

Arsenic–glutathione complexes—their stability in solution and during separation by different HPLC modes

Andrea Raab,^a Andrew A. Meharg,^b Marcel Jaspars,^a David R. Genney^b and Jörg Feldmann^{*a,b}

^aDepartment of Chemistry, College of Physical Sciences, University of Aberdeen, Meston Walk, Old Aberdeen, Scotland, UK AB24 3UE. E-mail: j.feldmann@abdn.ac.uk

^bSchool of Biosciences, University of Aberdeen, St Machar Drive, Aberdeen, Scotland, UK AB24 3UE

Received 11th July 2003, Accepted 24th September 2003

First published as an Advance Article on the web 21st October 2003

Complexes of arsenic compounds and glutathione are believed to play an essential part in the metabolism and transport of inorganic arsenic and its methylated species. Up to now, the evidence of their presence is mostly indirect. We studied the stability and chromatographic behaviour of glutathione complexes with trivalent arsenic: *i.e.* As^{III}(GS)₃, MA^{III}(GS)₂ and DMA^{III}(GS) under different conditions. Standard ion chromatography using PRP X-100 and carbonate or formic acid buffer disintegrated the complexes, while all three complexes are stable and separable by reversed phase chromatography (0.1% formic acid/acetonitrile gradient). As^{III}(GS)₃ and MA^{III}(GS)₂ were more stable than DMA^{III}(GS), which even under optimal conditions tended to degrade on the column at 25 °C. Chromatography at 6 °C can retain the integrity of the samples. These results shed more light on the interpretation of a vast number of previously published arsenic speciation studies, which have used chromatographic separation techniques with the assumption that the integrity of the arsenic species is guaranteed.

Introduction

Arsenic and its species are of great interest today due to their wide occurrence in the environment and their health impact. Oxygen and sulfur compounds of arsenic (inorganic arsenic species) without arsenic–carbon bonds are the most widespread in the abiotic environment. The uptake of these compounds into living cells often results in synthesis of organic arsenic species which contain between one and four arsenic–carbon bonds. This synthesis of organo-arsenicals results from the reduction of pentavalent to trivalent arsenic species followed by oxidative formation of an arsenic–carbon bond. For the formation of the next arsenic–carbon bond the arsenic species has to be reduced again to its trivalent form. The formation of dimethylarsinic acid (DMA(v)), the main excretion product of arsenic by humans, requires for example two reduction and two oxidative methylation steps. The exact mechanism of the reduction step is not yet known, but it is thought that in each case, sulfur-containing molecules, like glutathione (GSH) or thioredoxin (TR), and specific enzymes are involved. In mammalian metabolism, glutathione, the most dominant sulfhydryl-containing species in cells, is essential for the reduction of methylarsonic acid (MA(v)). The complex of GSH and arsenite (As(III)) may be required for enzymatic methylation of As(III) to MA(v).^{1,2} Oxidative formation of arsenic–carbon bonds, normally by methylation, is enzymatically controlled.¹

The uptake mechanism of inorganic arsenic depends on its valence. Arsenate is taken up *via* phosphate transporters and As(OH)₃ *via* glycerol transporters.³ Arsenic export from cells is known to take place *via* specific As(III) exporters.⁴ These exporters might transport arsenite as arsenic–glutathione complexes.⁴ Mono- and dimethylated arsenic species are probably excreted in a similar way. Biliary excretion studies of arsenic species in rats with and without down regulated GSH production have demonstrated the essential role of GSH for arsenic excretion by bile.^{5–7} Other studies demonstrate that bile contains mostly trivalent arsenicals and conclude that these

must be bound to glutathione because of the high affinity of trivalent arsenicals to sulfur.⁸ Experiments in cell culture using synthesised arsenic–glutathione complexes show that their toxicity is comparable to or higher than inorganic arsenic and that they are potent inhibitors of certain enzymes.¹

Up to now, evidence for the occurrence of arsenic–glutathione complexes in biological tissues is mostly derived indirectly by comparison of results from experiments with and without GSH inhibitors.^{5,6} In arsenic resistant earthworms (*Lumbricus rubellus*), arsenic speciation determined by standard methods for extraction (methanol–water) and separation (anion exchange chromatography) show that arsenic is present mainly as As(III); arsenobetaine and As(v) are minor compounds. X-ray absorption spectrometric analysis of these worms shows, however, that arsenic is bound *in vivo* to sulfur containing species, which are not detectable by HPLC-ICP-MS after extraction.⁹ The question of correct *in vivo* identification of arsenic species arising from the earthworm studies led us to investigate the stability of arsenic–glutathione complexes under different separation, extraction and storage conditions using HPLC-ESI-MS and HPLC-ICP-MS in an attempt to find conditions that enable identification and quantification of these complexes.

Over the years, anion and cation exchange chromatography, in addition to ion-pair chromatography, have evolved as the standard separation methods for inorganic and organic arsenicals and are usually used with an ICP-MS as an element specific detector. Since it is necessary to use both an element-specific and a molecular-specific detector for the correct identification of the arsenic–glutathione complexes, the mobile phase of the chosen separation method must be compatible with both ICP-MS and ESI-MS. Since ICP-MS is better suited for non-organic mobile phases and ESI-MS does not give good results using high salt concentrations or non-volatile mobile phases, compromise conditions have to be found and certain techniques can not be used. For these reasons, ion-pair chromatography was incompatible with ESI-MS and therefore not tested further as a separation

method for the complexes, despite successful use in combination with ICP-MS for determination of a whole range of arsenicals.¹⁰ For the same reason, phosphate buffers must also be avoided. Mobile phases found to be compatible with both detectors consist of carbonate, ammonia, formic (or acetic) acid or pyridine, with as little organic modifier (preferable methanol) as possible. Glutathione with pK_a values of 2.1, 3.5 and 9.6 can be expected to present as a mono- and/or divalent anion in solutions between pH 2 and 9. The formation of complexes does not involve peptide carbonyl groups and should consequently not vary the pK_a values to a great extent, which allows the use of anion exchange chromatography for the separation of the arsenic–glutathione complexes. Furthermore, cation exchange and more gentle separation techniques like size exclusion and reverse phase separation were also tested in our study.

Here, we describe the stability of three different arsenic glutathione complexes in solution at different pH values, temperatures, in the presence of matrix and under different chromatographic conditions.

Experimental

Chemicals

All reagents used were analytical grade or better quality. Deionised water (18 MΩ Elga, UK) was used throughout the experiments. Arsenic trioxide (As_2O_3) (BDH, UK), sodium arsenate ($Na_2HAsO_4 \cdot 7 H_2O$) (BDH, UK), disodium monomethyl arsonate (MA(V)) (ChemService, USA), sodium cacodylate (DMA(v)) (Strem, USA) and reduced glutathione (GSH) (Sigma) were used for the preparation of standard solutions and for synthesis of the complexes. Monomethylarsine oxide (MA(III)) and dimethylarsinous iodide (DMA(III)) were synthesised according to published procedures from MA(v) and DMA(v) by reduction with sulfur dioxide and, in the case of DMA(III), potassium iodide.¹¹ Methanol and ethanol (both BDH, UK) were used during synthesis and clean-up of complexes. D_2O used as solvent for the 1H experiments was purchased from Sigma (USA). For the preparation of the mobile phase solutions ortho-phosphoric acid (85% p.a. BDH, UK), ammonia (25% p.a. BDH, UK), citric acid (p.a. BDH, UK), ammonium carbonate (90% BDH, UK), pyridine (99+% BDH, UK), formic acid (100% p.a. BDH, UK), methanol (Fisher, UK) and acetonitrile (HPLC grade, Rathburn, UK) were used.

