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Review

# Sample preparation for arsenic speciation in terrestrial plants—A review

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ABSTRACT

Arsenic is an element widely present in nature. Additionally, it may be found as different species in several matrices and therefore it is one of the target elements in chemical speciation. Although the number of studies in terrestrial plants is low, compared to matrices such as fish or urine, this number is raising due to the fact that this type of matrix are closely related to the human food chain. In speciation analysis, sample preparation is a critical step and several extraction procedures present drawbacks. In this review, papers dealing with extraction procedures, analytical methods, and studies of species conservation in plants cultivated in terrestrial environment are critically discussed. Analytical procedures based on extractions using water or diluted acid solutions associated with HPLC–ICP–MS are good alternatives, owing to their versatility and sensitivity, even though less expensive strategies are shown as feasible choices.



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*Abbreviations:* AAS, atomic absorption spectrometry; AFS, atomic florescence spectrometry; As, arsenic; AsB, arsenobetaine; AsC, arsenocholine; CRM, certified reference material; DMA, dimethylarsinic acid; ESI–MS, electrospray ionization mass spectrometry; ESI-MS/MS, electrospray ionization tandem mass spectrometry; ESI–TOF–MS, electrospray time-of-flight mass spectrometry; ETAAS, electrothermal atomic absorption spectrometry; FAAS, flame atomic absorption spectrometry; GFAAS, graphite furnace atomic absorption spectrometry; HG, hydride generation; HPLC, high-performance liquid chromatography; HR, high resolution; IC, ion chromatography; ICP–MS, inductively coupled plasma mass spectrometry; ICP–OES, inductively coupled plasma optical emission spectrometry; LA, laser ablation; LC, liquid chromatography; LOD, limit of detection; MAE, microwave-assisted extraction; MMA, monomethylarsonic acid; MPE, modified protein extraction; *o*-APAA, *o*-aminophenylarsonic acid; *p*-APAA, *p*-aminophenylarsonic acid; PAA, phenylarsonic acid; PAO, phenylarsine oxide; PCs, phytochelatins; PCn, (γ-Glu-Cys)*n*-Gly ($n=2$–11); PLE, pressurized liquid extraction; RT, room temperature; SON, sonication solvent extraction; SEC, size exclusion chromatography; TFA, trifluoroacetic acid; TMA, tetramethylarsonium ion; TMAO, trimethylarsine oxide; UV, ultraviolet; XANES, X-ray absorption near edge structure spectroscopy; XRF, X-ray fluorescence.

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## 1. Introduction

Arsenic is an abundant element in Earth's crust, mainly as arsenopyrite mineral (FeAsS), its most important ore, and the natural occurrence of As is associated with volcanic deposit and geochemical environment [1–3]. On the other hand, anthropogenic sources of contamination are mostly due to mining activity, burning coal, copper smelting and the use of fertilizers and herbicides [4–7]. The most common forms of As in nature are arsenite ($As_2O_3$) and arsenate ($H_2AsO_4^-$ and $HAsSO_4$), i.e. As(III) and As(V), respectively, which lead to the presence of these species in plant tissues [8]. Consequently, the determination of As in terrestrial plants plays an important role because As occurs in some areas in high concentrations, either naturally or as a consequence of human activities.

Some plants have potential for phytoremediation, which is the property of absorbing elements present in soil. It can be easily seen as an advantage if they are essential elements or even if they are used in metals phytoremediation experiments [9,10]. However, if the absorption takes place in an excessive or accidental condition, the situation can easily turns into a dangerous health issue, if the contaminated plant is ingested by a human or inserted in the food chain. Thus, rice is the most intensively studied plant organism in As speciation, because of its high consumption by humans and also its great ability to accumulate arsenic in higher amounts than other grains. Additionally, the soil used for rice cultivation can also increase the concentration of As [11]. Although it is known that As is mainly found in plant tissues in its inorganic form, therefore toxic form, further researches are necessary to evaluate the response of the matrix for different extraction procedures.

Several extraction procedures are usually employed for As speciation in plants with particularities in solvent extractors, extraction devices, and instrumentation used for analysis [12–14]. Arsenic speciation studies become more attractive if they involve the mechanism of translocation of the analyte up to plant parts, since this is an aspect that adds important information[15]. The studies of As bound to phytochelatins (As–S) are mostly made on plants roots, once such binding occurs mainly in this part of the plant. The formation of this tripeptide is a defense mechanism of the plant organism, in order to avoid poisoning and translocation of the metal to the aerial parts of the plant [16,17]. Another crucial point in speciation analysis, is the conservation of species in order to ensure that the analytes obtained after extraction are as representative as possible of species present in the sample.

Although many studies on As speciation have been devoted to marine tissue samples, there is an increasing number of works evaluating the several species of this element present in earth-grown plants [18–22]. In this paper, a review on sample preparation for As speciation in terrestrial plants is presented by discussing previous studies that dealt with sample pretreatment, extraction procedure of analyte species, as well as separation and determination methods. Several procedures are employed for this sort of samples without establishing a standard procedure or protocol; therefore it is important to systematize this information and to look for general and simple analytical procedures. Furthermore, some adopted strategies have failures which often compromise the quality of the analysis; moreover, the most suitable procedure can be tailored to the type of sample.

