In general, after cleaning the algal material, the primary methods for water removal from samples are freeze-drying and thermal treatments, followed by homogenization to achieve a powdered form but the handling of different forms of algae (fresh, dried, or pelletized) required adjustments in processing. Fresh materials demand immediate analysis or freeze-drying to prevent degradation [22] and dried and pelletized samples are powdered using specific grinding equipment before homogenization [34].

6.2.2. Extraction

Efficient extraction techniques are fundamental in the analysis of arsenosugars from edible algae, serving as a crucial step to isolate these compounds from complex biological matrices. The extraction process not only determines the efficiency of analyte recovery but also impacts the subsequent steps of clean-up, separation, and quantification. Given the structural diversity and polar nature of arsenosugars, the choice of an extraction technique significantly influences the preservation of their integrity and the representativeness of the analytical sample. Methods such as microwave-assisted extraction (MAE), shaking, ultrasound-assisted extraction (UAE), and water bath extraction have been widely employed for this purpose. These techniques utilize varying mechanisms to disrupt cell matrices and mobilize arsenosugars into the extraction medium.

6.2.2.1. Extraction techniques

MAE has proven to be an efficient method for extracting arsenic species from edible algae. Different systems have been used for this process, including the MARS 5 microwave oven and the ETHOS 1 closed vessel microwave digestion system [3], which employ TFM-Teflon vessels. In these setups, algae are treated with deionized water (8 mL per 200 mg algae), and the mixture is heated at 90°C for 5 minutes, repeated three times. After this, the samples are subjected to centrifugation at 14,000 g for 10 minutes, and the supernatant is pooled and diluted to a final volume of 25 mL.

For specific applications, MAE was employed for tAs analysis in dried samples. It was the case of a report where aliquots of 0.2 g were digested with 8 mL of concentrated HNO_3 and 2 mL of H_2O_2, following a staged temperature program that reached 190°C [14]. Also, other systems, like the Milestone Ethos Touch Control, use a more aggressive digestion process involving also nitric acid and hydrogen peroxide [14]. In another report, for arsenic speciation, 0.1 g samples were extracted with 1% HNO_3 and 2% H_2O_2, heated to 95°C for 30 minutes, and then centrifuged to separate the supernatant for further analysis [35].

The addition of HNO_3 has been shown to improve extraction efficiency. Specifically, adding 0.2% nitric acid helps to preserve As(III) and As(V), and results in acceptable recoveries (80–110%) [36]. This technique has demonstrated satisfactory extraction efficiency for arsenic speciation, considering the complexity of the sample and the conversion of arsenic species.

UAE is another widely used technique for extracting arsenic species from seafood and algae. In UAE, MeOH-H_2O mixtures have proven effective, providing satisfactory recovery rates without altering the species of arsenic present. One study found that UAE, using a MeOH-H_2O mixture (1:1), followed by rotary evaporation and re-dissolution, was effective for arsenic speciation in algae [37].

Ultrasonic and microwave-assisted extractions are one of the main techniques used to extract arsenic species in seafood at present. They were widely used to extract arsenic species in edible seaweeds with a satisfactory recovery (>90%) and no species change by using MeOH-H_2O as extracting solvent [38].

Water baths are also commonly used to maintain a constant temperature during the extraction process, which is crucial for preserving the integrity of arsenic species and preventing their transformation. For example, in one study, arsenic species were extracted using 10 mL of 0.1 M HNO_3 and 3% H_2O_2, followed by heating in a water bath at 90°C for 1 hour while shaking at 100 rpm [19]. This method efficiently extracted arsenic species, especially when H_2O was used as a solvent.

Shaking is frequently used to promote the mixing of solvents and samples, ensuring that arsenic species are efficiently extracted from the matrix, and is often combined with other techniques, such as heating in a water bath, to improve extraction efficiency. In fact, this combination provides both the necessary temperature control to preserve arsenic species and the mechanical agitation needed for homogeneous mixing of the sample and solvent [14].

6.2.2.2. Total arsenic and arsenic speciation analysis methods

For total arsenic analysis, various techniques have been employed, most notably using concentrated HNO_3 and H_2O_2. This combination, under sequential heating (water bath), is effective for extracting tAs from algae [14]. Similarly, the Ultra WAVE system also utilizes nitric acid alone for tAs extraction [19]. Some studies use a combination of shaking and water bath techniques for total arsenic determination [37]. After extraction, tAs levels are typically analysed using methods such as ICP-MS or electrothermal atomic absorption spectroscopy, ensuring precise quantification of arsenic levels.

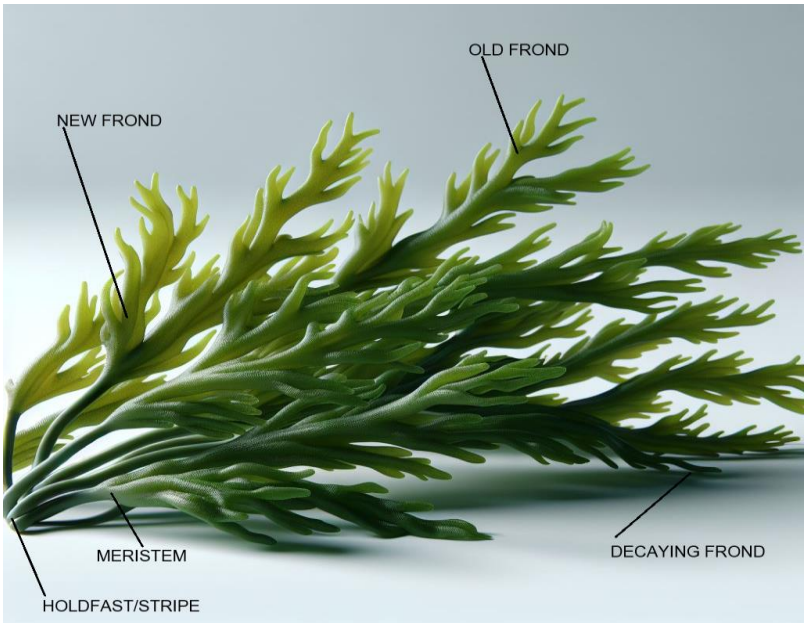
Arsenic speciation is crucial for understanding the different forms of arsenic present in algae. Speciation methods typically focus on As(III) and As(V) and often use deionized water heated to 90°C for extraction [3]. Studies have shown that in brown algae, arsenosugars are distributed unevenly across different parts of the organism, and specific extraction techniques are required to identify and quantify these species. One study with *Laminaria Digitata* demonstrated that freeze-drying improved extraction efficiency for arsenosugars [22].