1H -NMR

A Varian Unity Inova 400 was used for the 1H -NMR measurements with the parameters: relaxation delay 1 s, pulse 44.3 degrees, spectral width 5602.2 Hz at 400 MHz using a line broadening of 0.2 Hz at 25 °C. For the NMR measurements 38–48 mg substance was dissolved in degassed D_2O under nitrogen, the tubes were capped immediately and measurements took place within 2 h of preparation.

HPLC

The HP1100 HPLC system (Agilent Technologies, USA) with cooled autosampler and peltier controlled column-compartment was used throughout the experiments. For separations using columns other than the reversed phase column the column-compartment was set to ambient temperature, for reversed phase chromatography it was varied between 6 and 30 °C. The autosampler was cooled to 4 °C except where stated otherwise. The HPLC parameters used throughout the experiments were 1 mL min⁻¹ mobile phase flow and 100 μL sample volume. For flow injection measurements the sample volume was 20 μL. Post-column the flow was split in a ratio of

1:4 (1 part into the ICP-MS, 4 parts into the ESI-MS) using a microsplitter (Upchurch, UK).

ESI-MS

An HP1100 series LC/MSD (Agilent Technologies, USA) was used as a molecule-specific detector for post-column detection of the arsenic–glutathione complexes by their molecular peaks ($M+H^+$) or $(M+2H)^{2+}$. The MSD was used in the positive or negative ionization mode from m/z 120 to m/z 1000 or in the single ion mode with an API-electrospray head. The settings were: capillary voltage 4000 V, nebulizer pressure 40 psi, drying gas flow 12 L min⁻¹ at 350 °C, quadrupole temperature 100 °C and fragmentor voltage 100 V for positive ionization mode. For negative ionization mode the capillary voltage was 3000 V and the nebulizer pressure 35 psi, the other parameters remained the same. These parameters were optimised using a mixture of the glutathione complexes in the FIA mode of the instrument. The mass calibration was controlled regularly and when necessary optimised using the calibration solution supplied by Agilent (m/z 112 to 2233).

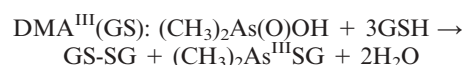
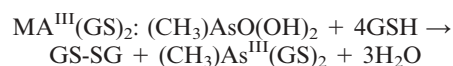
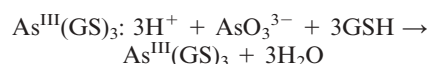
ICP-MS

An ICP-MS 7500 (Agilent Technologies, USA) was used for element specific detection of arsenic. The instrument was equipped with either a Babington nebulizer or a micro-concentric nebulizer (flow rate < 100 μL min⁻¹), a peltier cooled spray chamber and the option to use oxygen as an additional plasma gas. The instrument was mostly used in the soft extraction mode without oxygen, 3% oxygen was mixed into the nebulizer gas when acetonitrile was used in the mobile phase of the HPLC. The instrument settings were checked daily for arsenic sensitivity and optimised when necessary. The elements monitored were arsenic (m/z 75), tellurium (m/z 130), sulfur (m/z 34) and sulfur-oxide (m/z 48).

Synthesis of arsenic–glutathione complexes

Complexes were synthesised according to previously published procedures.¹² Briefly, the synthesis involved stirring the reagents for 12 h at room temperature under nitrogen. Tri(glutamylcysteinylglycyl)trithioarsenite ($As^{III}(GS)_3$) was synthesised by dissolving 117 mg $Na_2HAsO_4 \cdot 7H_2O$ and 0.5 g GSH in 1 mL water (0.38 mmol As to 1.6 mmol GSH). The reaction mixture was then mixed with 3 mL methanol and the resulting precipitate was filtered and dried at room temperature. For di(glutamylcysteinyl-glycyl)methyl-dithio-arsonite ($MA^{III}(GS)_2$) 56 mg MA(v) and 0.6 g GSH were dissolved in 2 mL H_2O (0.4 mmol As to 1.95 mmol GSH), after which the solution was mixed with 10 mL ethanol. The precipitated product was filtered and dried at room temperature. For γ-glutamylcysteinyl-glycyl dimethyl thioarsinite ($DMA^{III}(GS)_2$) 224 mg DMA(V) were mixed in 18 mL water with 1.6 g GSH (1.39 mmol As to 5.21 mmol GSH), after which the solvent was evaporated at room temperature in a rotor-evaporator. The resulting solid was extracted with methanol and the extract evaporated under reduced pressure in the rotor-evaporator. For the formation of $MA^{III}(GS)_2$ and $DMA^{III}(GS)_2$ complexes, a surplus of glutathione was used since the pentavalent arsenicals must be reduced before complex formation.

The reactions follow the equations:



Stability of arsenic–glutathione complexes in solution

In order to test the stability of $\text{As}^{\text{III}}(\text{GS})_3$, $\text{MA}^{\text{III}}(\text{GS})_2$ and $\text{DMA}^{\text{III}}(\text{GS})$ under pH conditions of possible mobile phases, their stability was studied in deionised water, 0.1% (v/v) formic acid pH 2.5 and 15 mM ammonium carbonate pH 8.3 using FIA-ESI-MS at room temperature. The mobile phase for FIA was the same as the solvent for the complexes. The solutions were stored in the autosampler at 4 °C or 25 °C and measured every 6 min.

Stability of arsenic–glutathione complexes under different chromatographic conditions

The synthesised arsenic complexes were separated using either a strong anion exchange PRP X 100 Hamilton (triethylammonium groups) (150 × 4.1 mm), a cation exchange Supelcosil LC-SCX (sulfonate groups) (250 × 4.1 mm), a silica based size exclusion column TSK G2000 SW (250 × 7.6 mm) or a Waters Spherisorb S5 ODS2 (250 × 4.6 mm) reversed phase column. The separation conditions tested are shown in Table 1. Each column was equilibrated with at least 300 mL mobile phase and the sample was injected three times for determination of retention times and reproducibility. Stock solutions of the complexes (*ca.* 1 mg mL⁻¹) were prepared every 3 d under nitrogen in 1% formic acid and stored at 1 °C. The working solutions were prepared daily from these stock solutions in 0.1% formic acid except where stated otherwise.

