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Speciation of volatile antimony compounds in culture headspace gases of *Cryptococcus humicolus* using solid phase microextraction and gas chromatography–mass spectrometry

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Direct analysis of the volatile antimony compounds stibine (SbH_3), monomethylantimony, dimethylantimony (Me_2Sb) and trimethylantimony (Me_3Sb) using solid phase microextraction (SPME) with polydimethylsiloxane fibres and gas chromatography–mass spectrometry (GC–MS) is described. The best analyte to background signal ratio was achieved using a 20 min extraction time. Antimony species were separated using a 3% phenylmethylsilicone capillary column operated at a column pressure of 70 kPa, a flow rate of 1.4 ml min^{-1} and temperature ramping from 30 to 36 °C at 0.1 °C min^{-1}. Cryogenic focusing of desorbed species was required to achieve resolution of antimony species. The optimized SPME–GC–MS method was applied to the analysis of headspace gases from cultures of *Cryptococcus humicolus* incubated with inorganic antimony(III) and (V) substrates. The headspace gases from biphasic (aerobic–anaerobic) biomass-concentrated culture incubations revealed the presence of SbH_3, Me_2Sb and Me_3Sb. Stibine was the major antimony species detected in cultures amended with inorganic antimony(V). Me_3Sb was the sole volatile antimony species detected when cultures were amended with antimony(III).

KEYWORDS: SPME; GC–MS; antimony; methylantimony; stibine; biomethylation; *Cryptococcus humicolus*

INTRODUCTION

Solid phase microextraction (SPME) gas chromatography–mass spectrometry (GC–MS) has been used extensively for the analysis of volatile organic compounds (VOCs).[1–4] SPME with GC–MS or inductively coupled plasma (ICP) MS detection has, in recent times, been applied to the analysis and speciation of derivatized involatile organometallic compounds of lead, tin and mercury.[5–7] As yet, there are no reports of the analysis of volatile organometallic compounds of environmental origin using SPME.

SPME is a simple, solventless technique that allows the rapid pre-concentration of trace compounds onto a fibre from samples. Volatile species may be sampled from the gaseous phase, whilst involatile species may be sampled from the aqueous phase, or derivatized to a volatile form and sampled from the headspace.[8] The technique excludes water from the extract, avoiding the analysis problems associated with water when headspace and aqueous samples are extracted by other means.[9,10] This is advantageous for the analysis of environmental samples and microbial cultures, where abundant quantities of water vapour may be present in headspace gases.

The SPME technique relies upon the equilibration of analytes between the liquid, and or the headspace gas and the stationary phase coating of the fibre. Equilibration, therefore, depends on the dissociation constant of the analyte and the thickness of the stationary phase. The amount of analyte adsorbed by the fibre is directly proportional to the concentration of the analyte in the

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sample when the system is at equilibrium. The relationship of these factors is described by Eqn. (1):[11]

$$n = \frac{C_0 V_1 V_2 K}{K V_1 + K_2 V_3 + V_2} \quad (1)$$

where n is the number of analyte moles sorbed by the fibre coating, C_0 is the initial analyte concentration in the aqueous phase, V_1, V_2 and V_3 are the volumes of the coating, aqueous phase and the headspace respectively, K_2 is the partition coefficient of the analyte between the headspace and the aqueous phases, and K (= K_1K_2) is the global partition coefficient of analytes between the fibre coating and the aqueous phase.

SPME is therefore regulated by partition coefficients and Henry's constants (which are temperature dependent),[11] and also the volume of aqueous sample and headspace volume and the time allowed for adsorption to occur.

There are several reports of antimony biomethylation by undefined communities of bacteria grown under anaerobic conditions.[12–14] Recently, Michalke *et al.*[15] reported the biomethylation of inorganic antimony by pure cultures of three methanogenic Archaea species, of the proteolytic bacteria *Clostridium collagenovorans* and of the sulfogen *Desulfovibrio vulgaris*. Volatile trimethylantimony was detected as the sole methylantimony species in headspace gases of these cultures. Michalke *et al.* also reported the detection of stibine in *Methanobacterium formicium* cultures amended with antimony trichloride.[15]

A number of groups have reported on the ability of the fungus *Scopulariopsis brevicaulis* to produce volatile antimony species from inorganic antimony substrate.[16–18] Jenkins *et al.*[16] reported the production of trimethylantimony from potassium antimony tartrate (PAT) by *Scopulariopsis brevicaulis* and noted the production of unknown volatile antimony species when cultures were amended with antimony trioxide or antimony pentoxide. Andrewes *et al.*[17] also reported the production of low concentrations of trimethylantimony, as well as trace concentrations of stibine, monomethylantimony and dimethylantimony, from PAT by this organism.