The average concentration of AsSugars and other water-soluble As species in each thallus section is shown in Table 1. There is a noticeable pattern of increasing As(V) concentration along the thallus, progressing from the holdfast and stipe material to the decaying frond. However, significant differences in extraction efficiency between fresh and freeze-dried materials are observed only in the old and decaying frond sections. For the holdfast/stipe region, which is challenging to homogenize due to its dense, fibrous structure, comparable levels of As(V) were extracted from both fresh and freeze-dried samples (0.78 and 0.63 mg/kg). In contrast, freeze-drying resulted in roughly a twofold increase in extraction efficiency for the old and decaying frond sections. Figure 4 illustrates the sectioning of a representative seaweed, highlighting the holdfast/stripe, the meristem, and the various types of fronds. The elevated As(V) levels in the older frond sections suggest that not all As(V) may be bound to the polysaccharides in the cell wall; some could be located within the cells. Therefore, freeze-drying might be necessary to fully release the As(V) present. However, even though a greater amount of arsenic was extracted from freeze-dried material, the distribution pattern of some AsSug within the thallus was different for both fresh and freeze-dried samples. This suggests that the extraction of this compound is primarily influenced by how well the sample has been homogenized. This information was also pointed out in another report with *Saccharina latissima* and *Alaria esculenta* samples [39]. The work elucidated that arsenic was not uniformly distributed within the two brown macroalgal species, with lower levels of total As found in the stipe/midrib compared to other thallus parts. The AsSug closely reflected the tAs content in the seaweed, primarily because sulfonate arsenosugar (SO_3-Sug) was the dominant arsenic species.

Table 1. The average concentration of AsSug and other water-soluble As species in each thallus section of *Laminaria Digitata* during both months comparing freeze-dried and fresh samples.

Month	Thallus section	Gly-Sug/As(III) [µg/g]	DMA [µg/g]	PO_4-Sug [µg/g]	SO_3-Sug [µg/g]	As(V) [µg/g]
February (freeze-dried)	Holdfast or stripe	3.19	0.19	4.21	25.38	1.82
	Meristem	3.12	0.22	9.89	41.92	2.19
	New frond	2.78	0.28	12.89	35.27	24.56
	Old frond	1.97	0.32	9.55	22.67	59.28
	Decaying frond	14.75	0.19	13.40	13.63	60.97
May (freeze-dried	Holdfast or stripe	11.45	0.05	5.55	20.78	0.78
	Meristem	15.88	0.07	5.72	33.26	2.46
	New frond	15.71	0.06	4.47	23.03	7.82
	Old frond	17.14	0.12	4.57	11.31	35.54
	Decaying frond	2.25	0.26	7.55	16.46	44.42
May (fresh)	Holdfast or stripe	6.41	0.03	3.79	14.13	0.63
	Meristem	11.69	0.03	6.40	24.18	1.65
	New frond	7.54	0.06	1.75	12.72	5.69
	Old frond	9.09	0.08	2.30	6.54	19.14
	Decaying frond	5.56	0.09	3.74	6.00	27.71

Figure 4. The sectioning of a seaweed representation.



These studies highlight the need to tailor extraction techniques to specific analytical goals. A report complements these findings by suggesting that the variability in arsenic extraction across different alga types is influenced by weather. For instance, a significantly higher total arsenic concentration was found in *Saccharina japonica* in the north (cold weather) compared to the south (warm weather) [31].

For water-soluble arsenic, the most effective extraction methods involved shaking, ultrasound-assisted extraction, or a combination of the two. After centrifugation, the dichloromethane (DCM) layer is collected and volatilized for tAs analysis, representing arsenosugar phospholipid concentrations, and the aqueous layer is collected for the analysis of water-soluble arsenic species [40]. Microwave-assisted extraction is also commonly employed in several studies [41], demonstrating efficiency in extracting arsenicals from various sample types.

Table 2. Overview of several extraction methods for arsenic speciation in edible seaweed.

Ref.	Extraction method		Extraction [%]
	tAs	Arsenic speciation	
[3]	Microwave digestion with HNO_3 and H_2O_2	0.2 g algae, 8 ml H_2O. Water bath at 90ºC for 5 min, 3 times. Centrifuged at 14000 g for 10 min.	49-93
[14]	0.2 g dried samples weighed to 0.1 mg in digestion vessels, 8 mL concentrated HNO_3 and 2 mL H_2O_2 are added. Digestion: 10 min from room temperature to 90ºC, 5 min at 90ºC, 10 min from 90ºC to 120ºC, 10 min from 120ºC to 190ºC and 10 min at 190ºC.	0.1 g dried pulverised samples weighed to 0.1 mg in 25 mL Teflon tubes. 10 ml H_2O added to each tube. End-over-end shaker at 30 rpm for 16 h at room temperature. The resulting mixtures are centrifuged.	69-99
[19]	0.2 g sample weighed into quartz digestion vessels, 2 mL concentrated (65%) HNO_3 is added. Ultra WAVE system and diluted with ultrapure H_2O to 25 mL.	iAs: 10 mL 0.1 M HNO_3 in 3% H_2O_2 is added and vortex mixing. Stand overnight and water bath for 1 h at 90ºC, shaking at 100 rpm. Cooled and centrifuged for 10 min at 3800 rpm. AsSug: 0.2 g sample weighed into 13 mL PP tubes and 5 mL (MeO:H_2O, 50% v/v) is added. Vortex mixing and water bath heating for 30 min (90ºC, shaking speed at 100 rpm). Cooled and centrifuged at 3800 rpm for 10 min.	
[40]	0.1 mM HNO_3. Microwave digestion.	0.1 g algae, 5ml DCM:MeOH 2:1. Centrifugation	
[42]	Microwave digestion.	0.25 g algae weighed into centrifuge tubes, 10 mL doubly deionized H_2O was added. End-over-end shaker at 30 rpm for 16 h at room temperature. Centrifuged at 3000 rpm for 20 min.	89
[21]		0.01 g algae, DCM:MeOH (2:1, v/v, 500 µL). Ultrasonic bath (15 min at 30°C), mixing on a rotatory cross (45 min at 22°C). Centrifuged (10 000 ×g, 10 min, 10°C). Dry pellets after lipid extraction extracted similarly but 20 mM NH_4HCO_3 employed as the solvent (pH 8.5 adjusted with NH3 500 µL).	
[43]	0.5 g algae into four Teflon microwave vessels and the fourth portion spiked with 1.0 g of a 36 µg/g arsenic solution. Concentrated HNO_3 (5 mL), 30% H_2O_2 (1 mL) added into the vessels, heated in a microwave at 250°C for 15 min with a 30-min linear ramp. Cooling to room temperature, transferred to PP centrifuge tubes, gravimetrically diluted to 50 g.	AsSug: 0.25 g sample weighed into 50 mL PP centrifuge tubes in triplicate. A fourth portion is weighed out from selected samples and spiked with solutions of arsenic species. Deionized H_2O (15 g) is added, vortexed and heated in a hot block at 90°C for 30 min with a 45 min linear ramp. Cooled to room temperature and centrifuged for 10 min at 3000 rpm. Nonpolar arsenic: The solids left from aqueous extraction are rinsed with deionized H_2O and kept in an oven at 70°C to dryness. Treated with 5 ml of a 2:1 (v/v) DCM:MeOH, mixed using a homogenizer and shaking for 60 min. Filtered into disposable borosilicate glass microwave vessels (VWR). Heated in a centrifugal evaporator at 45°C to remove the organic solvent.	58−101
[34]	0.5 g weighed into the digestion vessels. 8.0 mL 69% HNO_3 and 2.0 mL of 31% H_2O_2 are added. Digested following a temperature program up to 190ºC for 80 min.	0.1 g samples were weighed into 15 mL PP tubes, 10 mL doubly deionized H_2O was added. End-over-end shaker at 30 rpm for 16 h at room temperature. Suspensions centrifuged at 1855 rpm for 20 min.	79-91
[37]	0.1 g freeze-dried tissue weighed into 7 mL Teflon polytetrafluroacetate digestion vessels and 1 mL of concentrated HNO_3 added. MDS-2000 microwave oven with a time program consisting of three steps: 2 min at 600 W, 2 min at 0 W and 45 min at 450 W.	0.1–0.2 g sample for 3 h on a rotating wheel with 10 mL H_2O:MeOH (1:1 v/v). Centrifuged (5000 g) and supernatant collected. Pellet is further extracted with 3 × 10 mL H_2O:MeOH (1:1, v/v) and supernatant combined and roto evaporated to dryness at 30 °C. Residue is dissolved in 2 mL of H_2O. This extract is diluted with 10 mL deionised H_2O for further analysis. Arsenic concentrations in extracts determined by acid leaching 0.5 mL of extract with 0.1 mL of concentrated HNO_3 in a water bath at 90 °C for 2 h.	80 ± 1

