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Arsenic speciation in tree moss by mass spectrometry based hyphenated techniques

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ABSTRACT

A method based on ion-pair reversed phase high performance liquid chromatography (HPLC) hyphenated with inductively coupled plasma mass spectrometry (ICP-MS) was developed for arsenic speciation in extract of tree moss. Under the optimal conditions, the limit of detection of eight arsenic species including arsenite (As^{III}), arsenate (As^{V}), monomethylarsonic acid (MMA), dimethylarsonic acid (DMA), trimethylarsinoxide (TMAO), tetramethylarsonium (Tetra), arsenocholine (AsC) and arsenobetaine (AsB) is between 0.04 and 0.07 ng/mL, with a linear range of 0.2 − 500 ng/mL. Three unknown arsenic species (Unk1, Unk2 and Unk3) and six specific arsenic species (As^{III}, As^{V}, DMA, TMAO, Tetra and AsB) were detected in the extract of tree moss. Unk3 was identified as a kind of arsenosugars (2,3-dihydroxypropyl-5-deoxy-5(dimethylarsenoso)furanoside, arsenosugar X) by electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-qTOF-MS).

1. Introduction

Arsenic is a carcinogenic and mutagenic source with an average concentration of 5 μg/g in the earth's crust [1]. The emission of arsenic into the atmosphere are caused by natural phenomena such as weathering, biological activity, volcanic activity, and anthropogenic inputs. Then it is redistributed on the earth's surface by rain and dry fallout from atmosphere. The toxicity of arsenic species is related to its chemical forms, generally arsenite (As^{III}) > arsenate (As^{V}) > monomethylarsonic acid (MMA) > dimethylarsonic acid (DMA) > trimethylarsinoxide (TMAO), tetramethylarsonium (Tetra), arsenobetaine (AsB), and arsenocholine (AsC) [2].

Tree moss (*Ramalina fastigiata (Pers.) Ach.*) is a kind of lichen, which is a composite organism that arises from algae and/or cyanobacteria living among filaments of a fungus in a symbiotic relationship [3]. It spreads over the surface of stones and trees, and the extract of tree moss is applied to produce fragrant substances [4], and widely used in cigarette, food and perfume industry to get a fragrance of mixture of grass and tree. When the surrounding environment was polluted by arsenic, the tree moss would uptake arsenic and cause potential health risk. Nearing et al. [5] studied the uptake and transformation of arsenic during the vegetative life stage of terrestrial fungi. It was found that As^{V} could transform to TMAO in *Sparassis crispa*. It is also reported that arsenic could be accumulated in lichens [6], and the concentration of total arsenic is about ten micrograms per gram in dry lichen. Two kinds of arsenosugars were first identified by Edmonds *et al.* in brown kelp (*Ecklonia radiata*) [2], which may be the intermediates in the biological methylation of inorganic arsenic. In the extracts of terrestrial fungi and lichens from Yellowknife, Canada, Koch et al. [7] found various arsenic species including As^{III}, As^{V}, MMA, DMA, TMAO, Tetra, AsB and Arsenosugar X. The natural occurrence of arseno compounds in plants, lichens, fungi, algal species, and microorganisms was reviewed by Dembitsky et al. [8]. The above studies indicate that tree moss has the capability to accumulate arsenic and convert arsenic into organo-arsenic species. Due to the wide use of tree moss extracts as fragrant substances and possible toxicity caused by arsenic, arsenic speciation in the extracts of tree moss should be a vital step before it is used as an additive in products.

Hyphenated techniques are effective methods for elemental speciation. Various separation techniques, including gas chromatography (GC) [9,10], capillary electrophoresis (CE) [11,12] and high performance liquid chromatography (HPLC) [13,14], have been applied for the separation of arsenic species. Among them, HPLC is the most widely used method in arsenic speciation. Ultraviolet (UV) is the most commonly used detector for HPLC, but the limit of detection for arsenic species is relatively high. As element-specific detectors, atomic absorption spectrometry (AAS) [15], atomic fluorescence spectrometry (AFS) [9], inductively coupled plasma optical emission spectrometry (ICP-OES) [16] and inductively coupled plasma mass spectrometry (ICP-MS) can provide higher sensitivity for arsenic than UV detector. Compared with other elemental-specific techniques, ICP-MS exhibits high sensitivity, wide dynamic range, and good resistance to complex

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matrix. HPLC hyphenated with ICP-MS detection has become one of the most powerful techniques for arsenic speciation [17,18]. In HPLC analysis of arsenic species, ion exchange chromatography (IEC) [13,18] and ion pair reversed phase (IP-RP)-HPLC [14,19–21] are commonly used separation modes. In contrast to IEC, simultaneous separation of both charged and uncharged analytes can be achieved in RP-HPLC with the addition of ion pair reagents.

However, HPLC-ICP-MS only provides quantification information. For those unknown arsenic species observed in real samples, organic mass spectrometry is an essential tool to get the structure information. By using HPLC-ICP-MS together with electrospray ionization (ESI)-MS, Madsen et al. [22] identified four arsenosugars in an algal extract. Bluemlein et al. [23] analyzed arsenic peptides in an ornamental garden plant (*Thunbergia alata*) by LC-ES-MS/ICP-MS. Low-molecular-mass thio-organoarsenical compounds in the form of As^{III} -phytochelatins were found in root of *Thunbergia alata* exposed to arsenate. Therefore, hyphenated technique of HPLC-ICP-MS supplemented with ESI-MS would provide more useful information for arsenic speciation.

