

Cite this: *J. Anal. At. Spectrom.*, 2013, **28**, 843

Comparative oxidation state specific analysis of arsenic species by high-performance liquid chromatography-inductively coupled plasma-mass spectrometry and hydride generation-cryotrapping-atomic absorption spectrometry†

Jenna M. Currier,^a R. Jesse Saunders,^b Lan Ding,^b Wanda Bodnar,^c Peter Cable,^c Tomáš Matoušek,^d John T. Creed^e and Miroslav Stýblo^{*ab}

The formation of methylarsonous acid (MAs^{III}) and dimethylarsinous acid (DMAs^{III}) in the course of inorganic arsenic (iAs) metabolism plays an important role in the adverse effects of chronic exposure to iAs. High-performance liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC-ICP-MS) and hydride generation-cryotrapping-atomic absorption spectrometry (HG-CT-AAS) have been frequently used for the analysis of MAs^{III} and DMAs^{III} in biological samples. While HG-CT-AAS has consistently detected MAs^{III} and DMAs^{III}, HPLC-ICP-MS analyses have provided inconsistent and contradictory results. This study compares the capacities of both methods to detect and quantify MAs^{III} and DMAs^{III} in an *in vitro* methylation system consisting of recombinant human arsenic (+3 oxidation state) methyltransferase (AS3MT), *S*-adenosylmethionine as a methyl donor, a non-thiol reductant tris(2-carboxyethyl)phosphine, and arsenite (iAs^{III}) or MAs^{III} as substrate. The results show that reversed-phase HPLC-ICP-MS can identify and quantify MAs^{III} and DMAs^{III} in aqueous mixtures of biologically relevant arsenical standards. However, HPLC separation of the *in vitro* methylation mixture resulted in significant losses of MAs^{III}, and particularly DMAs^{III} with total arsenic recoveries $\leq 25\%$. Further analyses showed that MAs^{III} and DMAs^{III} bind to AS3MT or interact with other components of the methylation mixture, forming complexes that do not elute from the column. Oxidation of the mixture with H₂O₂ which converted trivalent arsenicals to their pentavalent analogs prior to HPLC separation increased total arsenic recoveries to $\sim 95\%$. In contrast, HG-CT-AAS analysis found large quantities of methylated trivalent arsenicals in mixtures incubated with either iAs^{III} or MAs^{III} and provided high ($\geq 72\%$) arsenic recoveries. These data suggest that an HPLC-based analysis of biological samples can underestimate MAs^{III} and DMAs^{III} concentrations and that controlling for arsenic species recovery is essential to avoid artifacts.

Received 21st December 2012

Accepted 27th March 2013

DOI: 10.1039/c3ja30380b

www.rsc.org/jaas

Introduction

Arsenic (As) is one of the most prevalent elements in the Earth's crust. Inorganic As (iAs) species, arsenite (iAs^{III}) and arsenate

(iAs^V) are common ground and surface water contaminants.¹ Millions of people worldwide who drink iAs-contaminated water are at risk of developing cancer and non-cancerous diseases, including hypertension, peripheral neuropathy or diabetes.^{2–5} However, the susceptibility to chronic iAs toxicity varies among individuals and depends, in part, on the efficiency and pattern of iAs metabolism. Thus, the analysis of iAs metabolites in biological matrices has become an essential tool for population studies examining the inter-individual differences in responses to iAs exposure, as well as for laboratory studies using animal or *in vitro* models for iAs toxicity and metabolism.

Once ingested, iAs is enzymatically methylated by As (+3 oxidation state) methyltransferase (AS3MT) in a sequence of *S*-adenosylmethionine-dependent reactions.⁶ Both tri- and pentavalent methylated oxoarsenicals are generated in this

^aCurriculum in Toxicology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7461, USA. E-mail: styblo@med.unc.edu; Fax: +1 919-843-0776; Tel: +1 919-966-5721

^bDepartment of Nutrition, Gillings School of Global Public Health, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7461, USA

^cDepartment of Environmental Sciences and Engineering, Gillings School of Global Public Health, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7461, USA

^dInstitute of Analytical Chemistry of the ASCR, v.v.i., Veveří 97, 602 00 Brno, Czech Republic

^eMicrobiological and Chemical Exposure Assessment Research Division, NERL, US EPA, Cincinnati, OH 45628, USA

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c3ja30380b

pathway, including methylarsonic acid (MAs^{V}), methylarsonous acid (MAs^{III}), dimethylarsinic acid (DMAs^{V}), and dimethylarsinous acid (DMAs^{III}) with trimethylarsine oxide (TMA^{VO}) being the final metabolite in some mammalian species.^{7–9} All these tri- and pentavalent oxoarsenicals have been detected in human urine and in biological samples collected in laboratory experiments.^{10–18} Recent studies have suggested that in addition to oxoarsenicals, a variety of sulfur-containing As species (thioarsenicals) can be produced in the course of iAs metabolism, possibly by intestinal bacteria or by reactions of oxoarsenicals with hydrogen sulfide in tissues.^{19–21} To date, only one of these thioarsenicals, dimethylmonothioarsinic acid (DMMTA), has been detected in urine of people exposed to iAs in drinking water.^{22,23} A growing body of evidence suggests that among all known metabolites of iAs, the methylated trivalent oxoarsenicals (MAs^{III} and DMAs^{III}) are the most biologically active and toxic species.^{24–27}

The methods used for speciation analysis of As in biological samples have been discussed in detail in several recent reviews.^{12,28–31} Most of these methods use high-performance liquid chromatography (HPLC) for separation of As species and various spectrometric techniques, including inductively coupled plasma-mass spectrometry (ICP-MS) for quantification of As in the chromatographic fractions. An alternative approach uses hydride generation (HG) to convert iAs and methylated As species in analyzed samples to gaseous arsine (AsH_3) with boiling point (b.p.) of -55°C and methyl-substituted arsines: methylarsine (CH_3AsH_2 , b.p. 2°C), dimethylarsine ($(\text{CH}_3)_2\text{AsH}$, b.p. 36°C) and trimethylarsine ($(\text{CH}_3)_3\text{As}$, b.p. 52°C). The generation of arsines in reaction with sodium borohydride is then followed by a cryotrapping (CT) step and separation of arsines by their boiling points. The HG-CT techniques typically use atomic absorption spectrometry (AAS), atomic fluorescence spectrometry, or ICP-MS for the detection and quantification of As in the separated arsines.^{12,29} In general, the HPLC-based methods are highly specific and can identify a wide spectrum of As species, including species that originate in foods and are not products of iAs metabolism in human body, e.g., arsenobetaine (AsB), arsenocholine (AsC),³² or arsenosugars.³⁰ The HG-based techniques are characterized by low detection limits, but have only limited specificity. A thorough method development and validation using appropriate standards and optimized HG conditions are required for these methods to reliably identify and quantify As species and to avoid artifacts associated with generation of a single form of arsine from two or more As species present in the analyzed samples.

A variety of HPLC-ICP-MS techniques have been used for separation of iAs metabolites and other As species in aqueous solutions and biological samples, mainly urine. Anion-exchange HPLC has been shown in some studies to separate tri- and pentavalent metabolites of iAs and As species originating in seafood, including AsB and AsC .^{10,30,33–35} Other studies used a combination of anion- and cation-exchange chromatography to achieve the same goal.¹⁶ On the other hand, reversed-phase HPLC has successfully separated AsC and the tri- and pentavalent metabolites of iAs, including MAs^{III} and DMAs^{III} with the use of a single column,^{36–40} but could not fully resolve AsB .⁴¹

Notably, while some researchers using HPLC for As speciation analysis found MAs^{III} and/or DMAs^{III} in biological samples, including human urine,^{10,16,35,36,38–40,42–45} others failed to detect these metabolites.^{28,46} What causes these inconsistent results is unclear, but the oxidation of MAs^{III} and DMAs^{III} in aqueous solutions and in human urine^{18,37,47} could play a major role. In addition, HPLC-based analysis typically involves sample preparation (extraction, filtration, etc.) which could produce oxidation state specific conversions and/or provide opportunities for unwanted analyte interaction with the matrix, chemicals or surfaces inherent within the analysis procedure.⁴⁶ Because surprisingly many of the published studies did not employ oxidation state specific procedural spikes or control for As-based mass balance to assess the impact of these undesirable artifacts, it is difficult to determine whether a procedure element was responsible for the analyte loss or conversion.