Stability of the arsenic–glutathione complexes during extraction

Since the determination of arsenic species in most biological matrices requires an extraction step before the separation of the species, the stability of arsenic–glutathione complexes under the extraction conditions needs to be evaluated. Methanol/water mixtures are one of the most often used solvents for the extraction of arsenicals from biological samples, in addition a mixture of water/acetonitrile (1 : 1 v/v) was tested. Due to the increased stability of the complexes in acidic solution, we also tested 1% (v/v) formic acid and 2% (v/v) trifluoroacetic acid (TFA) as extraction media. A non-contaminated sample of the grass *Holcus lanatus* (freeze-dried and powdered) was used as the test matrix, which was first mixed thoroughly with a mixture of the synthesized complexes (16–50 µg per 10 mg grass). These spiked samples were then extracted using 1% (v/v) formic acid, water/methanol (1 : 1 v/v), water/acetonitrile (1 : 1

or 2% (v/v) TFA (1 mL solvent per 10 mg sample). The samples were either stored at 4 °C for 24 h and then filtered or extracted using an ultrasonic bath three times for 10 min each; the samples were cooled to 4 °C for 30 min between each ultrasonic step.

Results and discussion

Synthesis of arsenic–glutathione complexes

To verify the success of the synthesis ¹H-NMR spectra of solutions of the arsenic–glutathione complexes in D₂O were measured (Table 2). The spectra were compared to GSH, DMA(v) and MA(v) spectra measured under the same conditions and to previously reported results.^{12,15} The main shift in the ¹H-NMR occurs at the cysteine H_β protons, the diastereomeric protons split into two multiplets and are shifted to a lower field when compared to GSH, as can be expected when an electronegative atom like arsenic is bound to the neighbouring sulfur. The protons at the methyl groups from DMA bound to GSH were shifted to a higher field and became diastereomeric due to the influence of the GSH. The chemical shifts found by Scott *et al.* for $\text{As}^{\text{III}}(\text{GS})_3$ and $\text{MA}^{\text{III}}(\text{GS})_2$ using 0.5 M phosphate buffer at pH 7.0 are similar to those found by us.¹²

From these NMR-data the amount of complex in the solution was calculated from the ¹H-signals of unbound glutathione and the complexed glutathione as 83% $\text{As}(\text{GS})_3$, 74% $\text{MA}^{\text{III}}(\text{GS})_2$ and 50% $\text{DMA}^{\text{III}}(\text{GS})$.

For additional verification of the complex formation, solutions of the complexes were injected into the ESI-MS using the FIA-mode. Mass spectra of the complexes are shown in Fig. 1a–f. Using the positive scan mode (Fig. 1a–c), $\text{As}^{\text{III}}(\text{GS})_3$ showed a strong molecular mass peak at *m/z* 994 [M + H]⁺ in addition to a signal at *m/z* 497.5 [M + 2H]²⁺. $\text{DMA}^{\text{III}}(\text{GS})$ showed signals at *m/z* 412 [M + H]⁺, *m/z* 823 [2M + H]⁺ and unbound DMA(v) at *m/z* 139 [M + H]⁺. The molecular signals of $\text{MA}^{\text{III}}(\text{GS})_2$ were at *m/z* 703 [M + H]⁺ and *m/z* 352 [M + 2H]²⁺ and, in addition, unbound MA(v) was detected at *m/z* 141 [M + H]⁺. Unbound, reduced glutathione (GSH) at *m/z* 308 [M + H]⁺ and oxidised glutathione (GSSG) *m/z* 613 [M + H]⁺ were detected in all solutions. Using the ESI-MS in negative mode (Fig. 1d–f) $\text{As}^{\text{III}}(\text{GS})_3$ showed the expected molecular peak at *m/z* 992 [M-H]⁻ in addition to GSH (*m/z* 306 [M-H]⁻) and GSSG (*m/z* 611 [M-H]⁻); $\text{MA}^{\text{III}}(\text{GS})_2$ had a molecular peak at *m/z* 701[M-H]⁻ in

Table 1 Chromatographic conditions

Reversed phase chromatography	column: Spherisorb S5 ODS2 (250 × 4.6 mm) eluent A: 1% formic acid in water eluent B: methanol gradient: 0–20 min up to 13% B, then 10 min 100% A or eluent A: 0.1% formic acid in water eluent B: acetonitrile gradient: 0–20 min up to 5–30% B, then 10 min 5% B
Strong anion exchange chromatography	column: PRP X 100 Hamilton (150 × 4.1 mm) mobile phase: 10 mM citric acid pH 2.0 or 20 mM ammonium carbonate pH 8.0
Strong cation exchange chromatography	column: Supelcosil LC-SCX (250 × 4.1 mm) mobile phase: 20 mM pyridine pH 2.5
Size exclusion chromatography	column: TSK G 2000 SW (300 × 7.6 mm) mobile phase: 20 mM pyridine pH 2.5 or 20 mM ammonium carbonate pH 8.0

Table 2 ¹H-NMR values for the protons at the H_β of the cysteine (δ in ppm) and the ¹H -signals for bound DMA and MA (δ ¹H (ppm) for GSH 2.78 m)

$\text{As}^{\text{III}}(\text{GS})_3$	$\text{MA}^{\text{III}}(\text{GS})_2$	$\text{DMA}^{\text{III}}(\text{GS})$
δ ¹ H(ppm), mult, J(par;Hz)	δ ¹ H(ppm), mult, J(par;Hz)	δ ¹ H(ppm), mult, J(par;Hz)
3.07 dd 14.1, 5.1	1.53 s	1.19 s 1.18 s
3.21 dd 14.4, 7.9	2.98 dd 14.0, 4.4	3.00 dd 14.4, 4.8
	3.11 dd 14.0, 7.9	3.12 dd 15.0, 7.2