## 2. Uptake and metabolism of arsenic in plant tissues

The main species monitored in studies of As speciation in plant are As(III), As(V), DMA and MMA [22,23], although there are studies that monitor the organic species of TMA, TMAO, AsB, AsC, and phenylarsenic compounds [24–27]. The major species present in plants are As(III) and As(V). However, in some sorts of plants or habitats traces of MMA and DMA can be found [28]. It is not well understood if all types of plants are able to methylate inorganic arsenic species, or even whether these organic species are taken up from soil by plants, already in the methylated form [29,30].

The plant capacity to absorb the elements present in the soil, for instance As, and the transport to the aerial parts like leaves and fruits depends on the properties of the soil, such as organic matter content, pH, ion competition, redox potential [31,32], microbiological activity [27], As species [33], presence of iron oxides [34], and also the sort of plant will affect the transport and accumulation of As [35].

In studies of As speciation in plant tissues, possibly the greatest goal is to understand how As is metabolized in plant cells, and not only to determine the concentration of each species. For complex matrices, many doubts are not well clarified, which show that despite previous works, new studies are always relevant to elucidate processes related to As metabolism. Several As species are found in plant samples, the presence of more than 14 species of complexed arsenic in sunflower samples were reported by Raab et al. [36].

According to Bergqvist and Greger [22], As accumulation in plants is determined by the habitat whereupon the plant was grown; plants cultivated in an environment that contains more water have higher accumulation power than those cultivated in dry soil. Algae, for instance, accumulates more As than terrestrial plants [37]. The uptake of As from soil to plant is associated with the sort of plant and how the element is available in soil [38]. Accumulation in terrestrial plants is very dependent on the As concentration in soil. Paradoxically, a study had shown that an increase in As concentration in soil caused a reduction of its accumulation in plant tissues [22].

Lomax et al. [39] have studied three plants (rice, tomato and red clover) aiming to investigate whether plants are able to synthesize methylated As species or if they just taken up these species produced by microorganisms [40]. When plants were cultivated in a habitat with MMA and DMA, they hold methylated species in their tissues. When exposed to an environment without methylated species, the three plants analyzed showed no MMA and DMA in their tissues and arsenite was the predominant species, although the plants were exposed to arsenate. It also became clear that the evaluated plants did not have the ability to transform methylated species in arsenite and that the methylation of As in soil does not depend on the plant presence.

On the other hand, Raab et al. [41] and Xu et al. [42] found traces of MMA and/or DMA in plants that were grown in a hydroponic solution without methylated arsenic species. It was shown the ability of some plants to perform the detox mechanism of methylation. These studies emphasize that the ability of As methylation can be associated to each particular plant organism. The plant tissue that is absorbing As will always play a role in the mechanism of translocation of As and interactions between element and matrix. Some seaweeds e.g. *Fucus spiralis* and *Hizikia fusiforme*, were not able to form complexes As–PC, unlike what happens in most terrestrial plants, although these seaweeds contain As(III) and As(V) in their tissues, which are known as strong inducers of phytochelatins formation [43].

Raab et al. [44] presented the first work detecting and quantifying complexes of As bound to phytochelatins in plant tissues, without changes of original species and involving HPLC–(ICP–MS)–(ESI–MS) instrumentation. In this procedure, organic and metal specific detector was used after extraction with 1% $vv^{-1}$ formic acid. It is emphasized that the analysis should be performed within a short period of time to achieve complex preservation.

Bluemlein et al. [45] reported that the binding of As–S in phytochelatin actually occur in some plants, such as *Thunbergia alata*, and that this binding is not formed during the extraction process. The authors also observed that even though the extraction procedure used was not the mildest possible, the As–S bindings were preserved.

The plant tissues have high ability to reduce As(V) to As(III) and this reduction is probably caused by agents such as ascorbic acid, glutathione, precursor of phytochelatins, or even the As (V) reductase [36]. Therefore, according to Xu et al., As(III) is the major species presents in roots of rice and tomato [42]. The presence of As(III) and As(V) in plants exposed to arsenate, indicates that inorganic species are more easily translocated than the As–PC complex. Raab et al. [36] described the formation of a MMA complex connected to phytochelatin 2 (PC2) in sunflower, indicating a new detoxification mechanisms in plant tissues that deserve more detailed studies.

Increased translocation of inorganic As from roots to leaves is an indicative that detoxification mechanism of phytochelatins formation is not being efficient, since phytochelatins are formed and tend to be retained in the roots. Therefore, the transport of inorganic As to leaves is favored when it is not bound to thiol groups (–S) of phytochelatins [46].