The aim of this work is to develop a new method by combining HPLC-ICP-MS with high resolution ESI-quadrupole time-of-flight (qTOF)-MS/MS for the identification and quantification of arsenic species in the extracts of tree moss. The extracts of tree moss sample were prepared by Soxhlet extraction prior to HPLC-ICP-MS analysis. For identification of unknown arsenic species by ESI-qTOF-MS/MS, the extracts of tree moss were further subjected to solid phase extraction and HPLC separation for the removal of complex matrix and purification, respectively.

2. Materials and methods

2.1. Materials and chemicals

Stock solutions (1.000 mg/mL as As) of eight arsenic standards (As^{III}, As^{V}, MMA, DMA, AsB, TMAO, Tetra and AsC) were prepared with $NaAsO_2$ (> 90%, Wako, Japan), $Na_2AsO_7{\cdot}H_2O$ (> 99%, Wako, Japan), $CH_3AsO_3Na_2$ (> 98.5%, J&K Chemical Ltd, China), $C_2H_6AsO_2Na{\cdot}H_2O$ (> 98.5%, Genebase Bioscience Co., Ltd , China), $C_5H_{14}AsBrO$ (> 95%, Wako, Japan), $(CH_3)_3AsO$ (> 95%, J&K Chemical Ltd, China), $(CH_3)_4AsI$ (> 97%, J&K Chemical Ltd, China) and $C_5H_{11}AsO_2$ (> 95%, Wako, Japan) in high purity water, respectively. The mixed standard solution was prepared by diluting the stock solution daily. All the concentration units stated in the whole manuscript are expressed as the concentration of As rather than that of As species. Malonic acid was obtained from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China), and 1-Butanesulfonic acid sodium (BSAS) was obtained from Tianjin Aoran Fine Chemical Research Institute (Tianjin, China). Tetramethylammonium hydroxide (TMAH) (~25% in H_2O) was obtained from Aladdin Industrial Corporation (Shanghai, China). High purity water was obtained by a Milli-Q water purification system (18.25 MΩ cm, Millipore, Molsheim, France). Other reagents used in the experiment were analytical grade unless otherwise mentioned. The containers employed in experiment were stored in 10% (v/v) nitric acid over 24 h, and rinsed with tap water and high purity water prior to use. Tree moss samples were kindly provided by Mr. Weiping Yang (Zhengzhou Institute of Tobacco, Zhengzhou, Henan, China).

2.2. Instruments

A CAPCELL PAK C18 column (250 mm × 4.6 mm, 5 μm particle size) was used for the separation of target arsenic species. The HPLC system consisted of an LC-10AD high pressure pump and a DGU-12A degasser (Shimadzu, Japan). A quadrupole ICP-MS (Agilent 7500a, Japan) with a Babington nebulizer was interfaced to HPLC via a minimum length piece of Teflon tubing (i.d. 0.5 mm, length 30 cm) with a finger-tight PEEK fitting. Optimization of the ICP-MS instrument (i.e. lens settings, sampling depth and carrier gas flow rate) was performed with conventional pneumatic nebulization (PN)-ICP-MS prior to being connected with HPLC. A semi-preparative C18 chromatographic column (250 mm × 10 mm, 10 μm particle size) was purchased from Soochow High Tech Chromatography CO., LTD. (Soochow, China) to enrich and purify unknown arsenic species. A microTOF-Q III MS with an ESI ion source (Bruker, German) was used for identification of unknown arsenic species. The operating conditions for HPLC-ICP-MS are summarized in Table 1. An electrically-heated thermostatic water bath (DF-101S, Keer, Wuhan) was used for Soxhlet extraction of arsenic species in tree moss. A rotary evaporator (RE-52AA, Yarong, Shanghai) was employed to remove the extraction solvent and enrich target analytes at 70 °C. Ultracentrifugation (ThermoFisher scientific, German) was applied to separate insoluble matter in the extract of tree moss. C18 solid phase extraction (SPE) cartridge (Agilent) was used to eliminate the impurities in tree moss extract.

Table 1
Operation conditions for HPLC-ICP-MS.

Instrumental conditions	
HPLC	
Column	CAPCELL-PAK C18 MG-II (250 mm × 4.6 mm, 5 μm)
Mobile phase A	10 mmol/L sodium butanesulfonate, 4 mmol/L TMAH and 4 mmol/L malonic acid, methanol/water (0.1/99.9, v/v), pH 3.0
Mobile phase B	5 mmol/L ammonium acetate, methanol/water (1/99, v/v), pH 7.0
Mobile phase C	methanol/water (0.1/99.9, v/v), pH 3.0
Flow rate	1.0 mL/min
Column temperature	ambient temperature
Injection volume	60 μL
ICP-MS	
Rf power	1150 W
Rf matching	6.2 V
Sampling depth	6.8 mm
Carrier gas	1.1 L/min
Time-resolved data acquisition	
Scanning mode	Peak-hopping
Dwell time	100 ms
Integration mode	Peak area
Detected isotope	^{75}As

2.3. Analytical procedure

The analysis procedure for arsenic speciation in tree moss is presented in Fig. 1. It can be divided into two parts, one is the quantification of specific arsenic species, and the other is the identification of the unknown arsenic species.