We report the development of an SPME–GC–MS method for the analysis of volatile organoantimony compounds, and the application of this method to the analysis of volatile antimony species produced by the fungus *Cryptococcus humicolus* when cultures are amended with antimony(III) and (V) compounds. This organism is a known biomethylator of inorganic arsenic and has been demonstrated to form trimethylarsenic in headspace gases.[19,20]

EXPERIMENTAL

Preparation of methylantimony standards

Stibine, monomethylantimony, dimethylantimony and trimethylantimony standards were prepared by hydride-generating trimethylantimony dichloride (kindly donated by Professor W. R. Cullen, University of British Columbia, Canada) in an oxygenated atmosphere to achieve dismutation of the trimethylated antimony species to the lower methylated compounds. Volatile hydrides of these species were generated in a 5 ml reaction vial containing 4.5 ml acidified sample (200 µl 10% (w/v) HCl, 4.05 ml deionized water containing trimethylantimony dichloride standard) by the injection of 250 µl 8% (w/v) sodium borohydride *via* the septum.

SPME

SPME was performed using polydimethylsiloxane fibres, 100 µm film thickness (Supelco, Castle Hill, New South Wales, Australia). SPME fibres were conditioned in the GC injection port for 1 h at the start of the analytical run, and subsequently for 20 min between each injection. Headspace extractions were performed at 25 °C from standards and liquid samples (culture media) that were agitated continuously using magnetic stirring. Sensitivity of SPME sampling of analytes from headspace gases has been demonstrated to be inversely related to the headspace volume.[21,22] The sample headspace was set at 0.5 ml for standards and 4 ml for samples, since these were the smallest headspace volumes that could practically be used. The ratio of aqueous phase to headspace volume was maintained for samples and standards.

The gauge setting (to adjust fibre length) on the manual fibre holder for headspace extractions was 1.8. Once the extraction period was complete, the gauge setting on the manual fibre holder was adjusted to 3.0 and the fibre was transferred to the GC injector port. The extraction period was varied to optimize extraction of species of interest with respect to background noise.

GC–MS analysis of methylantimony species

All analyses were performed on an HP 5890 gas chromatograph with a split–splitless injector, a carbon dioxide cryogenic focusing unit (SGE, Ringwood, Victoria, Australia), a flame ionization detector, and an HP 5970 mass spectrometer (Hewlett Packard, Blackburn, Victoria, Australia). The injector was operated in splitless mode with a delay time of 30 s. Separations were performed using a 30 m × 0.25 mm (0.25 µm film thickness) HP-5 (3% phenylmethylpolysiloxane) capillary column (Hewlett Packard). The inlet port and detector were maintained at 250 °C and 300 °C respectively. Helium (1.4 ml min^{-1}) was used as the carrier gas. Column head pressure was maintained at 70 kPa. Cryogenic focusing of volatile species was achieved by cooling the initial part of the column to −50 °C. After a sample run, the oven temperature was raised to 250 °C for 20 min to purge any materials retained on the column. The mass spectrometer was operated in electron-impact mode. Scanning was over the range 50–250 mass units.

Preparation of *C. humicolus* cultures

C. humicolus was maintained by routine sub-culture on

solidified YM medium prepared as described by Yamada *et al.*[23] Liquid YM medium was used for culture incubations.

The medium was prepared such that the final volume occupied 40% of total flask volume, e.g. for a 500 ml flask 200 ml of medium was used. The medium was amended with antimony(III) as PAT or antimony(V) as potassium hexahydroxyantimonate (PHHA) (Sigma–Aldrich, Poole, Dorset, UK), to give antimony concentrations of 50 mg l^{-1}, from sterile stock solutions that had been autoclaved. Culture inoculum was prepared from agar streak plates, incubated overnight at 28 °C. Culture was swabbed off agar plates and resuspended in 10 ml aliquots of fresh liquid medium to produce a turbid cell suspension with an absorbance of one unit at $\lambda = 600$ nm. Inoculum was added to culture medium at 0.5 ml per 100 ml. Cultures were incubated at 28 °C and 100 rpm in a Gallenkamp orbital incubator for 6 days. Aliquots (31 ml) of culture were transferred to 35 ml reaction vials containing a magnetic flea and sealed with a needle septum. Reaction vials were incubated anaerobically (up to 18 days), until SPME analysis was performed, in an orbital incubator (at 28 °C and 100 rpm) to minimize deposition of biomass in the reaction vials. Concentrated biomass cultures were prepared for SPME analysis by concentrating 6-day cultures tenfold by centrifugation (4000 rpm, 10 min), and incubating as described above. Control incubations were prepared by omitting inoculation of biomass.