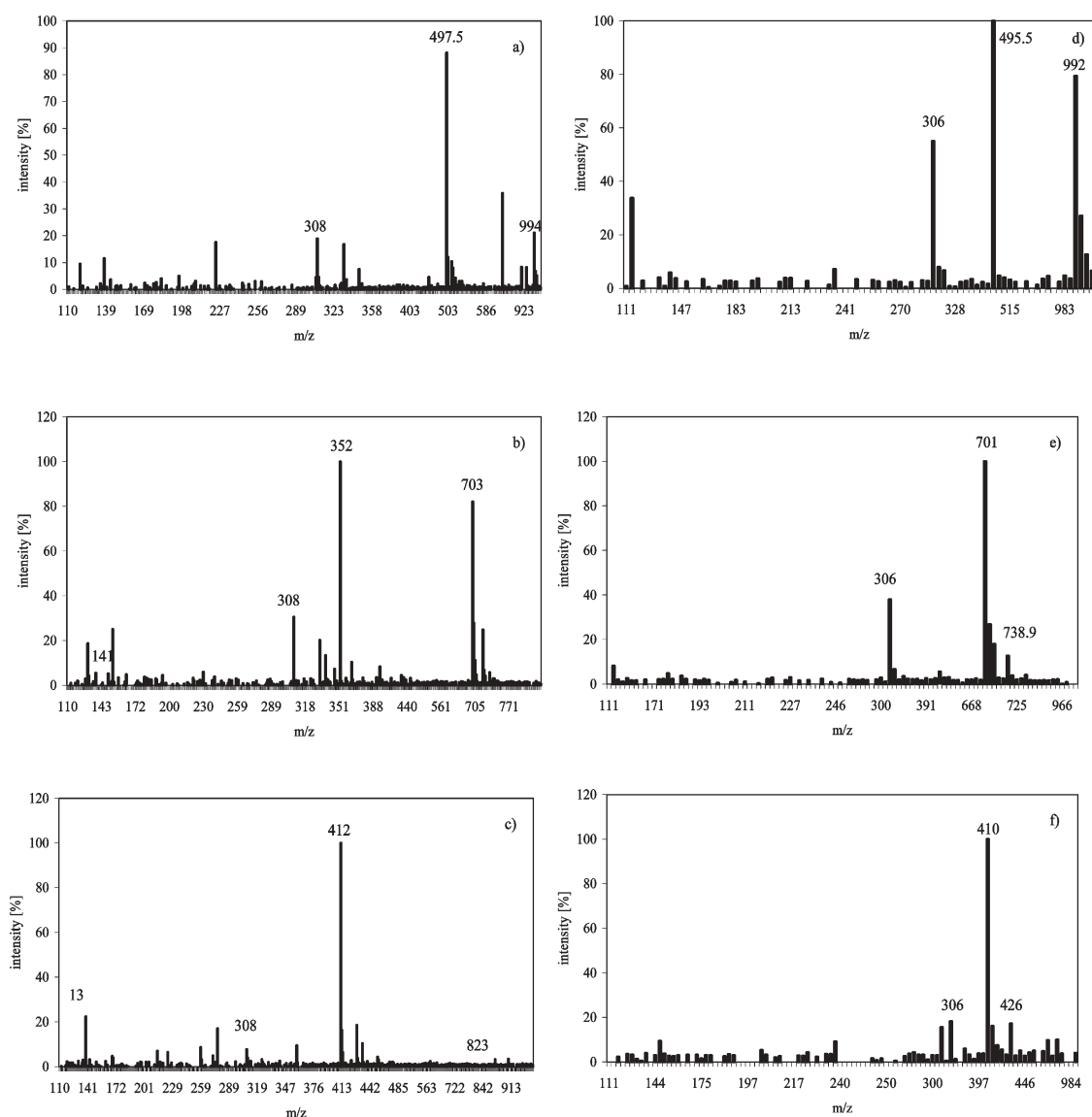


Fig. 1 Mass spectra using flow injection ESI-MS of $\text{As}^{\text{III}}(\text{GS})_3$, $\text{MA}^{\text{III}}(\text{GS})_2$ and $\text{DMA}^{\text{III}}(\text{GS})$ in 0.1% formic acid pH 2.5; positive ionization mode (a) $\text{As}^{\text{III}}(\text{GS})_3$, (b) $\text{MA}^{\text{III}}(\text{GS})_2$, (c) $\text{DMA}^{\text{III}}(\text{GS})$; negative mode, (d) $\text{As}^{\text{III}}(\text{GS})_3$, (e) $\text{MA}^{\text{III}}(\text{GS})_2$, (f) $\text{DMA}^{\text{III}}(\text{GS})$.

addition to unbound $\text{MA}(\text{v})$ at m/z 139 $[\text{M}-\text{H}]^-$ and $\text{DMA}^{\text{III}}(\text{GS})$ had a molecular peak at m/z 410 $[\text{M}-\text{H}]^-$ and unbound $\text{DMA}(\text{v})$ at m/z 137 $[\text{M}-\text{H}]^-$. $\text{As}(\text{III})$ and $\text{As}(\text{v})$ were not detected by ESI-MS under the chosen conditions. In formic acid solutions ($\text{pH} \leq 3$), $\text{As}^{\text{III}}(\text{GS})_3$ and $\text{MA}^{\text{III}}(\text{GS})_2$ preferably formed double charged molecule ions (Fig. 1a–c) while using water or methanol as mobile phase and solvent the single charged molecule ion was the dominant form (data not shown). Positive ionization was used throughout the rest of the experiments, since the negative mode was less sensitive. Neither the intermediates with one GSH attached to $\text{As}(\text{III})$ as $\text{MA}^{\text{III}}\text{GS}$ or two GSH attached to $\text{As}(\text{III})$ were detected nor the pentavalent arsenic glutathione complexes as proposed by Cullen and coworkers.^{13,14} Delnomdedieu *et al.* described the formation of the arsenic–glutathione complex as a two step process, where the first step is the reduction of pentavalent arsenical to its trivalent form, before complex formation takes place.¹⁵ They followed the complex formation with NMR at a glutathione concentration of 0.3 mol L^{-1} .

Stability of arsenic–glutathione complexes in solution

The recovery rates of the different arsenic glutathione complexes at two temperatures in three different mobile

phases/pHs are illustrated in Fig. 2a–c. All three complexes dissolved in ammonium carbonate buffer at pH 8.3 started to degrade in less than 5 min. After 10 min, less than 30% of the original signal was still detectable and complexes were completely degraded after 20 min at both 4°C and 25°C . No significant difference in the stability of the three complexes was detected.

When dissolved in deionised water (pH 6.5) and stored at 4°C for 6 h, $\text{DMA}^{\text{III}}(\text{GS})$ was the least stable complex with 60% loss, followed by $\text{As}^{\text{III}}(\text{GS})_3$ with *ca.* 55% loss and $\text{MA}^{\text{III}}(\text{GS})_2$ with *ca.* 40% loss. At 25°C , the stability of the complexes was drastically reduced with a loss of *ca.* 50% of $\text{MA}^{\text{III}}(\text{GS})_2$ and $\text{DMA}^{\text{III}}(\text{GS})$ after 15 min. $\text{As}^{\text{III}}(\text{GS})_3$ was somewhat more stable at 25°C , but the loss after 90 min was also more than 50% and after 6 h *ca.* 99% of all complexes were disintegrated.

At pH 2.5 in 0.1% (v/v) formic acid, the recovery of $\text{As}^{\text{III}}(\text{GS})_3$ was 95% and for $\text{DMA}^{\text{III}}(\text{GS})$ and $\text{MA}^{\text{III}}(\text{GS})_2$ 100% after 12 h. At 25°C the stability of the complexes was much less. $\text{MA}^{\text{III}}(\text{GS})_2$ and $\text{DMA}^{\text{III}}(\text{GS})$ seem to be less stable at higher temperatures in acidic solution than $\text{As}^{\text{III}}(\text{GS})_3$ of which *ca.* 80% was still present after 90 min. After 6 h storage at 25°C at pH 2.5, less than 1% of the complexes were still detectable. This confirms the results of Delnomdedieu *et al.*¹⁵ who found that $\text{As}^{\text{III}}(\text{GS})_3$ and $\text{DMA}^{\text{III}}(\text{GS})$ are stable in