Unlike HPLC, the HG-CT-based methods, specifically HG-CT-AAS and HG-CT-ICP-MS, have consistently detected MAs^{III} and DMAs^{III} in biological samples, including urine of individuals exposed to iAs in drinking water,^{15,18,48,49} tissues from mice exposed chronically to iAs,^{17,47} mammalian cells or tissue cultures treated *in vitro* with iAs,^{48,50,51} and *in vitro* systems in which iAs was methylated by recombinant rat or human AS3MT.^{50,52} However, because of a limited specificity of the HG-based techniques, results of these analyses have often been questioned and the detection of DMAs^{III} has been viewed by some as an artifact.^{28,53} The present study was designed to address these concerns. This study compares the capacities of previously described reversed-phase HPLC-ICP-MS^{37–40} and HG-CT-AAS^{52,54} techniques to detect and quantify MAs^{III} and DMAs^{III} in an *in vitro* system in which the methylated As metabolites are produced in reactions catalyzed by recombinant human AS3MT and which, because of its simplicity, limits possible artifacts. Results of this study suggest that analysis of biological samples by HPLC-based techniques can underestimate MAs^{III} or DMAs^{III} content due to the formation of stable complexes between these arsenicals and proteins or other endogenous substances and due to retention of these complexes on the chromatographic column.

Experimental

Arsenicals

The following arsenicals were used for method optimization and calibration: iAs^{III} ($\text{NaAs}^{\text{III}}\text{O}_2$) and iAs^{V} ($\text{Na}_2\text{HAS}^{\text{VO}}_4$), both >99% pure from Sigma-Aldrich (St. Louis, MO, USA); MAs^{V} ($\text{CH}_3\text{As}^{\text{VO}}(\text{ONa})_2$) and DMAs^{V} ($(\text{CH}_3)_2\text{As}^{\text{VO}}(\text{OH})$), both >98% pure from Chem Service (West Chester, PA); and custom-synthesized oxomethylarsine (CH_3AsO , $\text{MAs}^{\text{III}}\text{O}$; 96% pure), iododimethylarsine ($(\text{CH}_3)_2\text{AsI}$, $\text{DMAs}^{\text{III}}\text{I}$; 96% pure), TMA^{VO} (94.1% pure), AsC (99.6% pure), and AsB (99.9% pure), which were provided for this study by Professor William Cullen (University of British Columbia, Canada). The identity and purity of the custom synthesized arsenicals were determined by ^1H -NMR and mass spectrometry. DMMTA (92% pure) was synthesized in Dr Creed's laboratory as previously described.⁵⁵ In aqueous solutions, $\text{MAs}^{\text{III}}\text{O}$ and $\text{DMAs}^{\text{III}}\text{I}$ form the corresponding MAs^{III} and DMAs^{III} oxoanions.⁵⁶ Stock solutions of the pentavalent arsenicals,

DMMTA, AsC and AsB, were prepared in deionized water (electrical resistivity $>5 \text{ M}\Omega \text{ cm}$; further referred to as “water”) and stored at -80°C . Stock solutions of the methylated trivalent arsenicals were prepared in ice-cold water immediately before each experiment to limit oxidation.

Instrumentation

HG-CT-AAS. The speciation analysis of As by HG-CT-AAS was performed as previously described.^{50,54} The instrumentation, reagents and operating conditions are summarized in Table 1. Briefly, the HG-CT-AAS system consisted of custom made HG and CT units controlled by FIAS 400 flow injection accessory (Perkin-Elmer, Norwalk, CT) and coupled with AAnalyst 800 spectrometer (Perkin-Elmer) that was equipped with a multiple microflame quartz tube atomizer (multiatomizer).⁵⁴ Arsine and the methyl-substituted arsines from trivalent arsenicals (iAs^{III} , MAS^{III} and DMAS^{III}) and from $\text{TMA}^{\text{V}}\text{O}$ were generated directly in a buffered reaction mixture containing 0.75 M Tris (pH 6) and 1% NaBH_4 in 0.1% KOH (all from Sigma-Aldrich). To generate

arsines from both tri- and pentavalent arsenicals ($\text{iAs}^{\text{III}+\text{V}}$, $\text{MAS}^{\text{III}+\text{V}}$ and $\text{DMAS}^{\text{III}+\text{V}}$) at pH 6, standards or samples were pre-reduced with 2% L-cysteine hydrochloride (EMD Chemicals Inc., Gibbstown, NJ) for 1 hour prior to the HG step. Arsines were cryotrapped in a capillary U-tube filled with Chromosorb WAW-DCMS 45/60 (15% OV-3) (Sigma-Aldrich) submerged in liquid N_2 , and then separated by their boiling points upon heating prior to detection by the AA spectrometer. Concentrations of iAs^{V} , MAS^{V} , and DMAS^{V} were calculated as the differences between species concentrations obtained for the cysteine-treated and untreated sample aliquots.

HPLC-ICP-MS. The HPLC-ICP-MS analysis followed a previously described isocratic reversed-phase HPLC protocol.³⁹ The operating conditions are summarized in Table 1. The Prodigy ODS(3) C_{18} column ($150 \times 4.6 \text{ mm}$, Phenomenex, Torrance, CA) was heated to 30°C and eluted with a mobile phase containing tetrabutylammonium hydroxide (TBAH) (Acros Organic, Morris Plains, NJ), malonic acid (Sigma-Aldrich), and methanol (JT Baker Chemical Co., Phillipsburg, NJ). Ultrapure nitric acid (Fisher Scientific Co., Fair Lawn, NJ) was used to adjust pH of the mobile phase for optimal arsenical separation. The HPLC column was connected with PTFE tubing to the nebulizer of a 7500cx ICP-MS (Agilent Technologies, Palo Alto, CA).

In vitro methylation mixture

Recombinant human wild-type AS3MT which has methionine in position 287 was expressed and purified as previously described.⁵² Methylated arsenicals were generated enzymatically in a reaction mixture containing 100 mM Tris-HCl buffer (pH 7.4, Mediatech, Inc., Manassas, VA), recombinant AS3MT, 1 mM tris(2-carboxyethyl)phosphine (TCEP, Sigma), 1 mM S-adenosylmethionine (SAM, Sigma), and iAs^{III} or MAS^{III} as substrates. The actual measured concentrations of iAs^{III} or MAS^{III} in the mixtures prior to incubation were $0.93 \mu\text{M}$ (i.e., $10.4 \text{ As ng per } 150 \mu\text{L}$) and $1.09 \mu\text{M}$ (i.e., $12.2 \text{ ng As per } 150 \mu\text{L}$), respectively. The reaction mixtures with iAs^{III} contained $80 \mu\text{g mL}^{-1}$ of recombinant AS3MT and were incubated for 2 hours at 37°C . MAS^{III} is methylated by AS3MT more efficiently than iAs^{III} .⁵² Therefore, the mixtures containing MAS^{III} were incubated with $60 \mu\text{g mL}^{-1}$ of AS3MT for only 40 minutes. The mixtures were then chilled on ice and split into aliquots that were analyzed concurrently by HG-CT-AAS and HPLC-ICP-MS. Some of these aliquots were treated with cysteine or 3% H_2O_2 (for 4 hours) prior to analysis as described in Results and discussion. Sums of As species detected by HG-CT-AAS or HPLC-ICP-MS analyses were used to calculate As recovery as percentage of the known amount of As present in the *in vitro* methylation mixture prior to incubation. Spikes of arsenical standards into complete or incomplete reaction mixtures were used to assess interactions between the components of the mixture and the metabolites of iAs^{III} or MAS^{III} and effects of these interactions on results of the HG-CT-AAS and HPLC-ICP-MS analyses.