2.3.1. Quantification of specific arsenic species

2.3.1.1. Soxhlet extraction. To prepare the extracts of tree moss, benzene, petroleum ether and ethanol are the usually employed extraction solvents [24]. Here, ethanol was chosen as the extract solvent. Briefly, 5.38 g of tree moss and 200 mL extraction solvent consisting of ethanol and water (3:1, v/v) were added into a round bottomed flask. Soxhlet extraction was carried out for 3 h in water bath at 95 °C. After extraction, the majority of extraction solvent in the crude extract of tree moss was removed with the rotary evaporation apparatus. Next, the condensed crude extract was centrifuged at 12,000 rpm for 10 min and the supernatant was collected. The sediment after centrifugation was washed twice by high purity water with the assistance of vortex and then centrifuged to get the supernatant. All supernatant was merged as the extraction fraction and diluted to 25 mL with high purity water for following HPLC-ICP-MS analysis.

2.3.1.2. HPLC-ICP-MS. The operation conditions of HPLC for the separation of As^{III}, As^{V}, MMA, DMA, AsB, TMAO, Tetra and AsC was

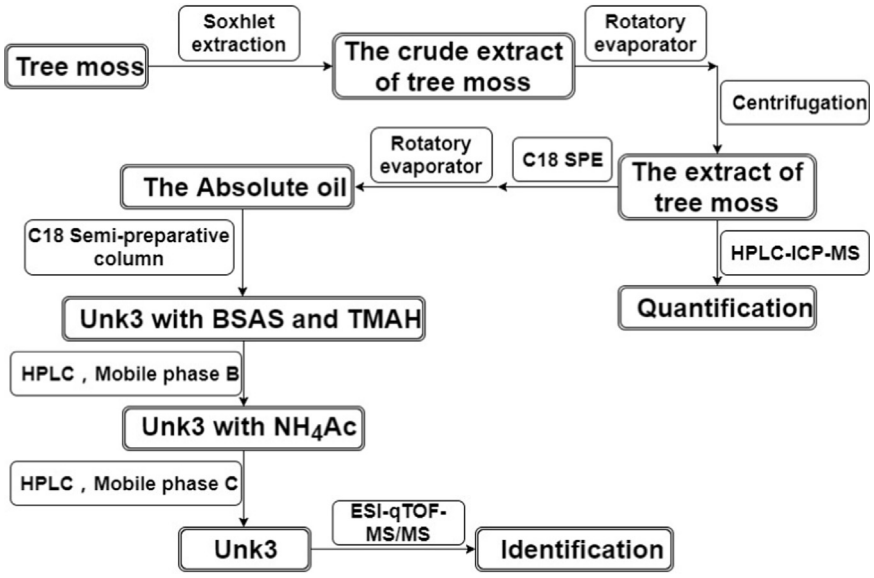

Fig. 1. The flowchart of arsenic speciation in tree moss. The conditions of mobile phase A, B and C are shown in Table 1.

according to Ref. [21] with minor modifications (mobile phase A). The optimal conditions for HPLC and ICP-MS are shown in Table 1. The extract of tree moss was diluted by mobile phase A in a ratio of 1:4 for arsenic speciation by HPLC-ICP-MS.

$^{40}Ar^{35}Cl$ would cause mass interference on ^{75}As in ICP-MS detection. While, it should be noted that Cl-containing reagents were avoided in our experiment; and chloric ion would be separated from target As species by using the ion-pair reversed phase (IP-RP)-HPLC. Moreover, in consideration of only two isotopes (35, 37) for Cl with the abundance ratio of about 3:1, the signal of $^{40}Ar^{37}Cl$ was monitored along with ^{75}As, and no obvious peaks were observed during the whole separation time range. It indicates that the mass interference of $^{40}Ar^{35}Cl$ on ^{75}As is ignorable herein.

2.3.1.3. Total arsenic analysis. The total arsenic in the original tree moss sample (Total, T), the extract of tree moss (Extracted fractions, E), and the residue after Soxhlet extraction (Remains after extraction, R) were all detected by ICP-MS after microwave acid digestion. As^{III} was used for the quantification of total arsenic by an external standard calibration strategy. Briefly, about 0.1 g of sample with 5 mL HNO_3 and 1 mL H_2O_2 was added to a PTFE pressure tank, maintained overnight and then subjected to microwave digestion. The microwave digestion program is as follows, 120 °C for 6 min, 150 °C for 6 min, and then 180 °C for 4 min. After digestion, the solution was evaporated to near dryness and then diluted to 8 mL with high purity water for PN-ICP-MS analysis.

2.3.2. Identification of unknown arsenic species

2.3.2.1. C18 clean-up procedure. The colored matrix in the supernatant obtained in Section 2.3.1 was removed by C18 SPE cartridge. Firstly, C18 SPE cartridge was activated by sequentially passing through 3 mL of CH_3OH and 3 mL of H_2O. Then 0.5 mL of the above-mentioned supernatant was loaded on the C18 SPE cartridge, and the effluent was collected. After that, 1.0 mL of H_2O was used to elute target analytes retained on the C18 SPE cartridge. C18 SPE cartridge could be cleaned by 3 mL of CH_3OH for next use. The effluent and the eluent were separately rotary evaporated to obtain the absolute oil.