6.2.2.3. Solvent trends for arsenic extraction

In general, studies reveal the critical role of solvent polarity in balancing extraction efficiency with species preservation [44]. H_2O, MeOH-H_2O mixtures, and HNO_3 have consistently been highlighted as the primary solvents for arsenic extraction. For tAs analysis, concentrated HNO_3 is favoured due to its oxidative strength, which helps to break down the algae matrix and release arsenic. In

contrast, for arsenic speciation, milder solvents [36, 19] like H_2O or diluted HNO_3 are preferred to prevent the degradation of arsenic species.

For polar arsenic species [40, 43], solvents like MeOH-H_2O and diluted HNO_3 have been found to be the most effective for extraction, while nonpolar arsenicals can be extracted using DCM:MeOH (2:1 v/v) mixtures and after extraction, the solution is separated into aqueous and organic layers, allowing for targeted analysis of nonpolar arsenic species.

Polar solvents, particularly MeOH-H_2O, have been shown to be compatible with arsenosugars, making them the solvent of choice for arsenic speciation across multiple studies [36, 38, 41]. Acidic environments can further enhance the release of arsenosugars while preserving the native arsenic species, as noted in other studies [45], but high concentrations of HNO_3 can risk altering species, requiring careful control of temperature and other conditions [38].

Regarding extraction solvents, H_2O and MeOH are the most used, either alone or mixed in different ratios depending on the matrix characteristics. MeOH is preferred because it extracts minimal amounts of non-arsenical compounds and can be easily removed through evaporation before instrumental analysis. However, MeOH is not very effective for extracting iAs and is seldom used on its own. Conversely, many natural arsenic species are polar and water-soluble, making H_2O an ideal solvent due to its ability to deeply penetrate the sample matrix. In addition to H_2O and MeOH, other extraction solutions, such as inorganic acids (notably H_3PO_4 and HNO_3) or buffer solutions, have been successfully applied [41].

It is worth noting that both MAE and UAE commonly use MeOH-H_2O mixtures as solvents, which highlights the versatility of this solvent for arsenic extraction.

Table 2 shows different extraction techniques for total arsenic and arsenic speciation along with different solvent trends.

6.2.2.4. More factors influencing extraction efficiency

Temperature and duration also play critical roles in extraction efficiency. MAE typically operates at elevated temperatures (90°C to 190°C) with short durations, enhancing extraction efficiency [3, 14, 36]. UAE, by contrast, operates at room temperature and relies on mechanical agitation to extract arsenic species [21, 37]. Sample preparation is equally important, as freeze-drying has been shown to improve arsenic recovery by homogenizing tough algal sections, enhancing As(V) extraction [22]. Water-soluble arsenicals exhibited higher extraction efficiency with aqueous MeOH at 90°C [46] and also with end-over-end shaking and water bath methods [34].

6.2.3. Clean-up

The clean-up process in analytical chemistry is a crucial preparatory step before separation techniques, such as HPLC or ICP-MS. This step ensures the removal of matrix interferences, concentration of analytes, and enhancement of signal clarity, which are essential for accurate quantification and speciation of target compounds like arsenosugars. The complexity of biological matrices, such as edible algae, necessitates optimized clean-up methods to isolate AsSug effectively without compromising their integrity. Various strategies, including filtration and SPE, have been utilized and compared for their efficacy. This section synthesizes insights from multiple studies to identify the most effective clean-up approach for arsenosugars in algae.

Several studies emphasize the utility of filtration in arsenosugar clean-up. Nylon syringe filters with pore sizes of 0.22 µm [3, 40] and 0.45 µm [42, 34] are commonly used to remove particulate matter and prevent column clogging during HPLC analysis. In another report, ash-free filter papers (Whatman 40) were employed for filtering digested samples before dilution, a technique particularly suited for handling highly processed matrices [14]. While filtration ensures the removal of suspended particles, it does not fractionate AsSug from other As species, which limits its specificity as a clean-up method.

SPE stands out as a sophisticated clean-up method for isolating arsenosugars. Using silica-based strong anion exchange and weak anion exchange (NH_2) cartridges, arsenosugars such as PO_4-Sug, SO_3-Sug, and sulfate arsenosugar (SO_4-Sug) have been successfully isolated with high recoveries [42]. The pH, ionic strength, and type of elution solvent significantly influence SPE performance. Therefore, the highly polar and anionic nature of arsenosugars demands careful optimization of the eluent polarity and acidity to maximize retention and recovery. Recoveries exceeding 75% are reported for SO_3-Sug and SO_4-Sug, while PO_4-Sug demonstrates lower recoveries of approximately 45% under various elution conditions. Despite these variations, SPE effectively

isolates arsenosugars as the sole arsenic species in the solution. Moreover, lyophilization post-SPE allows pre-concentration of analytes, further enhancing analytical sensitivity. This robust methodology also aligns with the goal of producing analytical standards for arsenosugars [42].

Based on reviewed studies, both filtration and SPE techniques offer unique advantages. Filtration is straightforward, cost-effective, and suitable for routine sample preparation, particularly when combined with centrifugation to remove particulates. However, it lacks the specificity required to isolate AsSug from complex matrices. In contrast, SPE provides a more targeted approach, enabling the separation and concentration of AsSug while minimizing co-elution with other arsenic species. The additional lyophilization step in SPE protocols further underscores its utility in preparing concentrated analytical standards.