Ultrafiltration

To examine binding of As species to AS3MT, the reaction mixtures with or without recombinant AS3MT were spiked with

Table 1 HG-CT-AAS and HPLC-ICP-MS operating conditions

HG-CT	Perkin-Elmer FIAS 400
Sample volume	500 μL
Buffer	0.75 M Tris-HCl (pH 6.0)
Reducing agent	1% NaBH_4 in 0.1% KOH
Carrier gases	He (75 mL min^{-1}); H_2 (15 mL min^{-1})
Column packing	Chromosorb WAW-DCMS 45/60 (15% OV-3)
Column heating	Ni80/Cr20 wire, 15Ω
AAS	Perkin-Elmer AAnalyst 800
Lamp	As electrodeless discharge (390 mA)
Wavelength	193.7 nm
Slit width	0.7 nm
Atomizer	Multiatomizer (900°C)
Outer gas	Air (35 mL min^{-1})
HPLC	Agilent 1260 Infinity series
Column	Phenomenex Prodigy 3μ ODS(3) 100A, $150 \times 4.60 \text{ mm}$
Temperature	30°C
Mobile phase	4.7 mM tetrabutylammonium hydroxide + 2 mM malonic acid + 4% methanol (pH 5.85)
Flow rate	1.5 mL min^{-1}
Injection volume	20 μL
ICP-MS	Agilent 7500cx series
Masses	75 (As), 77 (ArCl)
Integration time	0.1 s
RF power	1550 W
Skimmer cone	Ni
Spray chamber	Double-pass Scott-type
Sample depth	8 mm
S/C temperature	2°C
Plasma gas (Ar)	15 L min^{-1}
Carrier gas (Ar)	0.95 L min^{-1}
Make-up gas (Ar)	0.25 L min^{-1}
Cell gas (He)	4.0 L min^{-1}
Nebulizer	Micromist

iAs^{III}, MAs^{III}, or DMAs^{III} (1 μ M each) and incubated on ice for 5 minutes. After incubation, the reaction mixtures were filtered using Nanosep Omega spin columns with 3 kDa cut-off (Pall Life Sciences, Ann Arbor, MI) at 4 °C and 14 000 $\times g$ for 15 minutes. To ensure maximum As recovery, the columns were washed with 100 μ L of 100 mM Tris-HCl buffer (pH 7.4) and centrifuged for an additional 15 minutes at 4 °C. The filtrate and wash containing unbound arsenicals were analyzed by HG-CT-AAS after cysteine pre-treatment.

Statistical analysis

All statistical analyses were performed using the GraphPad Instat and GraphPad Prism software packages (GraphPad Software Inc., San Diego, CA). Linear regression and correlation analyses were employed to characterize the calibration curves. ANOVA followed by Bonferroni's multiple comparison posttest was used to determine significant differences in As concentration between the analytical methods and the effects of protein binding on As recovery. The differences with $p < 0.05$ were considered statistically significant.

Results and discussion

Method optimization

The method optimization focused on tri- and pentavalent As species that are substrates for human AS3MT or are known to be produced in the course of iAs methylation by AS3MT, including iAs^{III}, iAs^V, MAs^{III}, MAs^V, DMAs^{III}, DMAs^V.^{8,9} The following As species that are not products of AS3MT-catalyzed methylation of iAs but are commonly found in human urine were also included: TMAs^{VO}, DMMTA, AsC and AsB.

HG-CT-AAS. The HG-CT-AAS technique has been previously optimized and used for analysis of As species in biological systems as complex as tissue homogenates^{17,47} or as simple as *in vitro* methylation systems in which iAs was methylated by recombinant rat or human AS3MT.^{50,52} The oxidation state specific generation of arsine and methyl-substituted arsines from tri- and pentavalent iAs and methylated arsenicals in a buffered system at pH 6 has been described in details in our previous reports.^{50,54} In absence of cysteine pretreatment, arsines were generated almost exclusively from trivalent arsenicals (iAs^{III}, MAs^{III}, and DMAs^{III}) and from TMAs^{VO}. Only 3.5% of DMAs^V and 11.7% of DMMTA standards (2 ng As each) were converted to dimethylarsine under these conditions.⁵⁰ In the present study, 5.6% of DMAs^V standard (500 pg As) was converted to dimethylarsine in the absence of cysteine (Fig. 1A). Notably, DMMTA cannot be present in the *in vitro* methylation system used in this study because the reaction mixture does not contain H₂S or other donors of sulfur needed for DMMTA formation. Thus, arsine and methyl-substituted arsines generated from the *in vitro* methylation mixture can only represent iAs^{III}, iAs^V, MAs^{III}, MAs^V, DMAs^{III}, DMAs^V, or TMAs^{VO}. Consistent with previous reports,^{57,58} no arsines were detected by HG-CT-AAS analysis of either cysteine-treated or untreated aqueous solutions of AsC or AsB (data not shown).

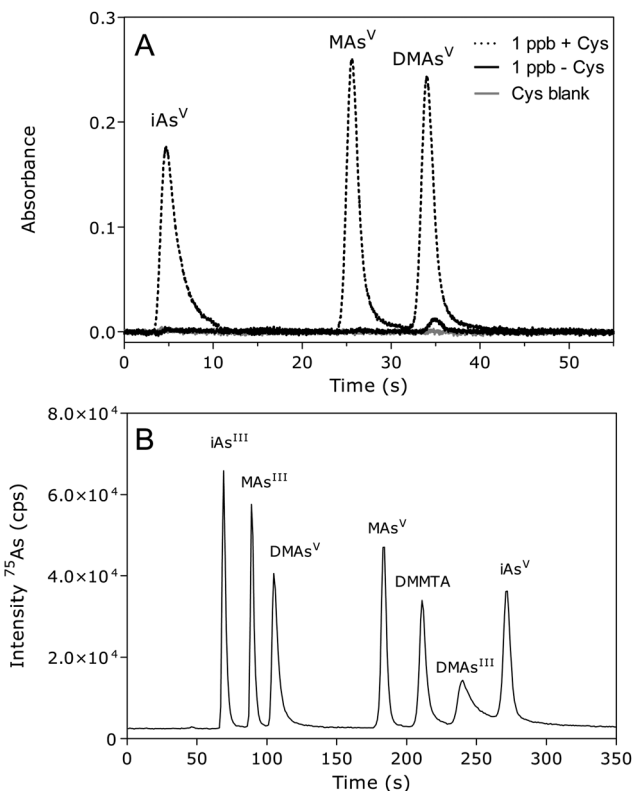


Fig. 1 The separation and detection of As standards by HG-CT-AAS (A) and by optimized HPLC-ICP-MS (B). For HG-CT-AAS, a mixture of three pentavalent oxoarsenicals (500 pg As each) in 500 μ L of water was analyzed directly (–Cys) or after reduction with 2% cysteine (+Cys); the blank containing only water and 2% cysteine (Cys blank) is also shown. For HPLC-ICP-MS, a mixture of six tri- and pentavalent oxoarsenicals and DMMTA (250 pg As each) was injected in 20 μ L of water. The HPLC-ICP-MS and HG-CT-AAS operating conditions are described in Table 1.

HPLC-ICP-MS. The reversed-phase C₁₈ column and mobile phases consisting of TBAH, malonic acid, and methanol have been used by other laboratories for the HPLC-ICP-MS analysis of As species in aqueous solutions, human urine, saliva or cells.^{38–40,59} This method was tested in the present study using a 150 \times 4.6 mm C₁₈ column heated to 30 °C. A nearly base-line separation of six oxoarsenicals (iAs^{III}, iAs^V, MAs^{III}, MAs^V, DMAs^{III}, and DMAs^V) and DMMTA was achieved with the mobile phase consisting of 4.7 mM TBAH, 2 mM malonic acid, and 4% methanol at pH 5.85 and with a flow rate of 1.5 mL min^{–1} (Fig. 1B). In this study, pH 5.6–5.8 or 5.9–6.0 resulted in an incomplete separation of iAs^V, DMAs^{III}, and DMMTA (data not shown). The optimized mobile phase was also used to examine elution profiles of TMAs^{VO}, AsC and AsB. The retention time for AsC was shorter than those for all the other As species; however, TMAs^{VO} and AsB co-eluted with iAs^{III} (Fig. S1, ESI†). The co-elution of TMAs^{VO} and AsB with iAs^{III} did not represent a problem for the current study because neither TMAs^{VO} nor AsB is a product of the methylation of iAs by human AS3MT. However, without further optimization, this HPLC technique could produce artifacts if used for analysis of biological samples that contain all three As species, *e.g.*, human urine.