Small concentration of As in plant tissues was observed when DMA was added to the nutrient solution, however, the translocation coefficient from roots to leaves for this cultivation was larger than those containing As(III), As(V) and MMA in the nutrient solution. In fact, phytochelatins were not found in the leaves, despite the As form added to nutrient solution. In the stems tissues, PC3 and PC4 were identified only for plants grown with arsenites. These same complexes were found in the roots, but only for plants grown in nutrient solution containing both arsenites and arsenates. It must be emphasized that the synthesis of phytochelatins was not observed in plants grown in the presence of MMA and DMA. The speciation analysis also revealed that arsenates were stored in the roots and the plants did not transport As(V) to stems and leaves[47].

Several studies have assessed the As translocation in rice plants, for different reasons, such as irrigation with water containing high doses of this element and the rice ability to tolerate As in its tissue. A trend is observed in the As distribution in the following parts of the rice plant: roots, stem, leaf and grain. The translocation of As in decreasing order of accumulation is: grain < stem≪broot [48–50]. Smith et al. [48] also highlighted that low concentrations of As in leaves often occurs due to its retention in plant roots and that phosphate may play an important role in this mechanism due to the competition on the transport of both. However, one question related to the tolerance of rice plants to high doses of As, since one cannot visually notice the toxicity caused by this element, remains not explained.

## 3. Sample preparation

In speciation procedures, sample preparation is one of the most critical step, where problems may occur, such as losses during sampling, contamination, interconversion between species, or even inefficient extraction of the analyte [51]. There are several options of solvents used for As speciation analysis in plant tissues, as well as mixtures of them. Among these, the most important ones are methanol, water, nitric acid, formic acid, enzyme solution, phosphate buffer and trisbuffer [23,52–56]. Similarly, extraction procedures are also numerous. Extraction may be accomplished by mechanical agitation and heating [23], ultrasonically assisted [57], pressurized liquid extraction [24] or assisted by microwave radiation [58], and even a combination of these procedures [59,60].

Regarding to sample pretreatment, the analyte can be extracted from fresh plants, lyophilized, frozen or even lyophilized and ground. Jedynak et al. [20], compared different pretreatments for mustard plants, by assessing the following conditions with or without using liquid nitrogen: fresh plant, frozen and dry. The extraction procedure was carried out using an ultrasonic bath and water as extractant. Taking into account the extraction efficiency and reproducibility of results, best results were obtained when the sample was dried and homogenized before extraction and without using liquid nitrogen. Alava et al. [61] emphasized that particle size plays a crucial role in the extraction efficiency of As. As expected, higher extraction efficiencies were achieved by decreasing particle sizes (finely ground to powder) for all types of rice tested when using water as the extraction solvent.

Bluemlein et al. [55] recommended fresh plant analysis for speciation studies of As-phytochelatin complex because the simple and common lyophilization process promoted the disintegration of As–PC complex. When fresh plant analysis is not possible, only freezing (−80 °C) should precede the analysis in order to achieve species preservation. There are still authors who chose to dry and grind plant samples without using liquid nitrogen and then store them at −20 °C for up to one year [57].

Schmidt et al. [24] reported that the extracted amount of DMA and arsenate did not depend whether the plant is fresh or ground, although the content of arsenite increased with milling. As expected, higher standard deviations were obtained without grinding. These researchers emphasized how difficult it is to validate procedures where the sample homogeneity is not reliable. Zhang et al. [62] did not have evidence of As–PC complex presence in the extracts when the plants were freeze dried and ground, thus they chose to use the fresh plant and to employ milder extraction conditions, once they have noticed the occurrence of an additional peak in the chromatogram. It is evident that irrespective of the treatment that is given to plant prior to extraction procedures, species must be preserved in their original form, and analyses must provide good reproducibility and repeatability.

Although solvents such as methanol and acetonitrile among organic solvents, are the most used in As compounds extraction, diluted acid solutions have been used as extractant of chemical species present in plant samples with different particularities. Acids solvents are recommended for extraction of As(III) connected to phytochelatins in plant tissues, based on the fact that the stability of the complex increases when decreasing pH. A good solvent with these characteristics is 1% $vv^{-1}$ formic acid, pH 2.2 and extraction at low temperature in order to determine the As–PC complex [55].

Pure acids or a mixture of them, concentrated or diluted, have been chosen as extracting solution [63]. Huang et al. [54] evaluated several extraction procedures, and the best performance was reached with 0.28 mol $L^{-1}$ $HNO_3$ at 95 °C for 90 min. The greater difficulty reported was the preservation of As(III) and As (V) during extraction, which was achieved only in a narrow range of $HNO_3$ concentrations. Due to the complexity of the matrix studied, the extraction procedure employed led to severe reductions of As(V) and oxidation of As(III) when working in the range of concentration below 0.28 mol $L^{-1}$ or higher than 0.70 mol $L^{-1}$, which reveals the extreme susceptibility of transformation between As(III) and As(V) during extraction. On the other hand, full recoveries of MMA and DMA were achieved for all evaluated concentrations of $HNO_3$. The predominance of As(III) in rice grains reinforcing the property of this grain to bioaccumulate the most toxic form of As may be inferred from these data. Furthermore, the need for further studies about As speciation in grains and plants is emphasized.