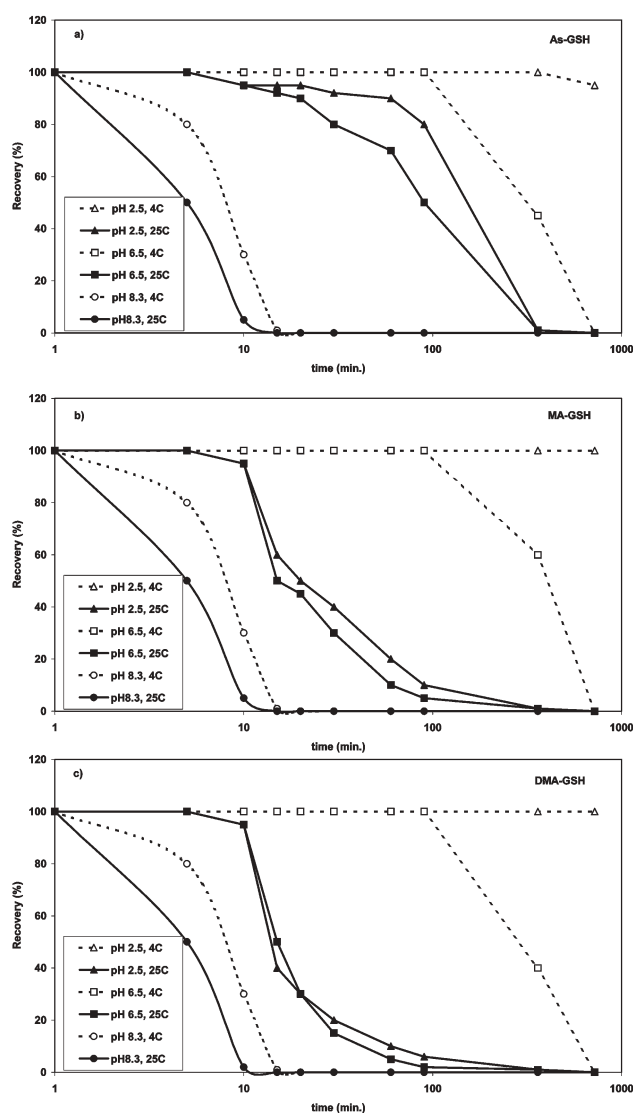


Fig. 2 Recovery of (a) $\text{As}^{\text{III}}(\text{GS})_3$, (b) $\text{MA}^{\text{III}}(\text{GS})_2$ and $\text{DMA}^{\text{III}}(\text{GS})$ (c) in solutions of different pH versus time in dependence of storage temperature. Signals (peak area of m/z 412 ($\text{DMA}^{\text{III}}(\text{GS})$), 703 ($\text{MA}^{\text{III}}(\text{GS})_2$) and 994 ($\text{As}^{\text{III}}(\text{GS})_3$)) was normalized to the measurement after 1 min, standard error *ca.* \pm 3%.

solutions with a pH ranging from 1.5 to 7.7 at ambient temperature using NMR measurements. The dissociation of the

complexes calculated by them was identical for $\text{DMA}^{\text{III}}(\text{GS})$ and $\text{As}^{\text{III}}(\text{GS})_3$. However, no indication was given of how long the complexes were stable. Kala *et al.*¹⁶ studied the stability of the arsenic–glutathione complexes in bile (pH 8.0) over time with HPLC-ESI-MS and found that $\text{As}^{\text{III}}(\text{GS})_3$ is the least stable with a half-life of 20 min, while $\text{MA}^{\text{III}}(\text{GS})_2$ and $\text{DMA}^{\text{III}}(\text{GS})$ were much more stable with a half-life of approximately 40 min. We found that the half-life of the complexes in ammonium carbonate solution at pH 8.3 was slightly shorter (between 5 to 10 min), one of the reasons might be that bile contains other molecules which can stabilise the complexes.

Stability of arsenic–glutathione complexes under different chromatographic conditions

A comparison of the different separation conditions and their ability to separate $\text{As}^{\text{III}}(\text{GS})_3$, $\text{MA}^{\text{III}}(\text{GS})_2$ and $\text{DMA}^{\text{III}}(\text{GS})$ is given in Table 1 and Table 3.

We tested the commonly used strong anion exchange column PRP X 100 from Hamilton with an alkaline and an acidic mobile phase (Fig. 3a and 3b). The use of 15 mM ammonium carbonate (pH 8.0) resulted in a total retardation of GSH, only unbound $\text{DMA}(\text{v})$, $\text{MA}(\text{v})$, $\text{As}(\text{III})$ and $\text{As}(\text{v})$ were detectable (Fig. 3a). Using 10 mM citric acid at pH 2.0, GSH, $\text{DMA}(\text{v})$, $\text{MA}(\text{v})$, $\text{As}(\text{III})$ and $\text{As}(\text{v})$ eluted from the column, but again there were no detectable complexes (Fig. 3b). The fact that $\text{MA}(\text{III})$ was not detectable by ICP-MS or ESI-MS and that the dissociation of the $\text{As}^{\text{III}}(\text{GS})_3$ complex resulted in partial oxidation of trivalent arsenic shows that strong, probably oxidising, interactions occur between the column matrix and the sample molecules. Suzuki *et al.*¹⁷ used 10 mM citric buffer pH 2.0 with a Shodex Asahipak ES-502N 7C column (anion exchange with diethylaminoethyl groups) in combination with ICP-MS detection. They found a slight shift in the retention time of the arsenic species when comparing the retention times of the “complexes” with the retention times of the unbound arsenic species. They did not use a molecule-specific detector so shifts in retention time can never be excluded, especially in a retention time window of only 4 min. Therefore the difference between their results and ours might either not exist because they did not detect the complexes themselves or the different ion exchange groups of the two columns might have a significant influence on the stability of the complexes during separation. Although slight tailing of the peaks indicates disintegration on the column, this tailing is minimal, which indicates that the complexes disintegrated very quickly at the very start of the separation.

Table 3 Retention time and chromatographic recovery of arsenic glutathione complexes during chromatographic separation for different columns as determined by ESI-MS (positive scan mode), separation conditions see Table 1

	GSH <i>m/z</i> 308	$\text{As}^{\text{III}}(\text{GS})_3$ <i>m/z</i> 994	$\text{MA}^{\text{III}}(\text{GS})_2$ <i>m/z</i> 703	$\text{DMA}^{\text{III}}(\text{GS})$ <i>m/z</i> 412	$\text{DMA}(\text{V})$ <i>m/z</i> 139	$\text{MA}(\text{V})$ <i>m/z</i> 141	recovery of total As in % of injected
RP ODS2 C18 acetonitrile gradient	4.5 min	9.6 min	11.3 min	14.3 min	3.5/13.4 min	2.8 min	100 \pm 3% total, 50% as complexes, 50% as $\text{DMA}^{\text{III}}(\text{GS})$, $\text{MA}^{\text{III}}(\text{GS})_2$ and $\text{As}(\text{GS})_3$
RP ODS2 C18 methanol gradient 1%	5.2 min	16.8 min	21.1 min	no (17.8 min DMA <i>m/z</i> 139)	3.9 min	3.0 min	97 \pm 5% total; 57% unbound As, 30% as $\text{As}(\text{GS})_3$, 11% as $\text{MA}^{\text{III}}(\text{GS})_2$
PRP X 100 pH 8	no	no	no	no	3.0 min	4.7 min	99 \pm 2% as $\text{As}(\text{III})$, $\text{DMA}(\text{V})$, $\text{MA}(\text{v})$ not bound to GSH
PRP X 100 pH 2.0	3.0 min	no	no	no	1.7 min	1.8 min	101 \pm 3% as $\text{As}(\text{III})$, $\text{DMA}(\text{v})$, $\text{MA}(\text{v})$ not bound to GSH
Supelcosil pH 2.5	3.8 min	3.9 min	4.0 min	4.4 min	4.2 min	4.0 min	100 \pm 5%, complexes coelute with unbound arsenic species
TSK G2000 SW pH 8	8.4 min	no	no	no	8.8 min	8.5 min	92 \pm 5% as $\text{As}(\text{III})$, $\text{DMA}(\text{v})$, $\text{MA}(\text{v})$ not bound to GSH
TSK G2000 SW pH 2.5	10.7 min	10.2 min	10.4 min	11.1 min	11.2 min	n.d.	90 \pm 5%, complexes in part coelute with unbound arsenic species