Calibration

Calibration curves were prepared only for the tri- and pentavalent oxoarsenicals that are products of As methylation by human AS3MT.

HG-CT-AAS. Aqueous solutions of the pentavalent As standards (iAs^V , $MAAs^V$, and $DMAAs^V$) pre-reduced with cysteine were used to generate 5-point calibration curves. The calibration procedure, including validation using the trivalent As standards has been described in our previous reports.^{50,54} Slopes of the calibration curves are provided in Table S1 (ESI[†]). We have previously shown that the slopes of calibration curves for As standards prepared in water and for As standards prepared in complex biological matrices (*e.g.*, cell lysates or tissue homogenates) do not significantly differ.^{17,48,50} Thus, the calibration curves for As standards prepared in water were used in the present study for quantification of As metabolites in the *in vitro* methylation mixtures analyzed by HG-CT-AAS.

HPLC-ICP-MS. Six-point calibration curves were prepared using solutions of tri- and pentavalent As standards in water or in the *in vitro* methylation mixture containing Tris-HCl buffer, SAM and TCEP, but not AS3MT. Consistent with some of the previously published data,^{37,40} slopes of the calibration curves for individual As standards varied (Table S1, ESI[†]). Specifically, the calibration slopes for $MAAs^{III}$ and $DMAAs^{III}$ in either solvent were lower than those for the other As standards, suggesting that these methylated trivalent arsenicals are partially retained

on the column. A lower calibration slope was also found for iAs^V standard prepared in the *in vitro* methylation mixture lacking AS3MT. Thus, the calibration curves for As standards prepared in the mixture containing Tris-HCl buffer, SAM and TCEP, but not AS3MT were used in the present study to quantify As metabolites detected by HPLC-ICP-MS.

Limits of detection (LODs)

Standard deviations (SDs) of the integrated peak areas corresponding to the retention times of As standards were determined for 10 injections of 500 μ L of water or 2% cysteine in water into the HG-CT-AAS system or 20 μ L of water into the HPLC-ICP-MS system. These injection volumes are consistent with the volumes of samples analyzed by HG-CT-AAS and HPLC-ICP-MS in this study. The LOD value was calculated for each As species as: $pg\ As\ mL^{-1} = (3 \times SD)/(\text{slope} \times \text{injection volume})$ (Table S2, ESI[†]). LOD values for HG-CT-AAS ranged from 24 to 57 pg As per mL; LODs for HPLC-ICP-MS were generally higher, ranging from 31 pg As per mL for $MAAs^{III}$ to 295 pg As per mL for iAs^V .

Speciation analysis of As in the *in vitro* methylation mixture

The *in vitro* mixtures were incubated with either iAs^{III} or $MAAs^{III}$ as described in Experimental. Two aliquots from each reaction mixture were immediately and simultaneously analyzed by

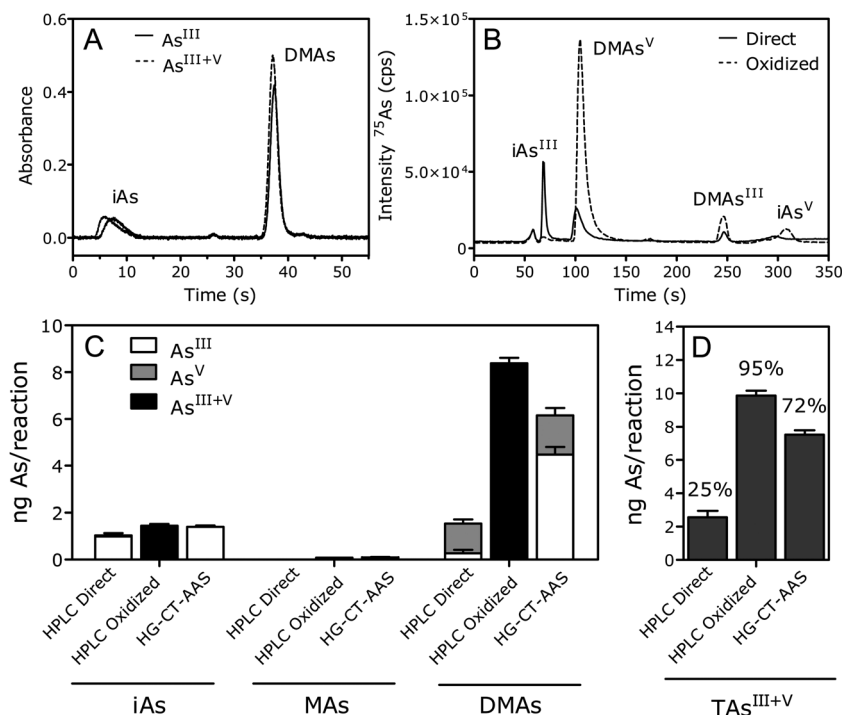


Fig. 2 Analyses of the complete *in vitro* methylation mixtures containing recombinant AS3MT ($80\ \mu g\ mL^{-1}$) incubated with iAs^{III} (10.4 ng) for 2 hours at $37\ ^\circ C$. Representative HG-CT-AAS (A) and HPLC-ICP-MS (B) chromatograms and amounts of As species detected (C) are shown. The recovery of As by either method is expressed in ng As and as percentage of the amount of As added into the reaction mixture prior to incubation (D). The mixtures were analyzed by HPLC-ICP-MS directly (HPLC direct) or after oxidation with 3% H_2O_2 (HPLC oxidized). HG-CT-AAS analysis was performed directly and after 2% cysteine pretreatment to measure As^{III} and As^{III+V} species, respectively. Pentavalent As species were calculated as the difference between these two analyses. Values are expressed as mean + SD for 4 separate reaction mixtures.

HG-CT-AAS and HPLC-ICP-MS. Here, one aliquot (20 μ L of the mixture) was diluted to 500 mL with ice-cold water and analyzed by HG-CT-AAS. The other 20 μ L aliquot was injected directly into the HPLC-ICP-MS system. Two additional aliquots (20 μ L each) were treated either with cysteine for determination of $\text{As}^{\text{III}+\text{V}}$ species by HG-CT-AAS or with H_2O_2 for analysis of $\text{As}^{\text{III}+\text{V}}$ species by HPLC-ICP-MS.

Analysis of the reaction mixture incubated with iAs^{III} . Representative chromatograms and amounts of As species detected in the mixture after incubation with iAs^{III} are shown in Fig. 2. HG-CT-AAS identified two methylated metabolites, DMAs^{III} and DMAs^{V} (Fig. 2A), which on average accounted for 43% and 16% of As added as iAs^{III} into the mixture prior to incubation; 13% of iAs^{III} remained unmethylated (Fig. 2C). The average sum of As species detected by HG-CT-AAS in the mixtures treated with cysteine was 7.5 ng, *i.e.*, 72% of the added As (Fig. 2D). In contrast, two major peaks recorded by the direct HPLC-ICP-MS analysis represented DMAs^{V} and iAs^{III} (Fig. 2B) and accounted for only 12% and 9.4% of the added As, respectively (Fig. 2C). DMAs^{III} was a minor metabolite (3% of the added As). Only $\sim 25\%$ of the added As was recovered by the direct HPLC-ICP-MS analysis (Fig. 2D). However, As recovery by HPLC-ICP-MS increased dramatically after oxidation of the reaction mixture with H_2O_2 , reaching on average 95%. Here, DMAs^{V} was the major As species detected, representing on average 7.1 ng As, *i.e.*, 68% of the added As. Notably, the amount of DMAs^{V} detected in the oxidized mixtures exceeded by 4.6

folds sums of DMAs^{III} + DMAs^{V} found by the direct HPLC-ICP-MS analysis, but was in a good agreement with the sum of DMAs^{III} + DMAs^{V} detected by HG-CT-AAS.