6.2.4. Separation

Techniques of separation play a pivotal role in speciation analysis prior to detection, ensuring accurate quantification and identification of arsenic species. Given the complex matrices of algae and the variety of arsenic compounds present, including both cationic and anionic forms, robust separation methodologies are required. Techniques such as HPLC, CZE, and GC have been extensively applied, often paired with advanced detectors like ICP-MS. This section compares these methods, focusing on the separation efficiency, stationary and mobile phase characteristics, and operational parameters.

6.2.4.1. Predominant techniques

HPLC emerges as the most widely used separation technique for arsenosugar speciation in algae due to its versatility and compatibility with various detection systems. HPLC, particularly when coupled with ICP-MS, has demonstrated the ability to separate a broad spectrum of arsenic species, including oxo-arsenosugars, iAs, and methylated forms. For instance, in a study using pentafluorophenyl (PFP) columns [47], 13 arsenic species were separated with high resolution, emphasizing HPLC's adaptability to complex mixtures. Other techniques such as UPLC/ESI-MS/MS [48] are used in some studies as a qualitative approach but these techniques are not quantitative and are therefore excluded from the validation process. UPLC/ESI-MS/MS methods provide complementary insights, especially when validated with CRM.

CZE and GC, though less common, are advantageous in specific contexts. CZE offers high separation efficiency for small ionic species but lacks the robustness needed for complex matrices like algae [15]. GC requires derivatization of arsenic species, which adds steps to sample preparation, limiting its routine use in arsenosugar analysis [13].

6.2.4.2. Stationary phases

The choice of column material and type is critical for achieving optimal separation. Anion-exchange columns like Hamilton PRP-X100 and zirconium-based columns are commonly used for anionic arsenic species. The PRP-X100 column, composed of polystyrene/divinylbenzene copolymer with trimethylammonium exchange groups, has been effective in separating As(III), As(V), MMA and DMA under isocratic conditions [41]. Zirconium columns, on the other hand, provide sharper peaks and faster separation for arsenosugars and are less influenced by matrix effects compared to PRP-X100 [37]. Cation-exchange chromatography employs columns like Zorbax 300-SCX, which have shown high efficiency in separating Gly-Sug, arsenobetaine, arsenocholine and other cationic species using pyridine-based mobile phases [14, 41]. These columns are particularly suitable for matrices where arsenobetaine and other cationic arsenic species dominate.

Reverse-phase chromatography, especially with PFP or C18 columns, has been applied to separate polar and less-polar arsenic compounds [47]. The unique dipole-dipole interactions of PFP columns enable strong retention of polar compounds without ion-pair reagents, making them a valuable alternative for comprehensive arsenic speciation. While standard C18 phases demonstrate hydrophobic interaction potential, PFP phases offer multiple interaction potentials. These include a relatively mild hydrophobic interaction potential (typically equivalent to C8 or lighter phases), strong dipole-dipole interactions arising from carbon-fluorine bonds, π-π interaction potential, charge-transfer interactions driven by the high electronegativity of fluorine groups, and ion-exchange interaction potential. These combined effects contribute to the distinctive properties of PFP phases.

6.2.4.3. Mobile phases and matrix effects

Mobile phase composition significantly impacts separation efficiency. In anion-exchange chromatography, phosphate buffers at varying concentrations and pH levels (20 mM ammonium dihydrogen phosphate, pH 5.6–6.0) [3, 41] are commonly employed to achieve optimal

resolution for inorganic and oxo-arsenosugars. However, phosphate buffers can contribute to signal drift in ICP-MS detection [41], leading to the adoption of alternatives like ammonium nitrate or bicarbonate, which stabilize retention times and reduce background noise.

For cation-exchange chromatography, aqueous pyridine solutions (10–20 mM, pH 2.3–3) adjusted with formic or hydrochloric acid have been effectively used to separate Gly-Sug, arsenobetaine, arsenocholine and related species [3, 14, 41]. The addition of MeOH to mobile phases in reverse-phase chromatography improves the separation of thio-Sug [41], addressing retention challenges posed by their hydrophobicity. Thioarsenosugars, and thio-arsenicals in general, are significantly less polar than their oxo-analogues, resulting in excessively long retention times and peak broadening under HPLC conditions typically used for oxo-arsenosugars. This behavior is attributed to the adsorption of thio-arsenicals onto the copolymer backbone of the PRPX100 column via hydrophobic interactions. However, in a study, it was found that adding MeOH to the mobile phase dramatically reduces the retention times of these compounds. This adjustment enables the separation of oxo- and thio-Sug in a single chromatographic run through gradient elution with ammonium bicarbonate or dihydrogen phosphate and increasing MeOH concentrations up to 50%.

Most researchers employ a sulfur (S)-based ion-pair reagent, such as sodium alkyl sulfonate for cationic arsenic species, combined with a different ion-pair reagent (tetramethylammonium hydroxide) for anionic arsenic species, along with a buffer solution and an organic modifier [47]. Ion-pairing reagents, while effective for specific arsenic species, introduce complexity and cost and are unsuitable for simultaneous detection of sulfur-containing species due to background interferences.

Matrix effects, such as co-elution and retention time shifts, present significant challenges in arsenosugar separation. An analysis of the results reveals that the quantification of Gly-Sug and PO_4-Sug using the zirconium column is sample-dependent. For macroalgae analysis, accurate Gly-Sug measurements are achieved in the absence of arsenobetaine since coelutes with arsenic cations on the PRP-X100 column, making the zirconium column suitable for cross-checking arsenobetaine concentrations in samples with low Gly-Sug content. PO_4-Sug and DMA exhibit similar retention times [37], making it impossible to quantify PO_4-Sug when significant amounts of DMA are present. Similarly, cation-exchange methods have highlighted matrix effects for arsenobetaine and dimethylarsenoacetate [41], necessitating additional steps for accurate identification.

6.2.5. Detection

Advanced detection techniques are essential for overcoming analytical challenges posed by the complex biological matrices of algae, the trace-level concentrations of arsenosugars, and the diverse range of arsenic species that can co-exist. This section comprehensively evaluates the state-of-the-art analytical methodologies for arsenosugar detection and quantification, providing a detailed comparison of their performance, limitations, and potential areas of improvement.

6.2.5.1. Comparative analysis of detection techniques

The accurate detection of arsenosugars in edible algae relies on advanced instrumentation capable of high sensitivity and selectivity. Various techniques have been extensively studied and applied in the field, including ICP-MS, ICP-AES, HG-AFS, HG-AAS and ESI-MS.

ICP-MS is widely recognized as one of the most versatile and sensitive techniques for arsenic detection, particularly when paired with chromatographic separation methods such as liquid chromatography (LC) or ion chromatography (IC). This combination facilitates the effective speciation and quantification of arsenosugars, even in highly complex matrices, enabling detailed analysis in environmental, biological, and industrial samples [14, 34, 49, 44]. Despite its exceptional sensitivity, ICP-MS is susceptible to matrix interferences, necessitating the use of advanced internal standards or collision/reaction cell technology to minimize spectral overlaps and ensure accurate results [49]. In comparison, ICP-AES has been successfully utilized for tAs determination across various biological matrices [3], but its inherently lower sensitivity relative to ICP-MS, coupled with its inability to resolve individual arsenic species without chromatographic separation, significantly limits its utility in speciation-focused analyses.