Schmidt et al. [24] studied As speciation in *Holcus lanatus* plants grown in contaminated soils. The authors employed a pressurized

liquid extraction (PLE) using water and water/methanol mixture as extractors. Eight As species were determined, as follows: As(III), As (V), MMA, DMA, TMA, TMAO, AsB e AsC. Additionally, to investigate the time of extraction, the number of extraction steps, and temperature, the authors also evaluated the stability of organic and inorganic species during extraction at temperatures ranging from 60 to 180 °C. They found high influence of sample pretreatment and good results were obtained for fresh samples, despite the low reproducibility for CRMs. The authors noticed that high temperatures had little influence on the stability of As species. They also observed the need for further studies to explain the different behaviors of arsenate and arsenite in subsequent extraction steps.

The great challenge of As speciation, as it has been highlighted, is to maintain the original characteristics of species during extraction and storage steps. Furthermore, extraction still must be quantitative, once an incomplete extraction prejudices the elucidation of As distribution in the studied matrix. Usually, the applied solvent for As extraction in marine tissues (animals and plants) is the methanol/water mixture, and it can be noticed that this approach is extensively explored for several reasons. In marine tissue, extraction with this kind of mixture proves to be very efficient in contrast to what occurs in tissues of terrestrial plants, since inorganic As species are more present in terrestrial plants [29,57].

Zheng et al. [64] observed that less than 10% of the total As in plants was extracted when methanol/water (9:1 v $v^{-1}$) mixture was used as solvent, emphasizing that new studies are required for searching extractors suitable for plants grown in terrestrial habitats. In this context, Zheng and Hintelmann [53] studied eight different extractors for extraction of organic and inorganic species. It is important to point out that methanol/water mixture is an effective extractor for organic species, even though, for inorganic species this efficiency decreases substantially. Consequently, its application to marine tissues is appropriated, once the organic forms are present in higher concentrations. This is because the inorganic arsenic in terrestrial plants is mainly bound to cell walls, lipids, lignin and insoluble cellulose, and the organic fraction, such as MMA, TMAO, DMA and others, are minor constituents in these tissues [25,64–66].

Mir et al. [57] examined the applicability of sequential extraction with water/methanol mixture followed by extraction with HCl. The basic idea of these authors was to extract organic and inorganic As species, since these extractants seem to be selective for these species, respectively. There was an increase in extraction efficiency using the sequential extraction method and a predominance of inorganic As in terrestrial plants samples was observed.

Larios et al. [58] used orthophosphoric acid for As speciation in plants grown in contaminated environment and evaluated procedures employed graphite heating block and microwave-assisted heating. These authors concluded that the best extraction conditions was reached in graphite block heated at 90 °C for 60 min, which led to an extraction efficiency of 80% for samples without species interconversion and recovery of 95% for Virginia tobacco Leaves. Arsenic(V) was the predominant specie followed by As(III) and only traces of DMA and MMA were found. The acid effectiveness on extraction procedure was mainly due to the ability of this solvent to break As–S bound, as observed when HCl was employed [65].

Five different phenylarsenic compounds, i.e. PAA, *o*-APAA, *p*-APAA, PAO, and roxarsone, were added to a model plant, *Tropaeolum majus*, for a study of As uptake, extractability, and metabolization of phenylated As compounds. The extractants used were water and phosphate buffer and parameters such as, sample pretreatment (intact and ground leaves), pH of sample solution, solvent composition, extraction temperature, and extraction time were investigated. The pH value of the extracting solution (7.7 or 9.0) as well as an elevated temperature (37 °C) did not affect the extraction yield. Significant higher extraction efficiency was achieved from ground leaves compared to intact leaves in case of buffer-assisted extractions. Altogether, the maximum extraction yield (90% for all the four species) was obtained for ground leaf material by a two-step extraction in 24 h using 0.1 mol $L^{-1}$phosphate buffer, pH 7.7 [26].

A study of different extractors (water, methanol-water, 10% $vv^{-1}$ HCl and NaOH 1 mol $L^{-1}$) showed better extraction efficiency for 1 mol $L^{-1}$ NaOH, once extractions performed with water or HCl were inefficient. However, the authors opted for an extraction using water-methanol (1:1 v $v^{-1}$) due to the easy oxidation of As (III) in basic medium. Extraction time (10, 20, 30, 40, 60 min) was also evaluated and the best efficiency was achieved after 20 min of extraction. They also emphasized the possible absorption of the analyte by the matrix after a long extraction time [67].

Sample preparation is such an important step in speciation analysis and often so specific, that depending on the plant part, the same extraction procedure can provide different responses. Smith et al. [48] compared two extraction procedures: modified protein extraction (MPE) and trifluoroacetic acid (TFA) extraction for roots, stem, leaf and grain of rice plants. The MPE extraction was inefficient (3–10%) for As extraction in roots, but was effective for other parts of the plant, however, the TFA procedure was effective in all parts of the plant. Arsenic(III) and As(V) were the main species found in rice roots, inorganic As and a small fraction of DMA were found in rice stems and leaves. Nevertheless, in rice grains the main specie found was DMA (84–94%), a result that has positive implications for human health due to the fact that DMA is a less toxic form than the inorganic ones. However, this evaluation is quite different for diverse studies, once they have estimated that As in the methylated form may be responsible for the percentage of 1–84% of total As found in the rice grains [63,68–70].