Analysis of the reaction mixture incubated with MAs^{III} . Results of this analysis are summarized in Fig. 3. The HG-CT-AAS analysis found DMAs^{III} to be the major product of MAs^{III} methylation, accounting on average for 52% of the added As; DMAs^{V} represented 10% of the added As. About 21.5% of the substrate remained unmethylated and was partially oxidized during the incubation to MAs^{V} (Fig. 3A and C). The recovery of As in the cysteine-treated mixtures was 83.4% (Fig. 3D). In comparison, DMAs^{V} was the only major species detected by the direct HPLC-ICP-MS analysis, representing 16.7% of added As. No DMAs^{III} and only traces of the unmethylated MAs^{III} were found. In the reaction mixtures oxidized with H_2O_2 , DMAs^{V} and MAs^{V} were the major species, accounting for 74% and 23% of added As. Here again, the total amounts of MAs^{V} and DMAs^{V} in the oxidized mixtures analyzed by HPLC-ICP-MS compared well with the sums of MAs^{III} + MAs^{V} and DMAs^{III} + DMAs^{V} detected by HG-CT-AAS. The average As recovery by HPLC-ICP-MS was 19.3% and 99% for the reaction mixtures before and after oxidation with H_2O_2 .

Taken together, these results suggest that DMAs^{III} is the major product of iAs^{III} and MAs^{III} methylation by recombinant AS3MT. However, the direct HPLC-ICP-MS analysis provides very low As recoveries (19–25%) and underestimates DMAs^{III} yields because a major portion of this metabolite either alone or in complexes with AS3MT protein or other components of the

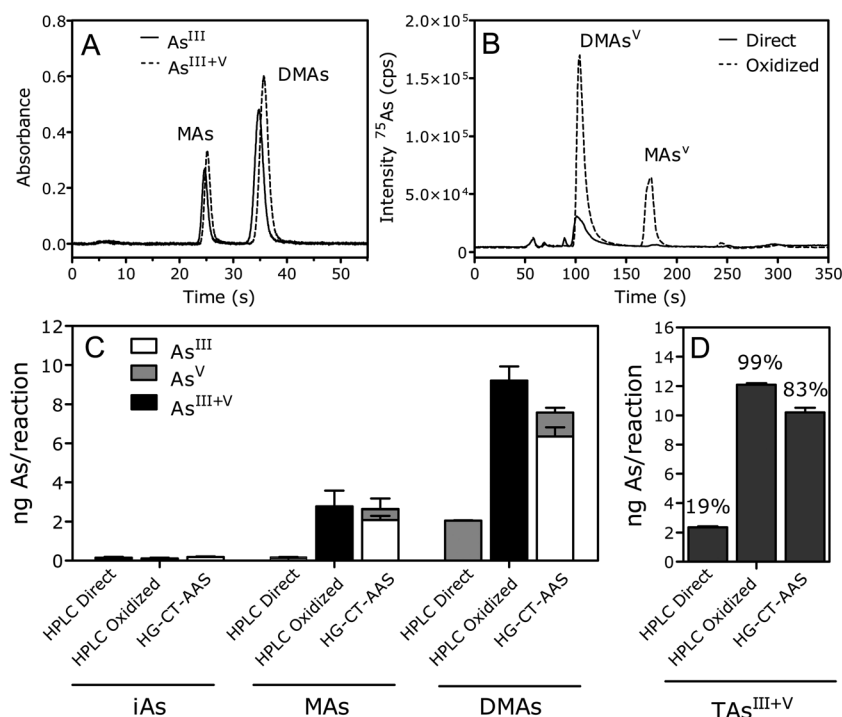


Fig. 3 Analyses of the complete *in vitro* methylation mixtures containing recombinant AS3MT (60 $\mu\text{g mL}^{-1}$) incubated with MAs^{III} (12.2 ng) for 40 min at 37 $^{\circ}\text{C}$. Representative HG-CT-AAS (A) and HPLC-ICP-MS (B) chromatograms and amounts of As species detected (C) are shown. The recovery of As by either method is expressed in ng As and as percentage of the amount of As added into the reaction mixture prior to incubation (D). The mixtures were analyzed by HPLC-ICP-MS directly (HPLC direct) or after oxidation with 3% H_2O_2 (HPLC oxidized). HG-CT-AAS analysis was performed directly and after 2% cysteine pretreatment to measure As^{III} and $\text{As}^{\text{III}+\text{V}}$ species, respectively. Pentavalent As species were calculated as the difference between these two analyses. Values are expressed as mean + SD for 4 separate reaction mixtures.

reaction mixture is lost on the chromatographic column. The interactions of DMAs^{III} with the components of the reaction mixture may also be responsible for the incomplete As recoveries (72–83.4%) during the HG-CT-AAS analysis. Notably, the HPLC-ICP-MS analysis detected DMAs^{III} also in the oxidized methylation mixture. This finding suggests that a longer incubation or a higher concentration of H_2O_2 may be needed to quantitatively release DMAs^{III} from its binding sites and/or to convert it into DMAs^{V} .

Interactions of As^{III} species with the components of the methylation mixture

This and other laboratories have previously shown that rat and human AS3MT require a reductant for activity.^{8,52} Although the reductant function is not entirely clear, it has been hypothesized that it is needed for maintenance or re-activation of the catalytically active AS3MT cysteines. Both thiol and non-thiol reductants have been used in the *in vitro* assay systems for the AS3MT catalyzed methylation of arsenicals. However, trivalent arsenicals are known to bind to thiol. Thus, we chose TCEP, a non-thiol reductant, for this study to limit the formation of thiol- As^{III} complexes which could interfere with As recovery or speciation. To investigate the interactions of DMAs^{III} and other trivalent oxoarsenicals with AS3MT, we compared HPLC profiles for the iAs^{III} , MAs^{III} and DMAs^{III} standards (0.5 μM each) that were incubated for 5 minutes in the complete reaction mixture (1 mM TCEP and 1 mM SAM in 100 mM Tris-HCl buffer) or in the Tris-HCl buffer in the presence or absence of recombinant AS3MT (60 $\mu\text{g mL}^{-1}$). The incubation was carried out at 0 °C to suppress the enzymatic activity of AS3MT. The presence of the recombinant protein in the complete reaction mixture dramatically changed the HPLC profiles and recoveries for all three arsenicals (Fig. 4). Only 47% of iAs^{III} and 6% of MAs^{III} were recovered from the mixtures containing AS3MT as compared to the mixtures that lacked the protein (Fig. 4B and C). However, presence of AS3MT had no significant effects on HPLC profiles and recoveries of the iAs^{III} or MAs^{III} standards that were incubated in the Tris-HCl buffer (Fig. S2A and B, ESI[†]), suggesting that the presence of SAM or TCEP facilitates the interactions of these arsenicals with the enzyme. In contrast, DMAs^{III} was detected only in the mixture with Tris-HCl buffer, but not in the complete reaction mixtures incubated either in the presence or absence of AS3MT (Fig. 4D). Incubation of DMAs^{III} with AS3MT in the Tris-HCl buffer in absence of SAM and TCEP decreased the area of DMAs^{III} to 21% (Fig. S2C, ESI[†]). Incubation of DMAs^{III} in the Tris-HCl buffer with either 1 mM SAM or 1 mM TCEP in the absence of AS3MT also decreased the DMAs^{III} signal, and thus recovery during the HPLC separation (Fig. S2C, ESI[†]). These results suggest that, unlike iAs^{III} or MAs^{III} , DMAs^{III} interacts not only with AS3MT, but also with SAM and TCEP, making a quantitative HPLC-ICP-MS analysis of this metabolite in the complete reaction mixture practically impossible.