Hydride generation techniques have long been used for arsenic detection due to their ability to selectively measure iAs at ultra-trace levels. HG-AFS is particularly effective for detecting low levels of As(III) and As(V) [3, 49], while HG-AAS offers simpler instrumentation but suffers from lower sensitivity. However, one notable limitation of these methods is the low hydride generation efficiency observed for arsenosugars, which often leads to the underrepresentation of these organic arsenic species in analytical studies [50].

Electrospray ionization mass spectrometry provides a complementary approach, enabling the structural characterization of arsenosugars with high-resolution capabilities. By leveraging time-of-flight mass spectrometry, researchers can accurately determine molecular formulas and isotopic patterns for arsenosugars [34].

There is a report [51] in which X-ray absorption near edge structure (XANES) analysis is useful for the characterization of arsenic speciation. However if the arsenic species are below 10% of the tAs, detection is not possible which indicates the method's accuracy is not the most suitable [51].

In conclusion, while ICP-MS is the gold standard for arsenosugar analysis, its combination with chromatographic separation is critical for accurate speciation. Hydride generation techniques and ESI-MS offer complementary insights but are hindered by limitations in sensitivity and standard availability.

6.2.5.2. Importance of separation prior to detection

Chromatographic separation is an indispensable step in arsenosugar analysis. Without proper separation, co-eluting species can significantly interfere with detection signals, leading to erroneous quantification. LC and IC are commonly used to isolate arsenic species before detection [34, 49]. For instance, the coupling of HPLC with ICP-MS (HPLC-ICP-MS) enables precise quantification of individual arsenosugars by preventing signal overlaps caused by complex biological matrices [49]. Similarly, IC-ICP-MS has been used to resolve sulfate and sulfonate arsenosugars in algal samples, providing insights into their distribution and prevalence [34]. Failure to employ chromatographic separation often results in overestimation of iAs, as AsSug and other oAs compounds can contribute to the detection signal. Techniques like hydride generation, which lack separation, are particularly prone to such interferences [49]. As a result, chromatographic separation not only enhances the specificity and accuracy of AsSug quantification but also ensures compliance with regulatory requirements for arsenic analysis.

6.2.5.3. Challenges

The analytical landscape for AsSug detection faces several persistent challenges. Among them, matrix interferences from complex biological samples are a major obstacle. Algal matrices contain high concentrations of salts, polysaccharides, and other organic compounds that complicate the analysis [49]. Additionally, the lack of CRM and pure arsenosugar standards [44] hinders reliable calibration across laboratories. Hydride generation methods, while effective for inorganic arsenic, exhibit poor efficiency for arsenosugars due to their structural complexity [50]. This necessitates further research into alternative derivatization strategies or novel reagents to improve detection efficiency. Moreover, advancements in coupling techniques, such as integrating nanoscale secondary ion mass spectrometry (NanoSIMS) with chromatographic methods, could provide unparalleled resolution and sensitivity for arsenosugar localization and quantification at the cellular level [35].

6.2.6. Quantification

The characterization and quantification of arsenic and arsenosugars in edible algae are critical due to their varying concentrations across algal species and their potential health implications. The present section discusses the quantitative distribution of total arsenic and arsenosugars in *Ochrophyta* (brown algae), *Rhodophyta* (red algae), and *Chlorophyta* (green algae), along with interspecies differences and similarities. Specific arsenosugar species predominant in each group are highlighted, and the factors influencing arsenic accumulation are discussed based on comparative analysis of recent studies. In Table 3, several quantification results of different algae are shown.

Table 3. Overview of results of several edible seaweed arsenic speciation.

Ref.	Algae group (*Scientific name*)	Sample	As species [µg/g]								
			As(III)	As(V)	MMA	DMA	Gly-Sug	PO_4-Sug	SO_3-Sug	SO_4-Sug	tAs
[3]	Brown (*Eisenia bicyclis*)	Arame	–	7.0 ± 0.1	–	0.48 ± 0.04	1.5 ± 0.1	2.04 ± 0.01	–	–	20 ± 2
	Brown (*Fucus vesiculosus*)	*Fucus*	–	11 ± 1	–	0.55 ± 0.07	2.6 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	7.2 ± 0.1	36 ± 2

Ref.	Algae group (*Scientific name*)	Sample	As species [µg/g]								
			As(III)	As(V)	MMA	DMA	Gly-Sug	PO_4-Sug	SO_3-Sug	SO_4-Sug	tAs
[3]	Brown (*Himanthalia elongata*)	Sea spaghetti	–	2.0 ± 0.1	–	–	4.5 ± 0.4	0.11 ± 0.01	4.2 ± 0.9	–	18 ± 2
	Brown (*Sargassum fusiforme*)	Hijiki	–	50.3 ± 0.4	–	0.44 ± 0.06	1.05 ± 0.03	0.4 ± 0.1	0.7 ± 0.1	2.7 ± 0.4	72 ± 1
	Brown (*Saccharina japonica*)	Kombu	–	24 ± 1	–	0.40 ± 0.04	3.1 ± 0.2	4.04 ± 0.04	–	–	39 ± 2
	Brown (*Laminaria digitata*)	Oarweed	–	77 ± 3	–	–	10.2 ± 0.7	3.5 ± 0.1	–	–	92 ± 2
	Brown (*Undaria pinnatifida*)	Wakame	–	4.5 ± 0.3	–	0.025 ± 0.007	2.68 ± 0.03	10.10 ± 0.05	–	–	40 ± 2
	Red (*Pyropia yezoensis*)	Nori	–	–	–	0.064 ± 0.005	1.02 ± 0.07	13 ± 1	–	–	14 ± 2
	LOD [µg/g]		0.019	0.028	0.027	0.007	0.030	0.048	0.062	0.076	
[14]	Brown (*Lessonia nigrescens*)	*Lessonia nigrescens*	105 ± 8	18.5 ± 2.0	–	–	–	–	–	–	119 ± 1
	Brown (*Durvillaea antarctica*)	*Durvillaea antarctica*	0.304 ± 0.090	0.117 ± 0.014	–	0.103 ± 0.001	2.53 ± 0.12	0.095 ± 0.004	6.13 ± 0.11	–	15.7 ± 0.2
[19]	Green (*Diacronema lutheri*)	*Diacronema lutheri*	0.26 ± 0.07	–	–	0.20 ± 0.02	–	–	–	0.12 ± 0.01	1.2 ± 0.1
[40]	Red (*Pyropia yezoensis*)	Nori	–	–	–	–	–	72-85% of tAs	–	–	12.4−35.4
	Brown (*Saccharina japonica*)	Kombu	–	–	–	–	–	16−19% of tAs	54% of tAs	–	26.4−56.5
[42]	Brown (*Fucus vesiculosus*)	*Fucus*	4.8 ± 0.3	1.7 ± 0.1	–	1.8 ± 0.1	–	4.1 ± 0.2	35 ± 1	13.4 ± 0.8	85 ± 3
	LOQ [µg/g]							0.1	0.4	0.6	0.04
[21]	Green (*Dunaliella tertiolecta*)	Batch mode	–	0.28 ± 0.09	–	0.04 ± 0.02	0.05 ± 0.02	0.03 ± 0.01	–	–	
	Green (*Dunaliella tertiolecta*)	Continuous mode	–	0.54 ± 0.17	–	0.10 ± 0.02	0.24 ± 0.05	0.04 ± 0.02	–	–	
	LOQ [µg/g]										0.3
[43]	Green (*Ulva* sp.)	Sea lettuce	–	0.031 ± 0.004	–	0.049 ± 0.004	0.132 ± 0.006	0.142 ± 0.017	0.018 ± 0.008	–	0.940 ± 0.205
	Red (*Porphyra umbilicalis*)	Purple laver	–	0.018 ± 0.013	–	0.381 ± 0.017	5.895 ± 0.258	16.542 ± 0.478	0.053 ± 0.020	–	47.557 ± 2.332
	Brown (*Ascophyllum nodosum*)	Knotted wrack	–	1.760 ± 0.164	–	0.621 ± 0.075	1.923 ± 0.197	3.253 ± 0.173	20.065 ± 1.599	2.168 ± 0.234	77.956 ± 3.794