Rahman et al. [71] tested the following extractors: water, 50% $vv^{-1}$ water/methanol, $NH_4H_2PO_4$, and protein extracting solution on shaking and microwave techniques, in samples of edible portion of spinach (e.g. amaranth and silverbeet). The authors noticed that, while the $NH_4H_2PO_4$ provided extraction of approximately the same amount of As(III) and As(V), As(III) was extracted twice as much with protein extracting solution, which shows the differences between the extractors on the solubilization of As(III), possibly complexed to phytochelatins. This study shows that protein extracting solution is a good extractor for the two samples evaluated, furthermore, As forms and the amount of them vary with the type of sample.

A study aimed to improve the extraction efficiency of arsenic in lichens, in which a comparison was made between two species of lichens, one corticolous (*Hypogymnia physodes* (L.) Nyl.) and other terricolous (*Cladonia rei Schaer.*). Extraction procedures were performed involving water at temperatures of 20, 60 and 90 °C and the following solvents: mixtures of methanol/water (9:1, 1:1 and 1:9 $vv^{-1}$), tris buffer, and acetone. The methanol/water mixtures showed lower extraction efficiency than pure water for samples of lichens. Acetone was the solvent with worst performance. Since a major fraction of As appeared to be associated with trapped soil particles, a sequential extraction procedure originally designed for soils was applied. The maximum values achieved without applying sequential extraction were low. For *H. physodes* the maximum extraction yield was 19.2% using hot water as extractant and for *C. rei*, 22.4% using tris buffer. Applying sequential extraction, the authors were able to extract about 45% of the total As from *H. physodes* and 83% from *C. rei* [72].

On-line continuous leaching was evaluated in order to obtain information of bio-accessible As species in rice. Advantages as faster and easier sample preparation, reduced risk of contamination and access to real time leaching data can be obtained from

this extraction procedure. The effectiveness of the on-line continuous leaching method was compared to a batch method. For both, sample pretreatment (cooked white rice, ground uncooked white rice and non-ground uncooked white rice) and extraction procedure (on-line and batch), the order of increasing leaching was as follows: intestinal juice < gastric juice < saliva. In cooked white rice the ascending order of occurrence of As species was: MMA < As(V) < As(III) < DMA, which shows that a considerable fraction of the As present in rice is bio-accessible and in a toxic form [73].

It is clear the occurrence of an increasing number of studies devoted to sample preparation for As speciation in plants grown in the terrestrial environment, mainly because its critical effect on speciation analysis and also because there is no standard procedure for As species extraction in plant samples. These studies highlighted diluted acid solutions as a good alternative as extractor, which presents low-toxicity, provides good extraction efficiency as well as the preservation of As species. In Table 1, some representative works of As speciation in different samples of terrestrial plants are compiled. It is highlighted the different behavior of the samples considering the employed extraction procedure.

### 3.1. Arsenic species stability

During sampling and sample storage process, several factors may promote or prevent species interconversion, such as microbial activity, temperature, storage container, light, matrix pH, and extracting solution. In plant samples, the species most susceptible to interconversion – As(III) and As(V) – are also the major species.

Most papers concerning stability of As species focus in water samples. There are not many studies devoted to plant samples and the few studies found are discussed here, but aiming to complement information As stability studies in standard solutions and in water samples were also included.

Standards of As(III) and As(V) were prepared in water and stored in separate vials, complete interconversion between them was observed and transformations occurred in a period of 36 h, which shows how susceptible to changes the inorganic As species are and the problems associated with storage [86]. Le et al. [87] described that in water samples As(V) is the predominant form and As(III) and As(V) interconversion are highly dependent on the matrix. Nevertheless, stable species in some cases are reduced, which emphasizes the importance of the medium in these transformation processes. Then, the authors suggested as an alternative the separation of the species before storage. If we think about liquid samples it maybe accomplished, however, for solid samples does not seem feasible.

Segura et al. [88] reported that in stored waste water samples, MMA and DMA species remained constant for months, but As(III) was converted to As(V) even at 4 °C and the instability of As(III) is attached to the matrix. The authors examined the stability of As (III) in raw wastewater and found that around pH 7, the stability was higher than at pH close to 1. The conversion of As(III) to As (V) raised when increasing storage temperature. Studies show that DMA, MMA and As(V) are stable during storage in samples of wastewater, however As(III) appeared to be unstable. Conversion of almost 100% of As(III) to As(V) was noticed during 120 h when mixed-stored at −21 °C, however, if stored in between 10 and 20 °C the species interconversion found was of only 5%. The storage of mixed-species solutions contributed to the interconversion, when As species were stored in single solution the species stability increased [89].