2.3.2.2. Enrichment and purification of unknown arsenic species. A semi-preparative C18 chromatographic column (Soochow High Tech Chromatography CO., LTD., 250 mm × 10 mm, 10 μm particle size) was employed to separate the unknown arsenic species (named as Unk3) in the absolute oil. Mobile phase A was used for an isocratic elution, and the fraction at the retention time in the range of 650–750 s was collected based on HPLC-ICP-MS analysis. Then the fraction was concentrated by rotary evaporation apparatus. Next, the sodium 1-butanesulfonate, TMAH and malonic acid in the fractions were removed by using a C18 chromatographic column (Sheseido, CAPCELL PAK C18 MG-II) with an isocratic elution program. Mobile phase B was adopted, and the fraction appearing at the retention time in the range of 250–300 s was collected and concentrated again. Then to remove the ammonium acetate in the fraction, the same C18 chromatographic column (Sheseido, CAPCELL PAK C18 MG-II) was used with another isocratic elution program. Mobile phase C was used and the fraction appearing at the retention time in the range of 330–390 s was collected. The employed three isocratic elution programs were all performed at the flow rate of 1.0 mL/min. Lastly, the final fraction without salt was concentrated for following ESI-qTOF-MS measurement.

2.3.2.3. ESI-qTOF-MS/MS analysis. Unk3 was identified by ESI-qTOF-MS/MS in a positive mode. The typical operating conditions in the MS mode is as follows: end plate offset 500 V, capillary voltage 4100 V, scan range 70–500 u, dry gas 4.0 L/min, dry temperature 180 °C. The fragments of $[M+H]^+$ ion were monitored in product ion scan mode. The collision energy was set as 18.0 V and the product ions were scanned in the range of interest within 30 s.

3. Results and discussion

3.1. Validation of HPLC-ICP-MS

It was reported that the arsenic species in lichen was mainly water soluble, which included anion (As^V, MMA, DMA *etc*.), cation (TMAO, Tetra, AsC) and neutral ion (As^{III}, AsB) species [7]. Herein, taking these eight arsenic species as model analytes firstly, the separation condition was optimized by using sodium butanesulfonate and TMAH as ion-pair reagents [21], and the separation chromatogram of them is shown in Fig. 2. The retention time was assigned to each As species by running single standards, and the signal peaks of As^{III}, As^V, MMA, DMA, AsB, TMAO, Tetra and AsC appeared at 134, 175, 186, 217, 243, 457, 518 and 550 s, respectively. Under the optimized experimental conditions, the analytical performance of HPLC-ICP-MS for analysis of target arsenic species was evaluated, and the results are shown in Table 2. The limits of detection (LODs, calculated as the concentration

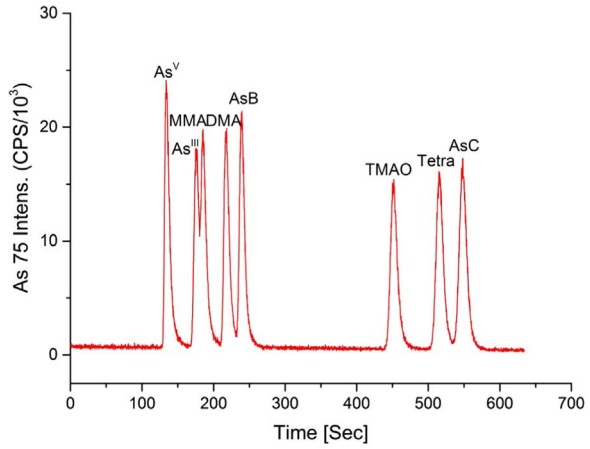

Fig. 2. The separation chromatogram of eight standard arsenic species by IP-RP-HPLC-ICP-MS (each of 10 ng/mL, as As).

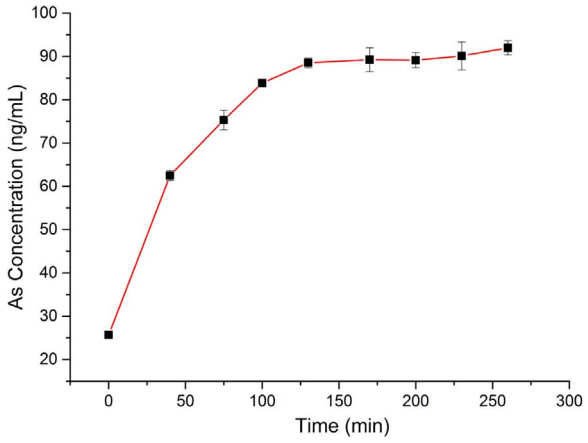

Fig. 3. Arsenic concentration in the extract by Soxhlet extraction over extraction time. (Conditions: mass of tree moss, 5 g; extraction solvent, ethanol and water (3:1, v/v). The zero point was the time when the ethanol was initially refluxed.).