Ref.	Algae group (*Scientific name*)	Sample	As species [µg/g]								
			As(III)	As(V)	MMA	DMA	Gly-Sug	PO_4-Sug	SO_3-Sug	SO_4-Sug	tAs
[43]	Red (*Chondrus crispus*)	Irish moss	–	0.160 ± 0.052	0.028 ± 0.005	0.494 ± 0.048	2.529 ± 0.283	0.158 ± 0.022	0.244 ± 0.042	0.270 ± 0.073	5.445 ± 0.854
	Red (*Palmaria palmata*)	Dulse	–	0.035 ± 0.020	0.022 ± 0.011	0.781 ± 0.168	1.952 ± 0.094	7.270 ± 0.957	0.077 ± 0.003	–	11.692 ± 0.930
[34]	Brown (*Fucus vesiculosus*)	In pellets	1.50 ± 0.04	0.49 ± 0.03	–	0.57 ± 0.02	0.31 ± 0.04	0.57 ± 0.03	16.90 ± 0.22	4.22 ± 0.33	51.77 ± 1.68
	Brown (*Fucus vesiculosus*)	Dried at low T	0.74 ± 0.15	–	–	0.53 ± 0.06	4.64 ± 0.34	0.63 ± 0.03	4.78 ± 0.20	9.15 ± 0.70	45.99 ± 0.81
	LOD [µg/g]		0.005	0.017	0.009	0.007	0.008	0.015	0.061	0.089	
	LOQ [µg/g]		0.016	0.058	0.031	0.025	0.028	0.05	0.205	0.297	
[37]	Brown (*Ascophyllum nodosum*)	Knotted wrack	–	1.07 ± 0.03	–	6.8 ± 0.2	6.4 ± 0.2	1.7 ± 0.1	6 ± 1	8 ± 1	31 ± 1
	Brown (*Ascophyllum nodosum*)	Knotted wrack	–	–	–	7 ± 1	–	–	7 ± 1	11 ± 1	33 ± 2

6.2.6.1. Total arsenic distribution

Brown algae generally exhibit the highest total arsenic concentrations compared to red and green algae. Reported total arsenic levels in brown algae range widely, from 15.3 to 150 mg/kg dry weight (dw) [3, 14, 40, 42, 31, 43, 34, 37]. For instance, *Sargassum henslowianum* demonstrates the highest recorded total arsenic concentration at 150 mg/kg dw, whereas *Padina australis* has a lower concentration of 15.3 mg/kg dw [31].

Studies consistently report that Hijiki (*Sargassum fusiforme*) accumulates substantial arsenic levels. *Laminaria* species, such as *Laminaria digitata*, exhibit tAs concentrations exceeding 100 mg/kg dw [35]. In some reports, this phenomenon is attributed to the structural properties of brown algae, particularly their polysaccharide-rich cell walls where alginate is abundant [43]. Polysaccharides like alginate have been reported to absorb metals and metalloids in marine environments. Alginates are known to accumulate mono and divalent cations like Na^+, K^+, Ca^{2+}, Zn^{2+}, and Cu^{2+}. However, inorganic arsenic, whether neutral ($As(OH)_3$) or anionic ($HAsO_4^{2-}$), does not bind to alginates in the same manner. Currently, it is unclear whether As(III) or As(V) binds to alginates or fucoidans, both abundant in the cell walls of brown seaweed. Fucoidans bind sulfates as sulfuric acid esters [35], but it is uncertain whether arsenic could bind as arsenate to carbohydrates in a similar way. Such arsenic acid esters would likely be very unstable, rapidly hydrolyzing back into iAs. Although taxonomy serves as a useful indicator, it cannot be considered an absolute rule, as some brown seaweeds, for instance, have been found to contain lower tAs levels than certain red algae. In addition to structural influences, environmental factors such as water salinity, pH, and temperature play roles in arsenic uptake and retention.

Regarding speciation, in the fronds of *Laminaria digitata*, the majority of the 117 mg/kg of arsenic is present as iAs (53%) and AsSug (32%), with only 1.5% of the total arsenic occurring as AsLip [35]. Arsenosugars represent a substantial fraction of tAs in brown algae. For example, in Kombu they constitute 67-84% of tAs [43].

Red algae exhibit intermediate tAs concentrations, ranging between 2.2 and 48 mg/kg dw [3, 40, 31, 43]. *Neopyropia* species, such as *N. yezoensis* and *N. haitanensis*, which are widely used in food products like Nori, have tAs levels of 39 mg/kg dw and 37 mg/kg dw respectively [31] but the maximum levels of arsenic in red algae has been found in Purple laver (*Porphyra umbilicalis*) [43]. Despite their lower tAs levels relative to brown algae, red algae can exhibit elevated arsenosugar percentages, such as 33-40% in Irish moss (*Chondrus crispus*), 45-64% in dulse (*Palmaria palmata*) and 66−79% in some laver types [43]. Green algae generally show the lowest tAs accumulation, with concentrations between 0.32 and 28.4 mg/kg dw [19, 21, 31, 43]. *Ulva* spp., a prominent green alga genus, demonstrates tAs concentrations

ranging from 1.5 to 6.3 mg/kg dw [31], consistent across samples collected from various geographic locations, including Greece, Spain, and the United States. This indicates a geographical variability that must be considered when analysing all the results quantitatively.

The relatively low arsenic content in green algae is attributed to their physiological and structural characteristics [43], which limit arsenic bioaccumulation. Despite their lower arsenic accumulation, green algae such as *Codium fragile* can exhibit relatively higher concentrations of 28.3 mg/kg dw [31], comparable to some red algae. *C. fragile* is known as an invasive species in marine ecosystems and is also used as feed for abalone. Consequently, its relatively high arsenic content could contribute to increased arsenic accumulation within the marine food web.