Binding of As^{III} species to AS3MT

The interactions of As species with AS3MT were further examined using ultrafiltration. Here, iAs^{III} , MAs^{III} , or DMAs^{III} (1 μM

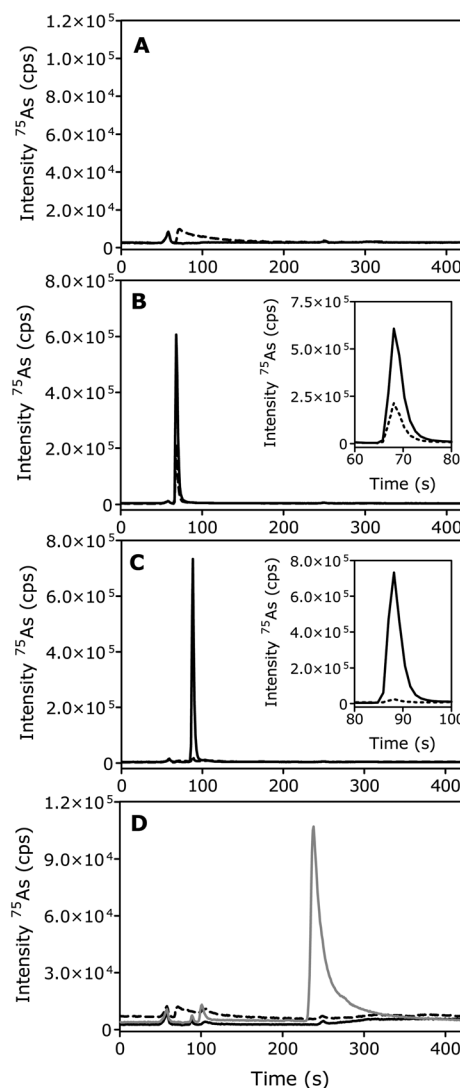


Fig. 4 The HPLC-ICP-MS profiles for trivalent arsenical standards spiked into the complete *in vitro* methylation mixture (1 mM TCEP, 1 mM SAM in 100 mM Tris-HCl buffer, pH 7.4) in absence (—) or presence (---) of recombinant AS3MT (60 $\mu\text{g mL}^{-1}$). Representative chromatograms for the methylation mixtures without As standards (A) and for the mixtures incubated at 0 °C for 5 minutes with 0.5 μM iAs^{III} (B), MAs^{III} (C), or DMAs^{III} (D) are shown. In panel D, 0.5 μM DMAs^{III} in Tris-HCl buffer (pH 7.4) (—) is also shown.

each) were incubated in the complete *in vitro* reaction mixture in presence or absence of recombinant AS3MT (60 $\mu\text{g mL}^{-1}$) at 0 °C for 5 minutes. After incubation, an aliquot of each reaction mixture was treated with cysteine and analyzed by HG-CT-AAS for $\text{As}^{\text{III+V}}$ species. A second aliquot was ultrafiltered using a Pall Omega 3k spin column with a 3 kDa cut-off filter membrane. The filtrate and wash (*i.e.*, the low-molecular weight fraction) were analyzed by HG-CT-AAS for $\text{As}^{\text{III+V}}$ species. Fig. 5 summarizes results of the analyses of the unfiltered aliquots and the ultrafiltrates. The amounts of arsenicals detected by HG-CT-AAS in the unfiltered aliquots of the mixtures containing AS3MT were lower as compared to the unfiltered aliquots of mixtures that did not contain the recombinant protein. However, this difference was statistically significant only for mixtures incubated with

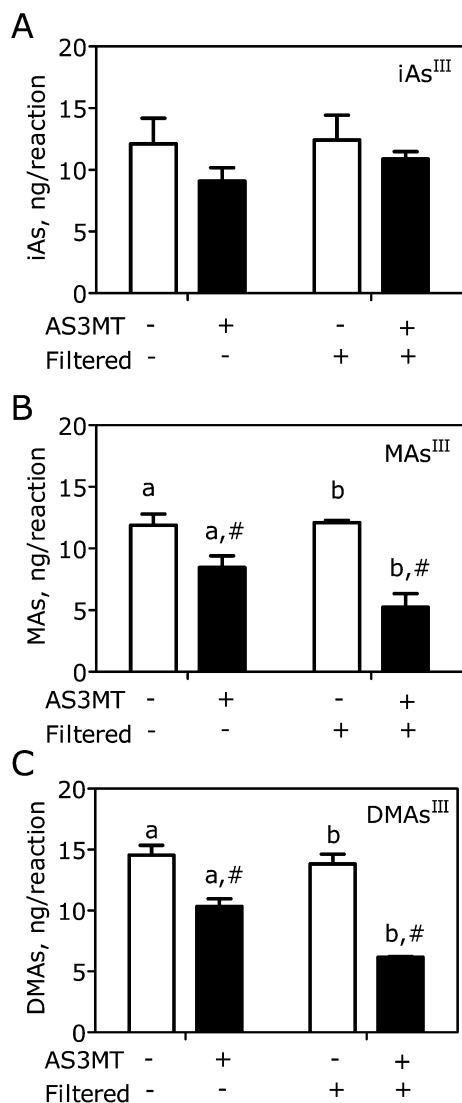


Fig. 5 The amounts of trivalent arsenical standards in the complete reaction mixture before and after ultrafiltration. The mixture (1 mM TCEP, 1 mM SAM in 100 mM Tris-HCl buffer, pH 7.4) was incubated at 0 °C for 5 minutes with 1 μ M iAs^{III} (A), MAs^{III} (B), or DMAs^{III} (C) in presence or absence of recombinant AS3MT (60 μ g mL⁻¹). The As^{III+V} species were analyzed by HG-CT-AAS in the mixtures before and after ultrafiltration using a 3 kDa cutoff membrane. Values are expressed as mean + SD for 3 separate mixtures. The effect of AS3MT protein (a and b) and of ultrafiltration (#) on the recovery of As were examined. Values labeled with the same letter or symbol are significantly different ($p < 0.05$).

MAs^{III} or DMAs^{III} (Fig. 5B and C), suggesting that interactions of these arsenicals with the recombinant protein limit the efficiency of hydride generation. The HG-CT-AAS analysis of ultrafiltrates confirmed that significant amounts of MAs^{III} or DMAs^{III} are bound to AS3MT. Specifically, the amounts of MAs^{III} or DMAs^{III} were significantly lower (by 38 and 40%, respectively) in ultrafiltrates from the AS3MT-containing mixtures as compared to the unfiltered AS3MT-containing mixtures. In contrast, ultrafiltration had no significant effects on the amount of iAs^{III} suggesting that iAs^{III} binds to AS3MT with lower affinity than the methylated trivalent species (Fig. 5A). These findings are consistent with results of a recent study by Marapakala and

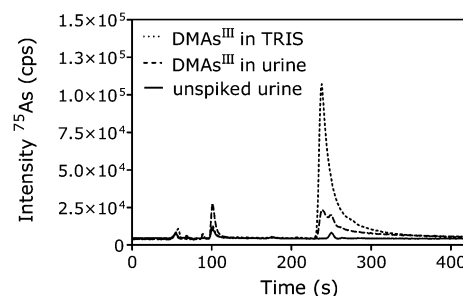


Fig. 6 The HPLC-ICP-MS profiles for 0.5 μ M DMAs^{III} standard spiked into 100 mM Tris-HCl buffer (pH 7.4) or into human urine from an unexposed subject.

associates who showed that MAs^{III} binds faster than iAs^{III} to CmArsM, a bacterial orthologue of AS3MT (binding of DMAs^{III} was not examined).⁶⁰ Notably, the losses of MAs^{III} or DMAs^{III} after ultrafiltration were not as high as those observed during the separation of the *in vitro* methylation mixtures by reversed-phase HPLC (Fig. 2 and 3), suggesting that the AS3MT-bound MAs^{III} or DMAs^{III} partially oxidized and detached from the recombinant protein during the ultrafiltration or that small complexes of arsenicals with TCEP and SAM which are not eluted from the HPLC column can pass through the filter.

AS3MT is a cysteine-rich protein.⁹ Some of its cysteine residues have been shown to play the catalytic role in the process of iAs methylation,^{61–63} possibly providing binding sites for the substrate or reaction intermediates. However, other cysteine-containing proteins and endogenous low-molecular weight thiols, like glutathione have been shown to bind As.^{64–69} Results of the present study suggest that by binding trivalent arsenicals, these proteins and thiols can interfere with quantitative speciation analysis of As by HPLC-based techniques. These results also show that DMAs^{III} interacts not only with AS3MT, but also with other components of the *in vitro* methylation mixture – SAM and TCEP. Thus, it is possible that interactions with these or other compounds may interfere with DMAs^{III} analysis in biological samples, including urine. To test this hypothesis, we compared HPLC-ICP-MS profiles for DMAs^{III} spiked into an ice-cold Tris-HCl buffer or human urine at final concentration of 0.5 μ M (Fig. 6). Notably, the peak area for DMAs^{III} in urine represented only 27% of the peak area in the buffer, suggesting that the urine matrix significantly impairs the ability of HPLC-ICP-MS to detect DMAs^{III} and that calibrating for aqueous standards when analyzing As species in urine could result in a substantial underestimation of DMAs^{III} content.