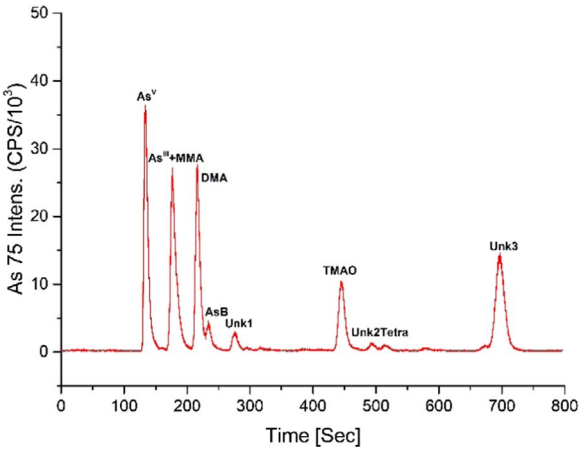

Fig. 4. Chromatograph of arsenic species in the extract of tree moss obtained by IP-RP-HPLC-ICP-MS.

corresponding to three times the standard deviation of 11 runs of the blank solution) were between 0.04 and 0.07 ng/mL as As, with a linear range of 0.2–500 ng/mL as As. The relative standard deviations (RSDs), calculated by 9 replicated analysis of arsenic standards with the concentration of 1 ng/mL as As, are in the range of 3.8–7.6%.

3.2. Optimization of Soxhlet extraction

The inorganic arsenic species and methylated arsenicals can be easily extracted by a mixture of water and ethanol in Soxhlet extraction [24]. While, the arsenic species binding to peptides or proteins are not easily released from cell of tree moss because high temperature and ethanol cannot destroy cytoderm of fungus and algae, which consists of chitin and cellulose. Methanol and water (1:1, v/v) was used to extract arsenic species from lichens by Iris Koch *et al.* [7], and the extraction efficiency ranged from 1.1% to 42% with different samples. Considering the toxicity of methanol, ethanol and water (3:1, v/v) was employed to get tree moss absolute from tree moss by using conventional Soxhlet extraction method. The process of Soxhlet extraction is investigated by monitoring the variation of arsenic concentration in the extract over extraction time and the result is shown in Fig. 3. The extract was diluted by 20-fold with high purity water for subsequent PN-ICP-MS analysis. As can be seen, the concentration of extracted arsenic species is increased with the increase of extraction time from 0 to 150 min, and levels off after 150 min. In the following experiment, the extraction time was chosen to be 180 min.

About 5 g of tree moss was extracted by a mixture of ethanol and water for 180 min. For the quantification of arsenic in tree moss, the residue and extract after Soxhlet extraction, about 0.1 g of corresponding sample was weighed and subjected to the microwave digestion as specified in “Total arsenic analysis” in Section 2.3.1. After acid digestion, the concentration of arsenic in tree moss (Total, T), residue (Remains after extraction, R) and extract (Extracted fractions, E) was analyzed by PN-ICP-MS. By using the formula of (the determined value × 8 mL) / sample mass, the concentration of arsenic in T, R, and E were determined to be 4.71 ± 0.10, 3.08 ± 0.10 and 1.03 ± 0.03 μg/g, respectively. The extraction efficiency of Soxhlet extraction (E/T) was calculated to be 21.9%. The Soxhlet extraction process was repeated for more than six times, and the extraction efficiency exhibited good reproducibility. Moreover, a recovery test was performed by spiking 0.398 μg As^{III} into 100 mg tree moss sample, followed by microwave digestion and ICP-MS detection. The recovery of 93.7% (n = 3) was obtained for As^{III}, which demonstrated the accuracy of the method.

Table 2
Analytical performance of HPLC-ICP-MS.

Analytes	Linear range (ng/mL, as As)	Linear equation	Coefficient of determination (R^2)	LODs (ng/mL, as As)	RSDs[a]
As^V	0.2 – 500	y = 27842.3x + 4098.3	0.9999	0.04	5.4
As^{III}		y = 19348.1x + 1388.0	0.9999	0.06	6.0
MMA		y = 24295.8x + 2262.5	0.9994	0.06	3.8
DMA		y = 24672.2x + 2065.3	0.9994	0.05	4.1
AsB		y = 25617.6x + 3068.9	0.9989	0.05	4.7
TMAO		y = 24554.6x + 4342.9	0.9979	0.07	4.2
Tetra		y = 26509.0x + 3715.4	0.9986	0.07	6.5
AsC		y = 26926.4x + 3566.1	0.9992	0.07	7.6

[a] (c = 1 ng/mL as As, n = 9, %).

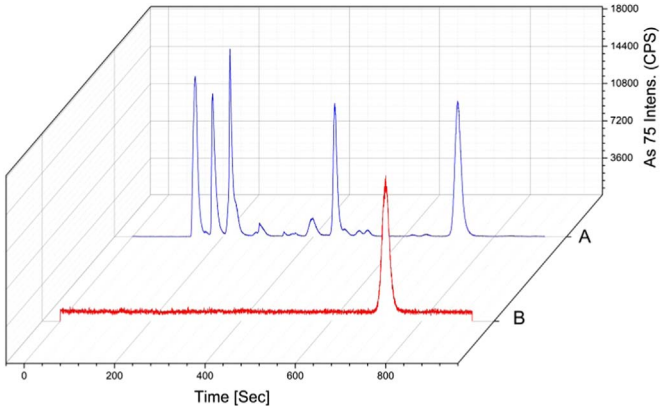

Fig. 5. Chromatogram of arsenic species in the extract of tree moss before (A) and after (B) the enrichment and purification process for Unk3.