These algae tend to exhibit lower iAs concentrations, often below 0.35 mg/kg dw [31], which may contribute to their reduced arsenic toxicity. Nevertheless, arsenosugar accumulation in green algae indicates a comparable metabolic pathway for arsenic detoxification as seen in other algae groups.

6.2.6.2. Arsenic speciation

Arsenic speciation and composition differ across various algae groups. For example, regarding red and green algae, the inorganic arsenic content in most edible seaweeds studied is less than 1 mg/kg dw. Its concentration in *Gracilaria* spp., *Meristotheca*, and *Eucheuma* (all red algae) is below 0.1 mg/kg dw. In contrast, slightly higher iAs levels are observed in *Neopyropia* (red), *Ulva* (green), *Gelidium* (red), and *Codium* (green), with concentrations ranging from 0.16 to 0.35 mg/kg dw [31]. Significant variability in iAs levels has been observed among brown algae, with a broad range of concentrations reported in species such as Arame, Fucus, Laminaria digitata, and Hijiki, where values have been found to span from 7 to 77 mg/kg [3, 33], reflecting considerable differences in their arsenic accumulation capacity.

Gly-Sug, SO_3-Sug, SO_4-Sug and PO_4-Sug are the predominant arsenosugars identified in seaweeds, although Gly-Sug has not been detected in Cyanobacteria. While SO_3-Sug and SO_4-Sug are well-known arsenosugars, they are not commonly found in edible seaweeds except for some brown algae such as *Fucus* [42, 34] and Knotted Wrack [43, 37]. Red algae exhibit higher PO_4-Sug proportions, as observed in *Palmaria palmata* (62% tAs) [43] and *Pyropia yezoensis* (up to 84% tAs) [40] although species like *Gracilaria* spp. and *Meristotheca* spp. show low extraction efficiencies for certain arsenosugars, resulting in undetected PO_4-Sug species [31]. Green algae typically accumulate Gly-Sug and PO_4-Sug, although in lower quantities compared to brown and red algae. *Diacronema lutheri* and *Ulva* spp. are examples of green algae in which arsenosugar concentrations range between 10-31% of tAs [19, 43]. Arsenic and arsenosugar concentrations within different algae groups are shown in Table 4.

Table 4. Arsenic and arsenosugar concentrations in 3 different groups of edible seaweed.

Algae group	Total arsenic range [mg/kg]	Inorganic arsenic range [mg/kg]	Arsenosugar fraction of tAs (%)	Predominant arsenosugars
Brown	15.3-150	7-77	32-84	Gly-Sug, SO_3-Sug, SO_4-Sug and PO_4-Sug
Red	2.2-48	< 1	33-84	Gly-Sug and especially PO_4-Sug
Green	0.32-28.4		10-31	Gly-Sug and PO_4-Sug

Although SO_3-Sug is not abundant, some minor quantities have been reported in algae with high agar content [31], such as *Gracilaria*, *G. furcata*, *Eucheuma*, and *G. divaricatum*. Arsenobetaine and arsenocholine are commonly present in marine animals and microorganisms, typically appearing at very low concentrations in algae, primarily influenced by the periphyton biofilm. Notably, arsenobetaine has been identified in certain species of *Sargassum fusiforme* and *Saccharina japonica*, as well as in the CRM *Fucus vesiculosus* (ERM CD200) [31], likely due to its trace amounts from periphyton organisms. However, it is important to highlight that the methodology used for arsenobetaine determination may lack selectivity. This compound is not retained on the chromatographic column and elutes close to the void volume, meaning this peak could also stem from unknown arsenic species not retained by the column. Therefore, the results should be interpreted with caution.

6.2.6.3. Certified reference materials

CRM provide essential benchmarks for validating arsenic quantification methods. Brown algae CRM, such as NIES No. 9 (107 mg/kg tAs) [3] and ERM CD200 (53 mg/kg tAs) [31], show higher tAs and arsenosugar diversity compared to green algae CRM like BCR 279 (1.4

mg/kg tAs) [37]. The speciation profiles of CRM mirror natural samples. For instance, SO3-Sug predominates in brown algae CRM (16.5 mg/kg in NIST 3232) [43], while this arsenosugar does not appear in red algae CRM like GBW 10023 [43]. These patterns corroborate observations in natural algae, underscoring the reliability of CRM in method calibration. In Table 5, several CRM are shown alongside the concentration of each of their arsenic species.

Table 5. Overview of results of several CRM arsenic speciation studies related to edible seaweed.

Ref.	Algae group	CRM code	Certified value [µg/g]								
			As(III)	As(V)	MMA	DMA	Gly-Sug	PO_4-Sug	SO_3-Sug	SO_4-Sug	tAs
[3]	Brown	NIES No. 9	–	70 ± 1	–	0.9 ± 0.1	1.0 ± 0.2	1.4 ± 0.2	–	7 ± 2	107 ± 2
[22]	Brown	CRM 7405-b	–	23.2 ± 1.6	–	–	0.70 ± 0.07	0.26 ± 0.07	0.18 ± 0.03	1.75 ± 0.06	45.2 ± 2.5
[31]	Brown	ERM CD200	0.14 ± 0.01 for total iAs		–	7.6 ± 0.2	0.28 ± 0.02	16.8 ± 0.3	–	5.6	53.1 ± 0.3
[31]	Red	GBW 10023	0.29 ± 0.04 for total iAs		–	0.69 ± 0.02	29.9 ± 0.2	0.26 ± 0.01	–	–	31.7 ± 0.4
[43]	Brown	NIST 3232	–	0.08 ± 0.02	–	1.097 ± 0.093	0.839 ± 0.015	4.895 ± 0.387	16.527 ± 3.668	0.056 ± 0.023	
[37]	Green	BCR 279	–	0.60 ± 0.02	–	0.11 ± 0.01	0.16 ± 0.01	0.26 ± 0.01	–	–	1.4 ± 0.2

6.3. ANALYTICAL QUALITY CONTROL

Analytical quality control and quality assurance are pivotal in arsenosugar analysis due to the complexity of these compounds and the matrices in which they are found. Critical parameters like the limit of detection (LOD) and the limit of quantification (LOQ) directly influence the reliability and sensitivity of the techniques employed. This section evaluates various techniques for arsenosugar analysis, focusing on their LOD, LOQ, associated strengths and weaknesses, and the role of CRM in ensuring data accuracy.

Table 3 highlights the LOD and LOQ of different arsenosugar detection methods. Techniques employing HPLC coupled with ICP-MS generally exhibit superior sensitivity, achieving LOD as low as 0.005 µg/g and LOQ of 0.016 µg/g for iAs [34]. Conversely, methods incorporating HPLC coupled with HG-AFS show slightly higher LOD, with values like 0.019 µg/g [3]. For specific arsenosugar species, such as Gly-Sug, methods achieve a range of LOD, from 0.008 µg/g using anion-exchange HPLC with ICP-MS [34] to approximately 0.030 µg/g using HPLC with HG-AFS [3]. The LOQ values follow a similar trend, indicating a direct relationship with the technique's detection limits.