Conclusions

Results of this study demonstrate that reversed phase HPLC-ICP-MS and HG-CT-AAS are almost equally effective when used for analysis of tri- and pentavalent iAs, MAs and DMAs in standard solutions prepared in water or Tris-HCl buffer. However, when these techniques are utilized for analysis of a simple biological system in which the methylated arsenicals are generated in enzymatic reactions, HG-CT-AAS produces much better As recoveries (mass balances) than does HPLC-ICP-MS. This difference in performance is not detector based but rather

is associated with losses of the methylated trivalent arsenicals during chromatographic separation. Spiking of the reaction mixture with standards provides evidence that MAS^{III} and DMAS^{III} interact with AS3MT and/or other components of the mixture to produce complexes that do not elute from the chromatographic column. These findings are consistent with results of Šlejkovec and associates who reported an on-column binding and losses of As species during analyses using an ion-exchange HPLC-ICP-MS.⁴⁶ The higher recoveries associated with HG-CT-AAS indicate that either the MAS^{III} -complexes and DMAS^{III} -complexes are unstable in the chemical environment associated with the HG step or the complexes produce the same hydrides as do free MAS^{III} and DMAS^{III} . In either case, if quantifying MAS^{III} or DMAS^{III} as a biomarker is an essential part of the study objectives then the existing HG-CT-AAS technique provides far less negative bias as compared to the existing HPLC-ICP-MS approach. This negative bias is not only present in the *in vitro* system used here to illustrate the problem but it should also be expected in other biological matrices which provide binding sites for MAS^{III} and DMAS^{III} , including human urine. The need to assess the losses of trivalent methylated arsenicals during HPLC-based analyses is clearly demonstrated in this study by almost complete recoveries of As from the reaction mixtures treated with H_2O_2 . Thus, laboratories that do not attempt to quantify species specific recoveries or quantify the chromatographic mass balance within the matrix are choosing not to estimate a source of uncertainty that may undermine the reliability and utility of the dataset.

Acknowledgements

This work was supported by NIH grant no. 2 R01 ES010845 to M.S., the UNC Nutrition Obesity Research Center grant no. DK056350, and by NIH grant no. P30ES010126 to the UNC Center for Environmental Health and Susceptibility. Additional support was provided by the Kontakt II program grant (project no. LH12040) from the Ministry of Education, Youth and Sports of the Czech Republic and the AS CR institutional fund RVO:68081715 to T.M. The investigation by J.C. was supported by a pre-doctoral traineeship (National Research Service Award T32 ES007126) from the National Institute of Environmental Health Sciences, NIH. The authors thank Dr William Cullen (University of British Columbia, Canada) for providing custom synthesized MAS^{III} and DMAS^{III} for this study. The views expressed in this article are those of the authors and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency. Mention of trade names, products, or services does not convey, and should not be interpreted as conveying, official EPA approval, endorsement, or recommendation.

References

- 1 P. L. Smedley and D. G. Kinniburgh, *Appl. Geochem.*, 2002, **17**, 517–568.
- 2 IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, *IARC Monogr. Eval. Carcinog. Risks Hum.*, 2004, **84**, 1–477.
- 3 C. H. Tseng, *J. Environ. Sci. Health, Part C: Environ. Carcinog. Ecotoxicol. Rev.*, 2007, **25**, 1–22.
- 4 C. H. Wang, C. K. Hsiao, C. L. Chen, L. I. Hsu, H. Y. Chiou, S. Y. Chen, Y. M. Hsueh, M. M. Wu and C. J. Chen, *Toxicol. Appl. Pharmacol.*, 2007, **222**, 315–326.
- 5 K. A. Thayer, J. J. Heindel, J. R. Bucher and M. A. Gallo, *Environ. Health Perspect.*, 2012, **120**, 779–789.
- 6 S. Lin, Q. Shi, F. B. Nix, M. Styblo, M. A. Beck, K. M. Herbin-Davis, L. L. Hall, J. B. Simeonsson and D. J. Thomas, *J. Biol. Chem.*, 2002, **277**, 10795–10803.
- 7 F. Challenger, C. Higginbottom and L. Ellis, *J. Chem. Soc.*, 1933, 95–101.
- 8 S. B. Waters, V. Devesa, L. M. Del Razo, M. Styblo and D. J. Thomas, *Chem. Res. Toxicol.*, 2004, **17**, 404–409.
- 9 D. J. Thomas, J. Li, S. B. Waters, W. Xing, B. M. Adair, Z. Drobna, V. Devesa and M. Styblo, *Exp. Biol. Med.*, 2007, **232**, 3–13.
- 10 B. Mandal, Y. Ogra and K. Suzuki, *Chem. Res. Toxicol.*, 2001, **14**, 371–378.
- 11 S. M. Cohen, L. L. Arnold, E. Uzvolgyi, M. Cano, M. St John, S. Yamamoto, X. Lu and X. C. Le, *Chem. Res. Toxicol.*, 2002, **15**, 1150–1157.
- 12 Z. Gong, X. Lu, M. Ma, C. Watt and X. Le, *Talanta*, 2002, **58**, 77–96.
- 13 B. Mandal, Y. Ogra, K. Anzai and K. Suzuki, *Toxicol. Appl. Pharmacol.*, 2004, **198**, 307–318.
- 14 M. Okina, K. Yoshida, K. Kuroda, H. Wanibuchi, S. Fukushima and G. Endo, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2004, **799**, 209–215.
- 15 O. L. Valenzuela, V. H. Borja-Aburto, G. G. Garcia-Vargas, M. B. Cruz-Gonzalez, E. A. Garcia-Montalvo, E. S. Calderon-Aranda and L. M. Del Razo, *Environ. Health Perspect.*, 2005, **113**, 250–254.
- 16 R. Xie, W. Johnson, S. Spayd, G. S. Hall and B. Buckley, *Anal. Chim. Acta*, 2006, **578**, 186–194.
- 17 J. M. Currier, M. Svoboda, D. P. de Moraes, T. Matousek, J. Dedina and M. Styblo, *Chem. Res. Toxicol.*, 2011, **24**, 478–480.
- 18 L. M. Del Razo, G. G. Garcia-Vargas, O. L. Valenzuela, E. Hernandez Castellanos, L. C. Sanchez-Pena, J. M. Currier, Z. Drobna, D. Loomis and M. Styblo, *Environ. Health*, 2011, **10**, 73.
- 19 H. Naranmandura, N. Suzuki, K. Iwata, S. Hirano and K. T. Suzuki, *Chem. Res. Toxicol.*, 2007, **20**, 616–624.
- 20 K. M. Kubachka, M. C. Kohan, S. D. Conklin, K. Herbin-Davis, J. T. Creed and D. J. Thomas, *J. Anal. At. Spectrom.*, 2009, **24**, 1062–1068.
- 21 T. S. Pinyayev, M. J. Kohan, K. Herbin-Davis, J. T. Creed and D. J. Thomas, *Chem. Res. Toxicol.*, 2011, **24**, 475–477.
- 22 R. Raml, A. Rumpler, W. Goessler, M. Vahter, L. Li, T. Ochi and K. A. Francesconi, *Toxicol. Appl. Pharmacol.*, 2007, **222**, 374–380.
- 23 F. Cubadda, F. Aureli, M. D'Amato, A. Raggi, A. C. Turco and A. Mantovani, *Pure Appl. Chem.*, 2012, **84**, 203–214.
- 24 D. J. Thomas, M. Styblo and S. Lin, *Toxicol. Appl. Pharmacol.*, 2001, **176**, 127–144.
- 25 S. Lin, L. M. Del Razo, M. Styblo, C. Wang, W. R. Cullen and D. J. Thomas, *Chem. Res. Toxicol.*, 2001, **14**, 305–311.