3.3. Analysis of arsenic species in the extract of tree moss

The arsenic species in the extract of tree moss is shown in Fig. 4. According to the retention time of eight arsenic species (Fig. 2), six arsenic species appearing at 133, 176, 217, 234, 444 , 515 s were identified to be As^{V}, As^{III} + MMA, DMA, AsB, TMAO, and Tetra, besides three unknown arsenic species (Unk1, Unk2 and Unk3) appearing at 276, 494, 697 s were found as well. Unk3 was separated and purified to an appropriate concentration (appr. 1 μg/g, without salt) before characterization by ESI-qTOF-MS/MS. While Unk1 and Unk2 were not identified because of low content in the supernatant and instability in process of identification.

3.3.1. Identification of unknown arsenic species

The chromatogram of arsenic species for the extract of tree moss obtained by HPLC-ICP-MS before and after the enrichment and purification process for Unk3 is shown in Fig. 5.

The obtained fraction of Unk3 without salt (50-fold enrichment) was diluted by CH_3OH in the ratio of 1:1 (v/v) for direct injection to ESI-qTOF-MS. Parent molecule ion peak was identified in the full scan mode, and the daughter ion peak was identified in the product ion scan mode. The analysis of Unk3 by ESI-qTOF-MS in positive mode is shown in Fig. 6**a.** All the potential interesting peaks in Unk3 including m/z 385.0139, 350.9898 and 329.0600 were subjected to tandem MS detection (product ion scan mode) for identification. The characteristic fragment ions containing arsenic were found for only m/z 329.0600, rather than m/z 385.0139 and 350.9898. It indicates that m/z 329.0600 is the arsenic-containing molecular in Unk3. In fractionation of Unk3, only one signal peak for arsenic species was detected by HPLC-ICP-MS (Fig. 5). But it does not mean that fractionation of Unk3 is a pure isolated compound. As a biological sample, matrix of tree moss should be complicated, so there would be some arsenic free compounds co-eluted with Unk3 which cannot be detected by ICP-MS with m/z 75 as the monitored isotope. This is probably the reason that in the full scan mass spectrum for Unk3 by ESI-qTOF-MS, m/z 329.0600 is accompanied by other main peaks (Fig. 6a).

A tandem MS was carried out to get more structure information about the ion of m/z 329.0600 (Fig. 6**b**). Based on the characteristic fragment ions of m/z 97.0497, 195.0084 and 237.0145, Unk3 was identified as arsenosugar X (2,3-dihydroxypropyl-5-deoxy-5(dimethylarsenoso) furanoside) based on literature [25,26] and the structure of arsenosugar X is shown in the insert of Fig. 6**b**. Dissociation pathway of precursor ions (m/z 329.0550) is shown in Fig. 6**c** and identification of arsenic species in the product ion scan mode is shown in Table 3.

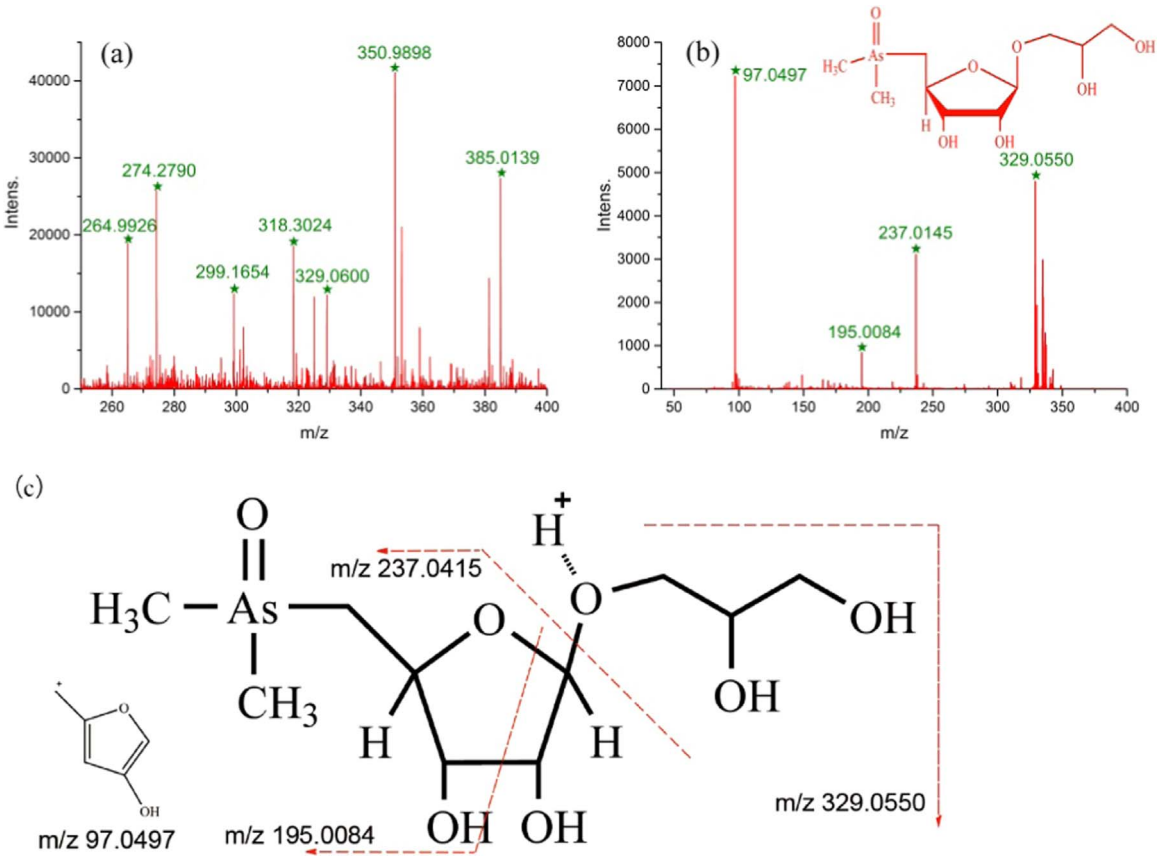

Fig. 6. Identification of Unk3. (a) Full scan mass spectrum for Unk3 obtained by ESI-qToF MS. (b) Product ion scan of m/z 329.0600. (c) Dissociation pathway of precursor ions (m/z 329.0550).