To ensure the reliability and accuracy of analytical data, it is essential to implement additional quality assurance and quality control measures. These include evaluating mass balance, extraction efficiency, and column recovery, as each provides critical insights into potential errors and procedural effectiveness [41]. Mass balance serves as a diagnostic tool to detect potential losses during analytical procedures, such as extraction or separation steps. Highlighting discrepancies helps pinpoint problematic stages in the workflow and facilitates corrective actions to improve overall accuracy. The efficiency of the extraction process is another pivotal factor in achieving reliable results.

Advanced techniques like MAE often yield higher recovery rates when compared to conventional methods such as shaking or UAE, making them preferable for challenging matrices. Column recovery is crucial for validating the alignment between the detected arsenosugar species and total arsenic concentrations. Inconsistencies in recoveries can signal issues with elution or retention within the chromatographic column. Monitoring and optimizing column performance is essential to maintaining the integrity of speciation analysis.

CRM play a crucial role in validating analytical methods for arsenosugar analysis. While most CRM certify tAs content, only a few provide data on individual arsenosugar species [41] (several examples in Table 5). This limitation restricts comprehensive method validation. The scarcity of CRM for arsenosugars is further compounded by challenges in synthesizing standards. Reported yields for

arsenosugar synthesis are low, making these standards expensive and difficult to obtain [34]. However, the availability of informative CRM, such as kelp dietary supplements certified for specific arsenosugars, offers alternative resources for quality control and quality assurance.

7. CONCLUSIONS

This review delves deeply into the analytical methodologies for arsenosugar characterization in edible algae, emphasizing the critical role of advanced techniques across all stages of the analytical process (processing, extraction, clean-up, separation, and detection). Each stage addresses specific challenges posed by the structural complexity of algae and the intricate nature of arsenosugars.

Processing methods form the foundation of reliable analytical workflows. Techniques like freeze-drying significantly enhance arsenosugar extraction efficiency by disrupting cell walls, particularly in algae with dense, fibrous structures like brown algae. Grinding is essential for achieving sample homogenization, ensuring reproducibility across analytical replicates. These preparatory steps are crucial for releasing both cell-wall-bound and intracellular arsenosugars.

Extraction is another critical stage, with methods such as microwave-assisted extraction and ultrasound-assisted extraction demonstrating high recovery rates for arsenosugars. MeOH-H_2O mixtures and diluted HNO_3 are the preferred solvents due to their ability to solubilize polar arsenosugars while preserving their native forms. The efficiency of these techniques is augmented using controlled conditions, such as temperature and duration, which are optimized to prevent arsenic species transformation.

Clean-up processes address the complexities of algal matrices, which contain polysaccharides, salts, and other organic compounds that interfere with detection. Filtration, using syringe or ash-free filters, is a straightforward approach for removing particulates but lacks the specificity needed for isolating arsenosugars. Solid-phase extraction, particularly with silica-based anion exchange cartridges, offers a more refined solution, enabling the targeted isolation of arsenosugars and minimizing co-elution with other arsenic species. Lyophilization following SPE further concentrates the analytes, enhancing detection sensitivity.

Separation and detection techniques are the cornerstone of arsenosugar analysis. HPLC-ICP-MS emerges as the gold standard for arsenosugar speciation, offering unparalleled precision and versatility. Advances in mobile phase composition, such as the incorporation of MeOH for thio-Sug, have enhanced separation efficiency. ICP-MS, despite its susceptibility to matrix interferences, remains the most sensitive detection method, particularly when paired with robust chromatographic separation. Complementary methods such as HG-AFS provide reliable detection for specific arsenic species at trace levels. INAA, which avoids the need for calibration standards, also shows potential for quantifying arsenosugars, particularly in challenging matrices.

The review also highlights the pressing need for analytical quality control measures. The absence of CRM and pure arsenosugar standards hampers standardization, undermining inter-laboratory reproducibility. Analytical quality control frameworks, including method validation and the development of CRM, are essential for ensuring the reliability of arsenosugar analysis.

From a toxicological standpoint, arsenosugars are considered less harmful than inorganic arsenic. However, their chronic effects remain uncertain, with studies indicating potential risks under specific conditions, such as high-dose consumption or structural alterations during digestion. Certain algae species, such as *Hizikia fusiformis*, pose significant health risks due to their high iAs content, often exceeding safety thresholds. Regulatory bodies in countries like France, Australia, and New Zealand have implemented limits for iAs in seaweed products, but arsenosugar-specific regulations are largely absent. This regulatory gap highlights the need for more comprehensive toxicity assessments and legislative frameworks to address arsenosugar exposure.

In summary, the integration of advanced techniques at each analytical stage has significantly improved arsenosugar characterization. However, persistent challenges, including matrix effects, limited CRM, and regulatory gaps, underscore the need for ongoing methodological refinements. Addressing these challenges will not only advance the field of arsenosugar research but also contribute to safeguarding public health by supporting the development of harmonized standards for arsenic in edible algae.

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9. ACRONYMS

As: Arsenic

AsLip: Arsenolipids

AsSug: Arsenosugar

BW: Body weight

CRM: Certified reference materials

CZE: Capillary zone electrophoresis

DC: Dichloromethane

Di: Daily algae intake

DMA: Dimethylarsinate

dw: Dry weight

EDI: Estimated daily intake

EFSA: European Food Safety Authority

ESI-MS: Electrospray ionization mass spectrometry

FAO: Food and Agriculture Organization of the United Nations

GC: Gas chromatography

Gly-Sug: Glycerol arsenosugars

HG-AAS: Hydride generation coupled to atomic absorption spectrometry

HG-AFS: Hydride generation coupled to atomic fluorescence spectrometry

HPLC: High-performance liquid chromatography

IC: Ion chromatography

ICP-AES: Inductively coupled plasma atomic emission spectroscopy

ICP-MS: Inductively coupled plasma mass spectrometry

iAs: Inorganic arsenic

INAA: Instrumental neutron activation analysis

LC: Liquid chromatography

LOD: Limit of detection

LOQ: Limit of quantification

MAE: Microwave-assisted extraction

meth-Sug: Methoxy arsenosugar

MMA: Monomethylarsonate

NanoSIMS: Nanoscale secondary ion mass spectrometry

oAs: Organic arsenic

PFP: Pentafluorophenyl

PO4-Sug: Phosphate arsenosugar

PP: Polypropylene

PRP: Polymeric reverse phase

Q-TOF: Quadrupole time-of-flight

SAC: Spindle assembly checkpoint

SCX: Strong cation exchange

SO3-Sug: Sulfonate arsenosugar

SO4-Sug: Sulfate arsenosugar

SPE: Solid-phase extraction

tAs: Total arsenic

TFM: Tetrafluoromethoxyl

thio-Sug: Thio-arsenosugars

UAE: Ultrasound-assisted extraction

UPLC: Ultra-performance liquid chromatography

WHO: World Health Organization

XANES: X-ray absorption near edge structure