According to Kumar and Riyazuddin [90] some key points can contribute to As(III) and As(V) interconversion during sampling and storage of water samples, such as oxidation of As(III) in presence of Fe(II)/Fe(III) promoted by photochemical reaction, adsorption of As species on Fe precipitates, and microbiological activities that can promote redox changes or mechanisms of methylation.

Iserte et al. [91] described interconversion between As(III) and As(V) and the oxidation process certainly occurred during the drying step of phosphoric acid employed for extraction, but interconversion were not observed among methylated species of arsenic. Abedin et al. [28] reported reduction of As(V) to As(III) when TFA (trifluoroacetic acid) was used as solvent at 100 °C; however, these authors believe that TFA is a better extractor than MeOH:$H_2O$ 1:1 v $v^{-1}$ mixture. Using methanol, they obtained a low recovery of about 10–20%, while with TFA extractor the efficiency was higher than 80%. It is not known why this interconversion happened, but Abedin et al. [28] believed to be due to low pH or due to extraction of organic compounds that changed the redox potential of the medium. Williams et al. [69] also observed conversion of arsenate to arsenite when using TFA as an extraction solvent for rice samples.

The extraction procedures for speciation are numerous as noted above, considering sample pretreatment, solvent, and extraction procedure. The sample preparation chosen should satisfy two main requirements: provide good extraction efficiency and preserve the integrity of all species. To achieve these objectives the extractant must be linked to the predominant species (organic or inorganic) in the sample matrix. Searching to adapt the procedure and make it more adjustable as possible, samples may show different responses to the extraction procedure employed, since they are complex and real samples. Alava et al. [61] highlighted a closed vessel MAE with water at 80 °C and a hold time of 30 min, as the best condition with respect to both effective extraction and As species preservation for rice samples.

In speciation analysis it is often preferable to have a less efficient extraction, although with species conservation than to seek for the maximum extraction condition. This sort of choice is quite common in studies that assess the binding of metal to protein, since non-conservation (i.e. denaturation) of this type of structure strongly compromises the procedure of speciation. Schmidt et al. [92] evaluated the binding capacity of arsenic species (DMA, MMA, As(III) and As(V)) to proteins using two-dimensional gel electrophoresis. Arsenic species were added to plants *T. majus* during the cultivation, and after harvesting them, samples were submitted to protein extraction, precipitation, washing, and, desalting that provided well-resolved and reproducible 1D- and 2D -GE maps. Plants grown with different As species showed As-protein binding stronger than the binding formed when As was spiked to the extract containing proteins. This study also showed that the type of As species influences the strength of protein binding.

One of the plant cell detoxification mechanisms is the formation of phytochelatins and despite numerous studies on this subject, there is still much to understand about this mechanism. In a study of As species stability in hydroponically cultivated plants, it was observed that the As(III) in the nutrient solution is rapidly converted to As(V) in the presence of white mustard's roots [47].

Rahman et al. [71] observed oxidation of As(III) to As(V), of around 30%, in $NH_4H_2PO_4$ medium after extraction using microwave radiation (90 °C/ 20 min), but they have not observed any changes between MMA and DMA. The authors cannot explain the cause of instability of these compounds, although it was believed it was due to the ammonia added to stabilize the pH. On the other hand, the species kept stable when were employed protein extraction solution at pH 5.6 and the same extraction procedure in microwave oven. These authors also studied the stability of As species during storage at 4 °C for 45 days, both $NH_4H_2PO_4$ and