Table 3
Identification of arsenic species in the product ion scan mode.

Compound[a]	MS^n	Measured	Calculated	Δ, ppm[b]	Species
Arsenosugar X(328)	MS	329.0550	329.0581	−9.4	$C_{10}H_{22}O_7As^+$
	MS^2	237.0145	237.0108	15.6	$C_7H_{14}O_4As^+$
$C_{10}H_{21}O_7As$	MS^2	195.0084	195.0002	42.0	$C_5H_{12}O_3As^+$
	MS^3	97.0497	97.0290	213.3	$C_5H_5O_2^+$

All units are in Dalton unless otherwise specified.
[a] The structural formulas for Arsenosugar X is shown in Fig. 6**c.**
[b] Δ is the difference between the measured and the calculated mass divided by the calculated mass and multiplied by 10^6.

Table 4
Analytical results and recoveries for arsenic species in the extract of tree moss.

Arsenic species	Found (ng/mL, as As)	Added (ng/mL, as As)	Measured (ng/mL, as As)	Recovery (%)	Tree moss (ng/g, as As)
As^V	48.0 ± 5.4	50.0	93.0 ± 4.0	94.9	223 ± 25
As^{III} + MMA	53.7 ± 5.6	50.0	99.0 ± 3.5	95.5	250 ± 26
DMA	52.0 ± 5.0	50.0	99.0 ± 3.5	97.1	242 ± 23
AsB	5.4 ± 0.5	6.25	11.6 ± 0.6	99.6	25.1 ± 2.3
TMAO	27.0 ± 0.5	25.0	47.9 ± 0.6	92.1	126 ± 2
Tetra	3.4 ± 0.1	6.25	9.25 ± 0.20	95.9	15.8 ± 0.5
AsC	–	6.25	5.55 ± 0.35	88.8	–
Arsenosugar X	42.9 ± 1.2	40.5	81.3 ± 0.4	97.5	199 ± 6

Raml et al. [27] reported the variability of arsenosugar X in human metabolism involving the ingestion of arsenosugar X by volunteer. The total, four-day, urinary excretion of arsenic for six volunteers ranged widely from 4% to 95%. The arsenic metabolites present in the urine also showed great variability that high arsenic excretion was accompanied by almost complete biotransformation of the ingested oxo-arsenosugar into a multitude of metabolites (> 10), whereas the subjects that excreted low amounts of arsenic produced low quantities of metabolites relative to unchanged oxo-arsenosugar and its thio-analogue. These results indicate that arsenosugars may have different metabolic way for each individual, so the toxicity of arsenosugars is still doubted.

3.3.2. Quantification of arsenic species

Eight arsenic species (As^{III}, As^V, MMA, DMA, AsB, TMAO, Tetra and arsenosugar X) were quantified in the extract of tree moss. The concentration of arsenic species in the extract of tree moss is shown in Table 4, along with the concentration of arsenic species in tree moss. As can be seen, the concentration of arsenic species in tree moss ranges from 15.8 ± 0.5–250 ± 26 ng/g and the concentration of total extracted arsenic species is 1081 ± 43.3 ng/g (1.08 ± 0.04 μg/g). The inorganic arsenic species account for about 44.7% of total arsenic in the extract of tree moss. The recovery test was carried out to validate the accuracy of method by spiking target arsenic species in the diluted extracts before injection into HPLC sample loop, and the results are also shown in Table 4. As can be seen, the recovery is in the range of 83.6–98.7%, indicating a good accuracy of the proposed method.

Arsenic species that can be analyzed by the proposed IR-RP-HPLC-ICP-MS is featured with good solubility in the mobile phase. In other words, these arsenic species are water soluble. As specified in Section 3.2, the concentration of arsenic in the extracts of tree moss obtained by PN-ICP-MS after microwave digestion is 1.03 ± 0.03 μg/g. It is consistent with that obtained by subjecting the extracts of tree moss to IR-RP-HPLC-ICP-MS measurement (1.08 ± 0.04 μg/g), based on a *t*-Test ($1.732 < t_{0.05/2,\ 2} = 4.303$). It can be concluded that the extracted species are water soluble arsenic species with low molecular weight, mainly consisting of As^{III}, As^V, MMA, DMA, AsB, TMAO, Tetra and arsenosugar X.