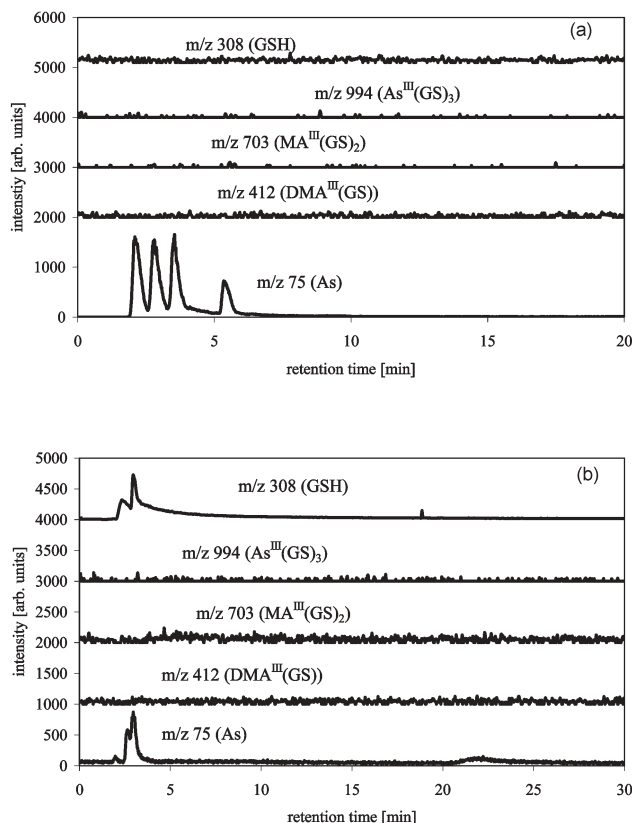


Fig. 3 HPLC-ICP-MS/ESI-MS chromatograms of $\text{As}^{\text{III}}(\text{GS})_3$, $\text{DMA}^{\text{III}}(\text{GS})$ and $\text{MA}^{\text{III}}(\text{GS})_2$ with PRP X 100 Hamilton (150×4.1 mm), buffer: 20 mM ammonium carbonate pH 8.0 (a), or 10 mM citric acid pH 2.0 (b), flow 1 mL min^{-1} , m/z 75 (As) measured by ICP-MS, m/z 412 ($\text{DMA}^{\text{III}}(\text{GS})$), 703 ($\text{MA}^{\text{III}}(\text{GS})_2$), 994 ($\text{As}^{\text{III}}(\text{GS})_3$) and 308 (GSH) measured by ESI-MS. Peaks 1–4 for m/z 75 are As^{III} , $\text{DMA}^{\text{III}}(\text{GS})$, $\text{MA}^{\text{III}}(\text{GS})_2$ and As^{V} .

Cation exchange chromatography was tested using a 20 mmol L^{-1} pyridine buffer at pH 2.5 with a silica-base Supelcosil LC-SCX column (Fig. 4). The complexes seem to elute intact from the column, but as expected, only a slight separation of $\text{As}^{\text{III}}(\text{GS})_3$ and $\text{MA}^{\text{III}}(\text{GS})_2$ from $\text{DMA}^{\text{III}}(\text{GS})$ was achieved since GSH is negatively charged at the pH used for the separation. However, the complexes co-eluted with the unbound arsenicals, therefore no information about the amount of disintegration of the complexes during separation can be given.

Size exclusion chromatography is theoretically the chromatographic technique with the least interactions between sample

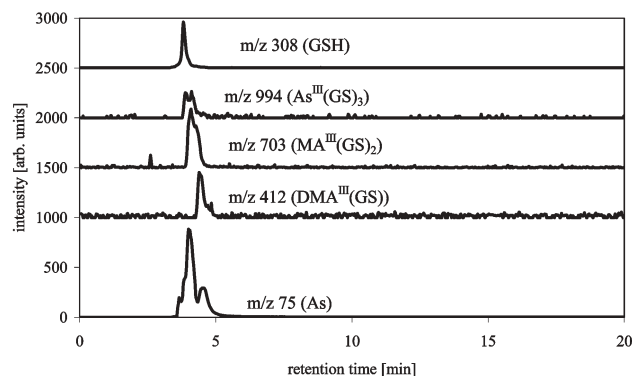


Fig. 4 HPLC-ICP-MS/ESI-MS chromatograms of $\text{As}^{\text{III}}(\text{GS})_3$, $\text{DMA}^{\text{III}}(\text{GS})$ and $\text{MA}^{\text{III}}(\text{GS})_2$ with Supelcosil LC-SCX (250×4.6 mm), buffer: 20 mM pyridine pH 2.5, flow 1 mL min^{-1} , m/z 75 (As) measured by ICP-MS, m/z 412 ($\text{DMA}^{\text{III}}(\text{GS})$), 703 ($\text{MA}^{\text{III}}(\text{GS})_2$), 994 ($\text{As}^{\text{III}}(\text{GS})_3$) and 308 (GSH) measured by ESI-MS.

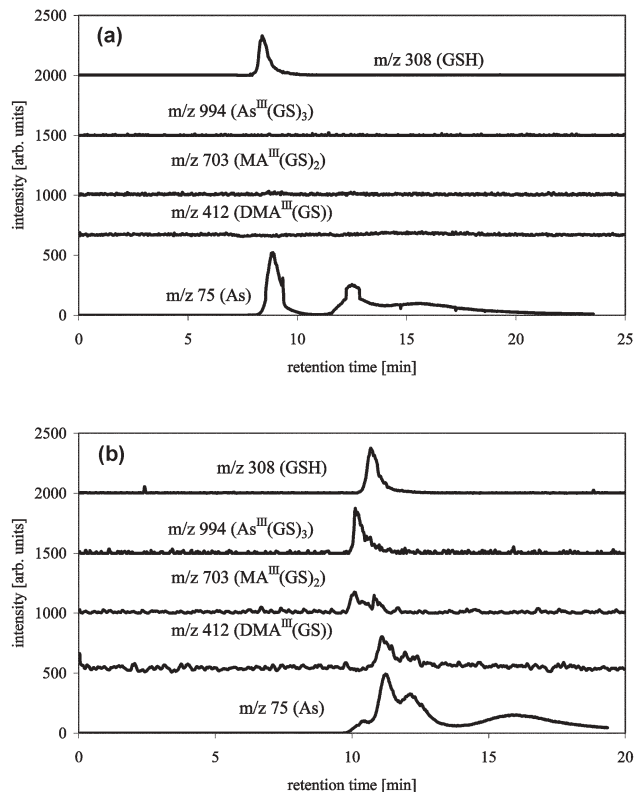


Fig. 5 HPLC-ICP-MS/ESI-MS chromatograms of $\text{As}^{\text{III}}(\text{GS})_3$, $\text{DMA}^{\text{III}}(\text{GS})$ and $\text{MA}^{\text{III}}(\text{GS})_2$ with TSK G2000 SW (300×7.6 mm), buffer: 15 mM ammonium carbonate pH 8.0 (a), or 20 mM pyridine pH 2.5 (b), flow 1 mL min^{-1} , m/z 75 (As) measured by ICP-MS, m/z 412 ($\text{DMA}^{\text{III}}(\text{GS})$), 703 ($\text{MA}^{\text{III}}(\text{GS})_2$), 994 ($\text{As}^{\text{III}}(\text{GS})_3$) and 308 (GSH) measured by ESI-MS.