**Table 1**
Representative sample preparation strategies for As speciation in terrestrial plants.

| Sample | Extraction procedure | Species | Method | Extraction yield (%) | Ref. |
|---|---|---|---|---|---|
| *Tropaeolum majus*[a] | RT, 4 or 16 h with: water; phosphate buffer, pH 7; cellulase in phosphate buffer, pH 7. SON, 15 min | As(III), As(V), MMA, DMA | IC/ICPMS | 80–92 | [56] |
| Carrot[b] | SON with water (60 °C, 10 min) | MMA(III), MMA(V), DMA, MMTA, As(III), As(V) | HPLC–ICP-MS and HR-ESI-MS | 75–95 | [74] |
| Apple[b] | (1)TFA (100 °C, 8 h); (2) α-Amylase treatment (pH 7.2, 37 °C, 12 h) followed by SON with water/acetonitrile (6 h) | DMA, MMA, As(V), As(III) | HPLC–ICP-MS | (1) 75<br>(2) 98 | [75] |
| Garlic[b] | SON with (1) $H_2SO_4$, (2) $HClO_4$, and (3) methanol:$H_2O$ (1:1 v $v^{-1}$) and reduction with KI | As(III), As (V) | HG-AFS | (1) 40–97<br>(2) 80<br>(3) 96 | [76] |
| Mushrooms[c] | (1) NaOH, shaking, RT, 30 min; (2) HCl, shaking, RT, 30 min; (3) Water, shaking, 60 °C, 30 min | As associated with proteins (1.3–51.5 kDa) | SEC-UV-ICP-MS | (1) 5.6–74.6<br>(2) 16.2–66.9<br>(3)6.2–62.6 | [77] |
| Wheat grain[d] | (1) SON with phosphate buffer (pH 6, 1 h); (2) MAE with 1% v $v^{-1}$ $HNO_3$ (100 °C) | As(III), As(V), MMA, DMA | HPLC–ICP-MS | (1) 59–75<br>(2) 57–218 | [78] |
| Wheat grain[d] | (1) SON with methanol/water (50 °C, 2 h); (2) $H_2O$ extraction with ultrasonic probe (3 min, 95 W); (3) MAE with $HNO_3$; (4) MAE with α-amylase | As(III), As(V), MMA, DMA | HPLC–ICP-MS | (1) 77<br>(2) 73<br>(3) 100<br>(4) 84 | [79] |
| Rice[d] | MAE with TFA/$H_2O_2$ (95 °C, 60 min) | As(III), As(V), MMA, DMA | HPLC–ICP-MS | 86–100 | [80] |
| Rice[d] | $HNO_3$ solution, heating blocks (95 °C, 90 min) | As(III), As(V), MMA, DMA | HPLC–ICP-MS | 99 | [81] |
| Rice[d] | Treatment with methanol: water (1:1 v $v^{-1}$), 55 °C, 10 h followed by shaking with water | As(III), As(V), MMA, DMA, AsB | LC-UV-HG-ICPMS | 66–73 | [82] |
| Terrestrial plant species[b] | MAE with modified protein extracting solution (90 °C, 20 min) | As(III), As(V), MMA, DMA | IC-ICP-MS | 104 | [83] |
| Pepper plant[c] (fruits, leaves, steams, roots) | Shaking, RT, 14 h with extractants: water, methanol/water, methanol, ammonium phosphate buffer | As(III), As(V), MMA, DMA | HPLC–HG-ICP-MS | 5–93 | [84] |
| Lichens[a] | Sequential extraction procedure: SON, RT, 30 min with (1) Milli-Q water, (2) $CaCl_2$ and (3) $H_3PO_4$ | As(III), As(V), MMA, DMA | HPLC–UV-HG-AFS | < 10 | [85] |
| Terrestrial plant[c] | Shaking, RT, 16 h with extractants: water, methanol/water mixtures | As(III), As(V), MMA, DMA, TMAO, TETRA | HPLC–ICP-MS | 3.0–41.4 | [21] |
| Sunflower (*Helianthus annuus*)[e] | Extracted with 1% v $v^{-1}$ formic acid (solid:liquid ratio 1: 3) for 90 min at 1 °C | As(III), As(V), MMA, DMA, As(III)–$PC_3$, GS–As(III)–$PC_2$, [As(III)-$(PC_2)_2$],($\gamma$-Glu-Cys)$_2$-Gly $CH_3As$ (MA(III)–$PC_2$ | HPLC–(ICP-MS)-(ESI-MS) | 60–90 | [36] |

Numbers (1)–(4) are different procedures applied for the same sample.

[a] Fresh sample.
[b] Freeze dried sample.
[c] Dried and ground.
[d] Round.
[e] Ground under liquid nitrogen.

protein extraction solutions remained stable for 45 days, but with a trend to decrease the concentrations of species, which shows that the time between extraction and analysis of species should be as short as possible.

## 4. Analytical methods

Although several hyphenated methods have been used in speciation analysis, HPLC–ICP–MS is one of the most popular ones and this is especially due to its low detection limits and versatility to determine both volatile and non-volatile species. In this case, one can combine a technique with high separation power, such as high performance liquid chromatography (HPLC), to a detection technique which offers high sensitivity, robustness and a wide linear dynamic range, such as inductively coupled plasma mass spectrometry (ICP–MS) [51,52,93–96].

The use of HG coupled to chromatographic techniques in As speciation was tested as high performance liquid chromatography – ultraviolet photo-oxidation – hydride generation atomic – fluorescence spectrometry (HPLC–UV-HG-AFS) for As species determination inedible alga [97]. This same system was used for determination of As(III), As(V), MMAA, DMAA, AsB, TMAO, glycerol-ribose and phosphate-ribose in lichens extracts [72]. Furthermore, speciation of As(III), As(V), MMA and DMA was performed in bush samples by HPLC-HG-AFS [67].

Highly sensitive and selective instrumentation as mass spectrometry (MS) is employed in order to obtain information about the metal-binding molecule. The main advantages of this type of coupling is the relevance of information provided and the fact that it is not necessary using standards, not often existents due to complexity and variety of compounds that are present in some matrices. A set of organoarsenicals and degradation products of phenylarsonic acid and o-arsanilic acid were isolated and identified in soil samples using HPLC–ICP–MS, ESI–MS, ESI–MS/MS and ESI–TOF–MS [98]. The As(III)–thiol complexes or As–phytochelatins (As–PC) and the mechanisms of As tolerance have been extensively studied in terrestrial plants by HPLC–ICP–MS and ESI–MS without phytochelatins standards [46,99,100]. These studies generally aim to understand the mechanism of complexes formation, As–PC, to be able to explain the high tolerance of plants to As toxicity, as well as to study metal accumulation and the translocation throughout the plant organism.