3.4. Discussion on possible arsenic metabolism in tree moss

Tree moss is a homobium consisting of fungus and algae and/or cyanobacteria, which belongs to lichens. Lichens are among the best biogenic dust collectors and can be used in detailed studies of airborne pollutants, which may contain multiple heavy metals such as arsenic, mercury, copper and lead. Methylation is an important process for organism to protect itself from arsenic toxicity. S-adenosyl methionine

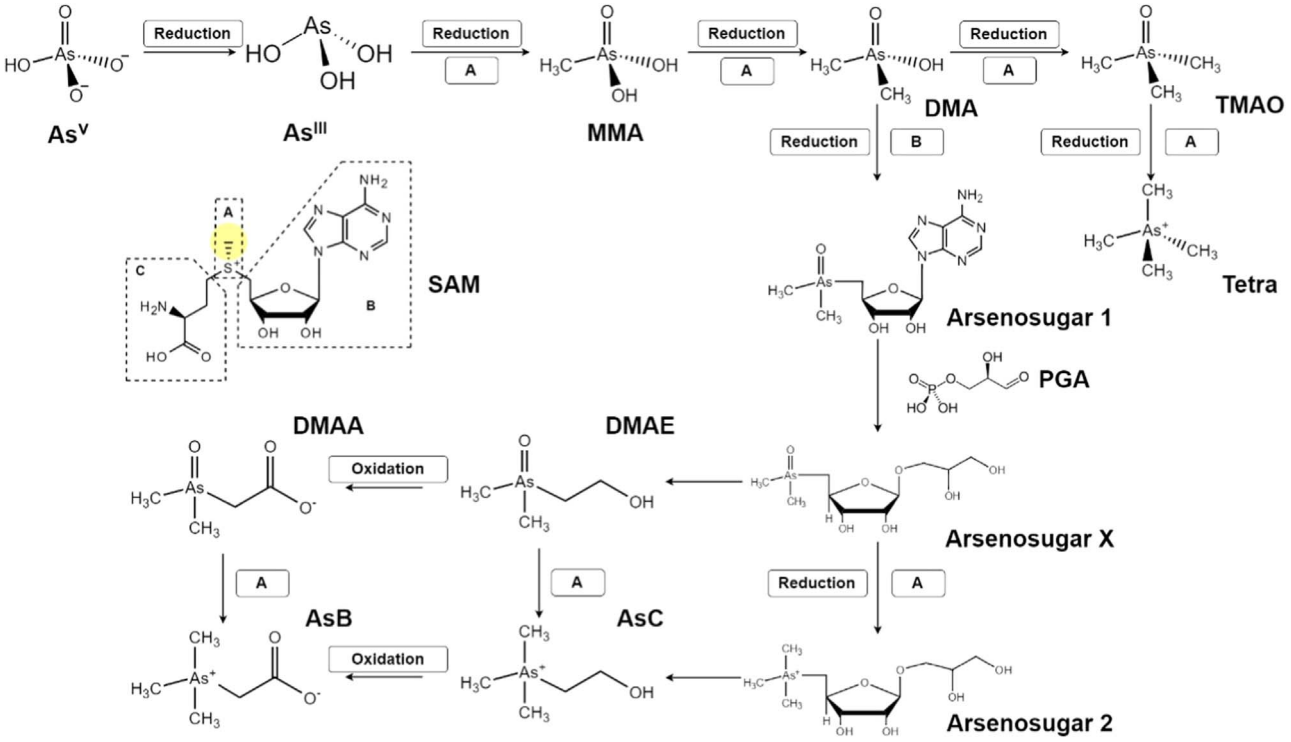

Fig. 7. The possible arsenic metabolism in tree moss body.

(SAM) is an important methyl donor in all eukaryotic cell [28]. In this work, As^{V}, As^{III}, MMA, DMA, TMAO, Tetra, arsenosugar X and AsB are found in the extract of tree moss, and a possible arsenic metabolism process is presented in Fig. 7 based on arsenic metabolism in algae [29].

Tree moss may take in inorganic arsenic from dust and water from surrounding environment and then the process of methylation occurs in tree moss body. Arsenate was reduced to arsenite in cell and then a methyl of SAM was transferred to arsenite to form MMA. Then another active methyl was transferred to MMA and DMA forms. TMAO and Tetra are further methylation products of DMA, and Tetra is rare in tree moss because of sterically hindered methyl in TMAO possibly. Meanwhile, the part of adenosine in SAM was transferred to arsenic atom in DMA to form dimethylarsinoylriboside (Arsenosugar 1) to some extent. Then glycosidation between arsenosugar 1 and 3-phosphoglycerate (PGA) to form arsenosugar X and a methyl from SAM was transferred to arsenosuagr X to form trimethylarsinoylriboside (Arsenosugar 2). The cleavage of the C-C bond of the sugar ring of arsenosugar X can result in the formation of dimethyloxarsylethanol (DMAE), which can transform to AsB through a step of oxidation and methylation. Trimethylarsinoylribosides would transform into AsC by a ring opening reaction, and then AsB can form through an oxidation of AsC [30].

4. Conclusion

In this work, we developed a method involving HPLC-ICP-MS and ESI-qTOF-MS/MS for the identification and quantification of arsenic species in tree moss. We found six arsenic species and three unknown arsenic species based on the retention time. Unk3 was characterized as arsenosugar X and seven arsenic species were quantified. Two unknown arsenic species cannot be identified because of low content and their instability in the process of separation and enrichment. A possible arsenic metabolism was proposed to explain the obtained results on arsenic species found in tree moss body based on its physiological structure and relevant literatures.

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