and column matrix. A TSK G2000 SW column was tested for the separation of the complexes using mobile phases of ammonium carbonate (pH 8.0) or pyridine (pH 2.5). Using a mobile phase of pH 8.0, the complexes were not stable; GSH, $\text{DMA}^{\text{III}}(\text{GS})$ and $\text{MA}^{\text{III}}(\text{GS})_2$ coelute and As^{III} and As^{V} elute later as can be seen from the ICP-MS data (Fig. 5a). Using acidic conditions the complexes elute from the column with a slight separation in the order $\text{As}^{\text{III}}(\text{GS})_3$, $\text{MA}^{\text{III}}(\text{GS})_2$, $\text{DMA}^{\text{III}}(\text{GS}) \approx \text{GSH}/\text{GSSG}$, and inorganic arsenic (Fig. 5b). The same experiments were performed using a polymer based TSK G2000 PW with identical results (data not shown). The column matrix, whether it is silica or polymer, has obviously no influence on the stability of the complexes.

Using reverse phase chromatography $\text{As}^{\text{III}}(\text{GS})_3$ and $\text{MA}^{\text{III}}(\text{GS})_2$ were separable on a C18 ODS2 column using a methanol–1% formic acid gradient (column-compartment at 30°C) (Fig. 6a). $\text{DMA}^{\text{III}}(\text{GS})$ disintegrated on the column using these separation conditions. This disintegration on the column can be followed by observing $\text{DMA}^{\text{III}}(\text{GS})$ on m/z 139, which shows an additional very broad peak that more or less coelutes with $\text{As}^{\text{III}}(\text{GS})_3$ and $\text{MA}^{\text{III}}(\text{GS})_2$. This can also be seen in the ^{75}As trace of the ICP-MS (Fig. 6a) as a broad hump under the signals of $\text{As}^{\text{III}}(\text{GS})_3$ and $\text{MA}^{\text{III}}(\text{GS})_2$. Changing the gradient and/or the concentration of formic acid (0.1% and 2% were also tested) did not improve recovery of $\text{DMA}^{\text{III}}(\text{GS})$ on m/z 412. The only separation condition, so far, which allows separation and detection of all three arsenic–glutathione complexes, is an acetonitrile–0.1% formic acid gradient on a C18 column (Fig. 6b) similar to that described previously.¹⁶ The use of a gradient from 5–30% acetonitrile made the addition of 3% oxygen to the plasma gas necessary. The change from methanol to acetonitrile as the organic solvent improved the recovery of $\text{DMA}^{\text{III}}(\text{GS})$, but even with acetonitrile $\text{DMA}^{\text{III}}(\text{GS})$ partially disintegrated on the column, as can be seen on m/z 139 ($\text{DMA}^{\text{III}}(\text{GS})$) and the ^{75}As trace of the ICP-MS.

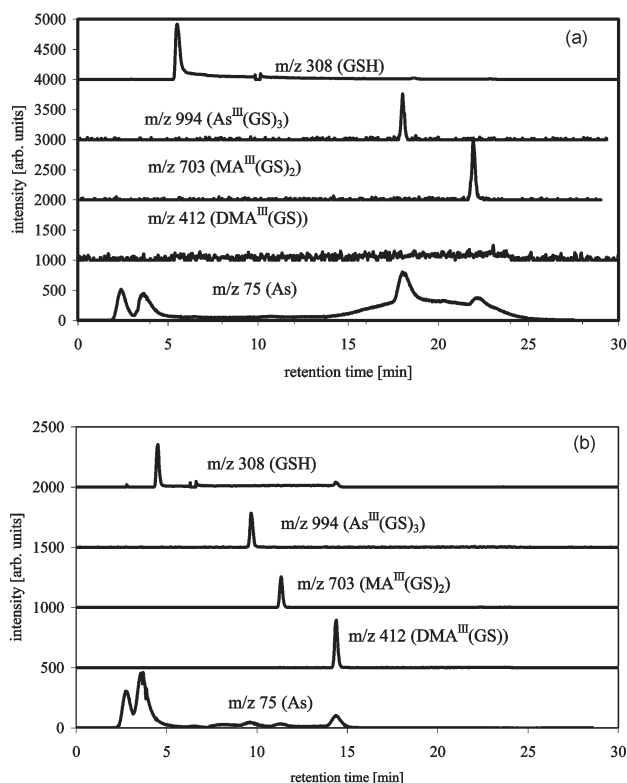


Fig. 6 HPLC-ICP-MS/ESI-MS chromatograms of $\text{As}^{\text{III}}(\text{GS})_3$, $\text{DMA}^{\text{III}}(\text{GS})$ and $\text{MA}^{\text{III}}(\text{GS})_2$ with Waters ODS2 C18, buffer A: 1% formic acid, buffer B: methanol, flow 1 mL min^{-1} , gradient 0–20 min 0–13% B, 20–30 min 0% B (a), or buffer A: 0.1% formic acid, buffer B: acetonitrile, flow 1 mL min^{-1} , gradient 0–20 min 5–30% B, 20–30 min 5% B, column-oven at 6°C (b); m/z 75 (As) measured by ICP-MS, m/z 412 ($\text{DMA}^{\text{III}}(\text{GS})$), 703 ($\text{MA}^{\text{III}}(\text{GS})_2$), 994 ($\text{As}^{\text{III}}(\text{GS})_3$) and 308 (GSH) measured by ESI-MS.

The stability of $\text{DMA}^{\text{III}}(\text{GS})$ during the separation was improved by cooling the column-compartment to 6°C (Fig. 7). Furthermore, a signal at the mass of the oxidised form of $\text{DMA}^{\text{III}}(\text{GS})$ was detectable, whether this oxidation takes place in the interface region of the ESI-MS or on the column needs to be studied further. The instability of trivalent DMA compounds seems to confirm the results from others, e.g., Gong *et al.*¹⁸ found that $\text{DMA}(\text{III})$ was less stable than $\text{MA}(\text{III})$ in urine. Overall, the chromatographic recovery of arsenic is reasonably good. However, under certain conditions, either the arsenic complexes disintegrate on the column giving non-Gaussian peaks or simply fall apart immediately, so that the complexes elute as different arsenic species, which can lead to misidentification and misinterpretation of the species.