Methods less usual as two-dimensional gel electrophoresis (2D-GE) provides high resolution and offers the possibility to detect metal- or metalloid bindings to the separated protein spots by means of laser ablation and ICP–MS [92].

To prevent interconversion between inorganic species, some studies search for detection techniques not requiring analyte extraction such as synchrotron-based spectroscopic methods (e.g. X-ray, XANES, XRF). These techniques can perform in situ analysis, eventually its greatest advantage, e.g. they can determine by image analysis the location of As in plant structure, which adds great contribution to studies of As uptake [1,101,102].

Although several methods have been used in the past and still nowadays for As speciation analysis in plants, the most used are undoubtedly the coupling HPLC–ICP–MS or LC-ICP–MS. Therefore, their use in matrices such as carrots [74], mustard [47], rice [39,61], apple [75], grains [78], and terrestrial plants in general [23,58,103] probably will continue for As speciation due to their versatility and sensitivity for separation and detection of As species in plant tissues.

## 5. Conclusions

Speciation studies have aimed samples of plants since there is close proximity to humans through the food chain and plants are capable of absorbing potentially toxic elements present in soils. Simple extraction procedures involving water or even diluted acids are good alternatives once they promote efficient extraction, involving solvents less toxic or even non-toxic. The time between the extraction procedure and analysis must be as short as possible in order to avoid interconversion between species, especially in binding of As to phytochelatins studies. Most suitable separation techniques involve liquid chromatography, HPLC or LC, and ICP–MS as detector has been widely appropriate. However, techniques as ESI–MS, ESI–MS/MS or ESI–TOF–MS, can provide important information about metal-molecule bindings.

The Web of Science was searched in order to obtain summary information about As speciation in plants covering the period of 2000–2012. The output information was combined in Fig. 1.

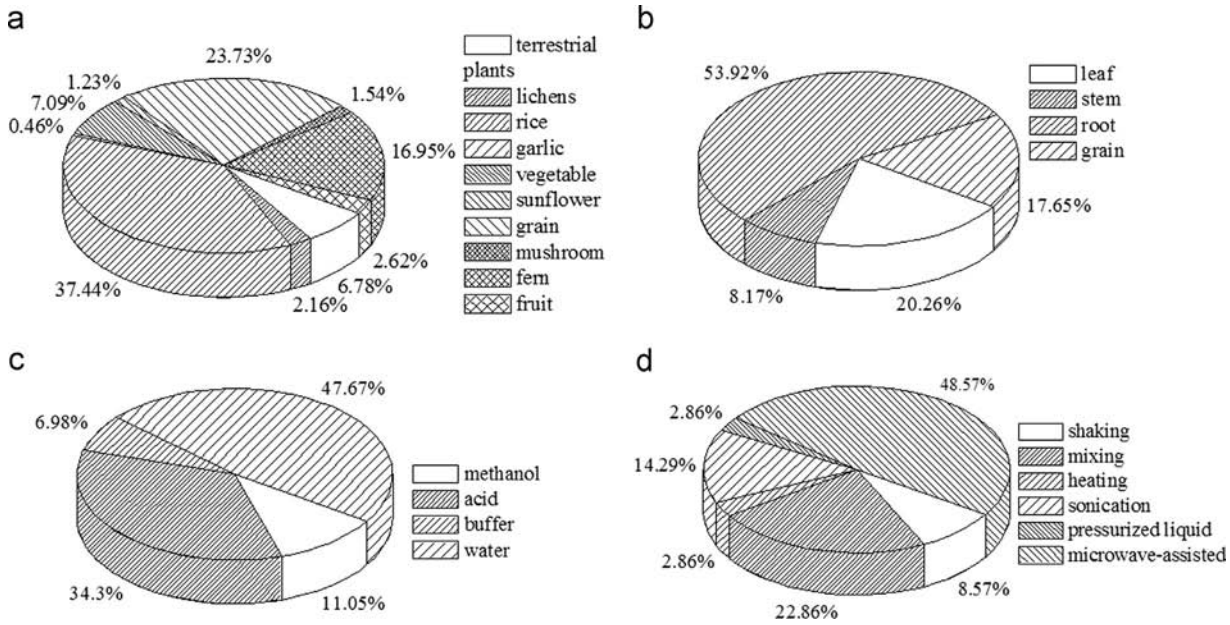


**Fig.1.** Papers published in the selected topics according to the Web of Science Database covering the period of 2000–2012.

For Fig. 1a the keywords "arsenic speciation" were maintained and the words shown in Fig. 1a were added separately. The same was done for Fig.1b. For Fig. 1c and d the keyword maintained were "arsenic speciation plant extraction" and also the words shown in these figures were added separately. The search performed shows that for As speciation in plants, rice is the most studied sample and if we think about plant's parts, the root has been the main focus. In terms of sample preparation, the Web of Science search indicates that the increasing order of solvent use is: water > acid > methanol > buffer. Finally, MAE is the most used procedure.

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