- 26 Z. Drobna, I. Jaspers, D. J. Thomas and M. Styblo, *FASEB J.*, 2003, **17**, 67–69.
- 27 T. C. Wang, K. Y. Jan, A. S. Wang and J. R. Gurr, *Mutat. Res.*, 2007, **615**, 75–86.
- 28 K. Francesconi and D. Kuehnelt, *Analyst*, 2004, **129**, 373–395.
- 29 A. L. Lindberg, W. Goessler, M. Grandner, B. Nermell and M. Vahter, *Toxicol. Lett.*, 2007, **168**, 310–318.
- 30 K. Hsu, C. Sun and Y. Huang, *Kaohsiung J. Med. Sci.*, 2011, **27**, 382–389.
- 31 I. Komorowicz and D. Baralkiewicz, *Talanta*, 2011, **84**, 247–261.
- 32 B. S. Choi, S. J. Choi, D. W. Kim, M. Huang, N. Y. Kim, K. S. Park, C. Y. Kim, H. M. Lee, Y. N. Yum, E. S. Han, T. S. Kang, I. J. Yu and J. D. Park, *Arch. Environ. Contam. Toxicol.*, 2010, **58**, 222–229.
- 33 K. T. Suzuki, B. K. Mandal and Y. Ogra, *Talanta*, 2002, **58**, 111–119.
- 34 C. B'Hymer and J. Caruso, *J. Chromatogr., A*, 2004, **1045**, 1–13.
- 35 A. Raab and J. Feldmann, *Anal. Bioanal. Chem.*, 2005, **381**, 332–338.
- 36 X. Le, X. Lu, M. Ma, W. Cullen, H. Aposhian and B. Zheng, *Anal. Chem.*, 2000, **72**, 5172–5177.
- 37 Z. L. Gong, X. F. Lu, W. R. Cullen and X. C. Le, *J. Anal. At. Spectrom.*, 2001, **16**, 1409–1413.
- 38 Z. Wang, J. Zhou, X. Lu, Z. Gong and X. Le, *Chem. Res. Toxicol.*, 2004, **17**, 95–103.
- 39 S. Rabieh, A. V. Hirner and J. Matschullat, *J. Anal. At. Spectrom.*, 2008, **23**, 544–549.
- 40 J. Hippler, R. Zdrenka, R. A. D. Reichel, D. G. Weber, P. Rozynek, G. Johnen, E. Dopp and A. V. Hirner, *J. Anal. At. Spectrom.*, 2011, **26**, 2396–2403.
- 41 F. Pan, J. F. Tyson and P. C. Uden, *J. Anal. At. Spectrom.*, 2007, **22**, 931–937.
- 42 X. Le, M. Ma, X. Lu, W. Cullen, H. Aposhian and B. Zheng, *Environ. Health Perspect.*, 2000, **108**, 1015–1018.
- 43 H. Aposhian, E. Gurzau, X. Le, A. Gurzau, S. Healy, X. Lu, M. Ma, L. Yip, R. Zakharyan, R. Maiorino, R. Dart, M. Tircus, D. Gonzalez-Ramirez, D. Morgan, D. Avram and M. Aposhian, *Chem. Res. Toxicol.*, 2000, **13**, 693–697.
- 44 H. Aposhian, B. Zheng, M. Aposhian, X. Le, M. Cebrian, W. Cullen, R. Zakharyan, H. Ma, R. Dart, Z. Cheng, P. Andrewes, L. Yip, G. O'Malley, R. Maiorino, W. Van Voorhies, S. Healy and A. Titcomb, *Toxicol. Appl. Pharmacol.*, 2000, **165**, 74–83.
- 45 K. T. Suzuki, T. Tomita, Y. Ogra and M. Ohmichi, *Chem. Res. Toxicol.*, 2001, **14**, 1604–1611.
- 46 Z. Šlejko, I. Falnoga, W. Goessler, J. T. van Elteren, R. Raml, H. Podgornik and P. Cernelc, *Anal. Chim. Acta*, 2008, **607**, 83–91.
- 47 J. M. Currier, M. Svoboda, T. Matousek, J. Dedina and M. Styblo, *Metallomics*, 2011, **3**, 1347–1354.
- 48 L. M. Del Razo, M. Styblo, W. R. Cullen and D. J. Thomas, *Toxicol. Appl. Pharmacol.*, 2001, **174**, 282–293.
- 49 O. L. Valenzuela, Z. Drobna, E. Hernandez-Castellanos, L. C. Sanchez-Pena, G. G. Garcia-Vargas, V. H. Borja-Aburto, M. Styblo and L. M. Del Razo, *Toxicol. Appl. Pharmacol.*, 2009, **239**, 200–207.
- 50 A. Hernandez-Zavala, T. Matousek, Z. Drobna, D. S. Paul, F. Walton, B. M. Adair, J. Dedina, D. J. Thomas and M. Styblo, *J. Anal. At. Spectrom.*, 2008, **23**, 342–351.
- 51 C. Douillet, J. Currier, R. J. Saunders, W. M. Bodnar, T. Matoušek and M. Stýblo, *Toxicol. Appl. Pharmacol.*, 2013, **267**, 11–15.
- 52 L. Ding, R. J. Saunders, Z. Drobna, F. S. Walton, P. Xun, D. J. Thomas and M. Styblo, *Toxicol. Appl. Pharmacol.*, 2012, **264**, 121–130.
- 53 H. R. Hansen, A. Raab, M. Jaspers, B. F. Milne and J. Feldmann, *Chem. Res. Toxicol.*, 2004, **17**, 1086–1091.
- 54 T. Matousek, A. Hernandez-Zavala, M. Svoboda, L. Langrova, B. M. Adair, Z. Drobna, D. J. Thomas, M. Styblo and J. Dedina, *Spectrochim. Acta, Part B*, 2008, **63**, 396–406.
- 55 M. Fricke, M. Zeller, W. Cullen, M. Witkowski and J. Creed, *Anal. Chim. Acta*, 2007, **583**, 78–83.
- 56 M. Styblo, S. V. Serves, W. R. Cullen and D. J. Thomas, *Chem. Res. Toxicol.*, 1997, **10**, 27–33.
- 57 A. J. Murer, A. Abildtrup, O. M. Poulsen and J. M. Christensen, *Talanta*, 1992, **39**, 469–474.
- 58 X. LE, W. Cullen and K. Reimer, *Appl. Organomet. Chem.*, 1992, **6**, 161–171.
- 59 A. Shraim, N. Sekaran, C. Anuradha and S. Hirano, *Appl. Organomet. Chem.*, 2002, **16**, 202–209.
- 60 K. Marapakala, J. Qin and B. P. Rosen, *Biochemistry*, 2012, **51**, 944–951.
- 61 D. E. Fomenko, S. M. Marino and V. N. Gladyshev, *Mol. Cells*, 2008, **26**, 228.
- 62 X. Song, Z. Geng, J. Zhu, C. Li, X. Hu, N. Bian, X. Zhang and Z. Wang, *Chem.-Biol. Interact.*, 2009, **179**, 321–328.
- 63 X. Song, Z. Geng, X. Li, Q. Zhao, X. Hu, X. Zhang and Z. Wang, *Biochimie*, 2011, **93**, 369–375.
- 64 J. Gailer, G. N. George, I. J. Pickering, R. C. Prince, H. S. Younis and J. J. Winzerling, *Chem. Res. Toxicol.*, 2002, **15**, 1466–1471.
- 65 S. V. Kala, G. Kala, C. I. Prater, A. C. Sartorelli and M. W. Lieberman, *Chem. Res. Toxicol.*, 2004, **17**, 243–249.
- 66 N. A. Rey, O. W. Howarth and E. C. Pereira-Maia, *J. Inorg. Biochem.*, 2004, **98**, 1151–1159.
- 67 X. Cui, Y. Kobayashi, T. Hayakawa and S. Hirano, *Toxicol. Sci.*, 2004, **82**, 478–487.
- 68 A. M. Spuches, H. G. Kruszyna, A. M. Rich and D. E. Wilcox, *Inorg. Chem.*, 2005, **44**, 2964–2972.
- 69 A. J. Percy and J. Gailer, *Bioinorg. Chem. Appl.*, 2008, **2008**, 539082.