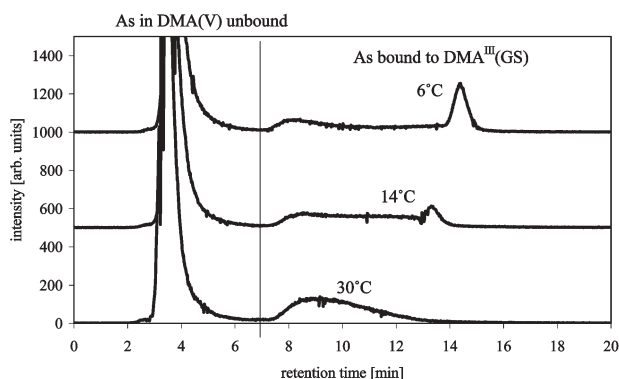


Fig. 7 Effect of column temperature on the separation/recovery of $\text{DMA}^{\text{III}}(\text{GS})$ with Waters ODS 2 C18, buffer A: 0.1% formic acid, buffer B: acetonitrile, flow 1 mL min^{-1} , gradient 0–20 min 5–30% B, 20–30 min 5% B, m/z 75 (As) measured by ICP-MS.

Detection limits

All experiments to date have used arsenic concentrations of the different arsenic species between $16\text{--}8000 \mu\text{g mL}^{-1}$. The detection limits for ESI-MS in the positive scan mode using an acetonitrile gradient are $30 \mu\text{g mL}^{-1}$ for $\text{As}^{\text{III}}(\text{GS})_3$, $10 \mu\text{g mL}^{-1}$ for $\text{MA}^{\text{III}}(\text{GS})_2$ and $40 \mu\text{g mL}^{-1}$ for $\text{DMA}^{\text{III}}(\text{GS})$ based on three times the standard deviation of the blank signal of the biggest molecular peak. Detection limits for the ESI-MS in SIM mode (single ion monitoring) are about a factor of five better than with the scan mode and detection limits in the negative mode are worse than in the positive mode (factor of 5 to 10). Detection limits for arsenic using the ICP-MS in the on-line mode with a flow splitter in the ratio 1 : 4 were around 1 ng mL^{-1} .

Stability of the complexes during extraction

The different extraction methods produced different results. Stability of the complexes was quantified by using the molecular peaks of the different complexes using ESI-MS. When TFA was used, neither method allowed successful identification of any of the complexes. Using the ultrasonic bath method at ambient temperature with the previously cooled sample, all other extractions (water–methanol, water–acetonitrile or 1% formic acid) show disintegration of $\text{DMA}^{\text{III}}(\text{GS})$, confirming again the instability of this complex. However, $\text{As}^{\text{III}}(\text{GS})_3$, $\text{MA}^{\text{III}}(\text{GS})_2$ and $\text{DMA}^{\text{III}}(\text{GS})$ were all detectable in the extracts made by storing the extracts (water–methanol, water–acetonitrile or 1% formic acid) at 4°C . The recovery of the spiked $\text{DMA}^{\text{III}}(\text{GS})$ was 75%, while $\text{As}^{\text{III}}(\text{GS})_3$ had a recovery of 80% and $\text{MA}^{\text{III}}(\text{GS})_2$ of 95% using methanol–water or water–acetonitrile as solvent. The recovery of the complexes is slightly lower with 60% of the spiked $\text{MA}^{\text{III}}(\text{GS})_2$ and 50% of $\text{As}^{\text{III}}(\text{GS})_3$ and $\text{DMA}^{\text{III}}(\text{GS})$ in 1% formic acid solution.

Conclusion

All these experiments about the formation, stability, separation and detection of $\text{As}^{\text{III}}(\text{GS})_3$, $\text{MA}^{\text{III}}(\text{GS})_2$ and $\text{DMA}^{\text{III}}(\text{GS})$ show that biological samples that may contain these complexes must be treated very carefully. They must be kept permanently cooled in an acidic environment and measured as soon as possible if their detection is expected to be successful. Many papers have published studies on arsenic speciation in which the integrity of the arsenic species has been assumed or tested. Our results indicate that many previously published results must be reinterpreted, since possible arsenic glutathione complexes have not successfully been detected due to the harsh conditions used in “routine” analysis of arsenic species in biological extracts.

Acknowledgements

This project has been supported by BBSRC (REI 2002,18479) and AR thanks the College of Physical Sciences for their financial support.

References

- 1 R. A. Zakharyan and H. V. Aposhian, *Chem. Res. Toxicol.*, 1999, **12**, 1278.
- 2 E. Wildfang, R. A. Zakharyan and H. V. Aposhian, *Toxicol. Appl. Pharmacol.*, 1998, **152**, 366.
- 3 O. I. Sanders, C. Rensing, M. Kuroda, B. Mitra and B. P. Rosen, *J. Bacteriol.*, 1997, **179**, 3365.
- 4 B. P. Rosen, *Trends Microbiol.*, 1999, **7**, 207.
- 5 Z. Gregus and A. Gyurasics, *Biochem. Pharmacol.*, 2000, **59**, 1375.
- 6 A. Gyurasics, F. Varga and Z. Gregus, *Biochem. Pharmacol.*, 1991, **42**, 465.

- 7 A. Gyurasics, F. Varga and Z. Gregus, *Biochem. Pharmacol.*, 1991, **41**, 937.
- 8 L. Csanaky and Z. Gregus, *Comp. Biochem. Physiol., C*, 2002, **131**, 355.
- 9 C. J. Langdon, A. A. Meharg, J. Feldmann, T. Balgar, J. Charnock, M. Farquhar, T. G. Pearce, K. T. Semple and J. Cotter-Howells, *J. Environ. Monit.*, 2002, **4**, 603.
- 10 X. C. Le, X. F. Lu, M. S. Ma, W. R. Cullen, H. V. Aposhian and B. S. Zheng, *Anal. Chem.*, 2000, **72**, 5172.
- 11 M. J. Mass, A. Tennant, B. C. Roop, W. R. Cullen, M. Styblo, D. J. Thomas and A. D. Kligerman, *Chem. Res. Toxicol.*, 2001, **14**, 355.
- 12 N. Scott, K. M. Hatlelid, N. E. Mackenzie and D. E. Carter, *Chem. Res. Toxicol.*, 1993, **6**, 102.
- 13 W. R. Cullen, B. C. McBride and J. Reglinski, *J. Inorg. Biochem.*, 1984, **21**, 45.
- 14 W. R. Cullen, B. C. McBride and J. Reglinski, *J. Inorg. Biochem.*, 1984, **21**, 179.
- 15 M. Delnomdedieu, M. M. Basti, J. D. Otvos and D. J. Thomas, *Chem.-Biol. Interact.*, 1994, **90**, 139.
- 16 S. V. Kala, M. W. Neely, G. Kala, C. I. Prater, D. W. Atwood, J. S. Rice and M. W. Lieberman, *J. Biol. Chem.*, 2000, **275**, 33404.
- 17 K. T. Suzuki, T. Tomita, Y. Ogra and M. Ohmichi, *Chem. Res. Toxicol.*, 2001, **14**, 1604.
- 18 Z. L. Gong, X. F. Lu, W. R. Cullen and X. C. Le, *J. Anal. At. Spectrom.*, 2001, **16**, 1409.