GC–atomic absorption spectrometry (AAS) analysis of volatile antimony species

Headspace gases from *C. humicolus* cultures were also analysed by GC–AAS. Concentrated cultures were prepared as described above for SPME–GC–MS analysis. After the incubation period was complete, headspace gases were purged using a flow of helium (40 ml min^{-1}) for 10 min through a liquid-nitrogen-cooled 50 cm × 4 mm i.d. glass column packed with PT 10% OV101 on 80/100 mesh Chromosorb-W-HP (Alltech Associates, Deerfield, Illinois, USA) wound with nickel–chromium resistance wire (10.04 Ω m^{-1}). After purging, the liquid nitrogen was removed and the column was heated electrothermally by constant voltage application of 12 V, to a final temperature of 90 °C. Volatile species were eluted from the column according to their boiling point and analysed using a Perkin Elmer (Beaconsfield, Bucks., UK) PE1300 atomic absorption spectrometer, as described previously.[24]

RESULTS AND DISCUSSION

Optimization of SPME parameters

SPME headspace extractions were performed at 25 °C, since higher temperatures generally favour extraction of involatile species.[8,25] Increasing the extraction period from 5 to 20 min selectively increased the amount of methylantimony analyte detected over background signal by GC–MS (Fig. 1). Further increasing the extraction period to 40 min again increased the amount of methylantimony detected, but also significantly increased the background signal. The background signal was identified as being siloxanes, phthalates and hexanedioc acid esters, which most likely arose from the plastic septa system used with the reaction vial. The contaminating peaks did not arise from the fibre itself, since analysis of blank fibres did not reveal the presence of such compounds.

The extraction time of an analyte by SPME is directly related to the analyte partition coefficients (K_1 and K_2), which generally increase with increasing molecular weight.[22] Increasing the extraction period will therefore result in

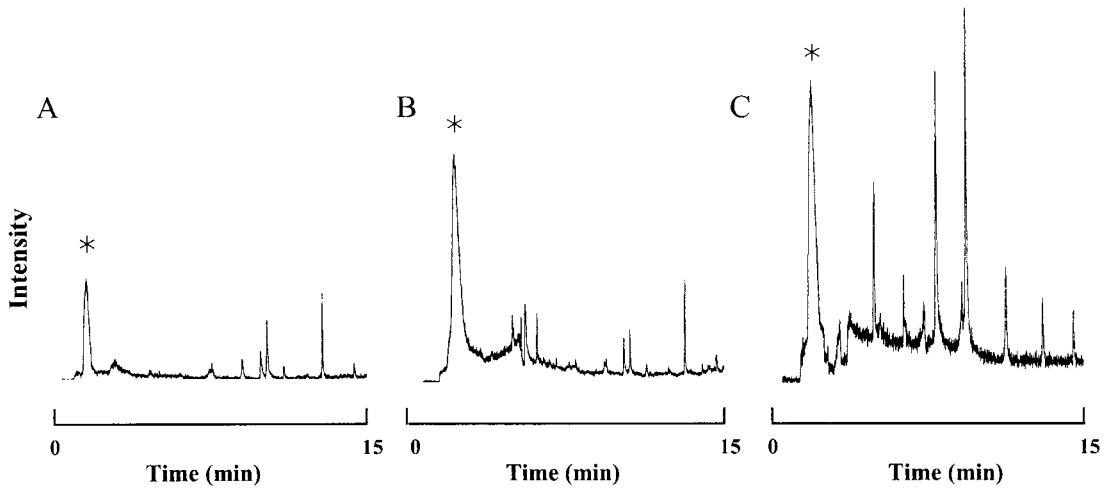

Figure 1. SPME–GC–MS chromatograms demonstrating effect of extraction time upon extraction of trimethylantimony from headspace of a reaction vial containing sodium borohydride derivatized trimethylantimony dichloride. Extraction periods are (A) 5 min, (B) 20 min and (C) 40 min. The peak marked * represents trimethylantimony.

selective enhancement of the extraction of higher molecular weight compounds, such as the plasticizers observed here.

Possible carryover between subsequent analyses, i.e. the incomplete desorption of sample analyte from SPME fibres, was investigated. Analysis of consecutive fibre blanks following extraction of a 40 μg Sb l^{-1} derivatized trimethylantimony chloride solution did not reveal the presence of retained stibine or methylantimony species. This indicates that the volatile antimony species were efficiently desorbed from the fibre under the experimental conditions used. Cleaning of fibres between extractions was performed (20 min at 250 °C) to fully desorb recalcitrant higher molecular weight compounds, such as siloxanes and phthalates, from the fibre.

Optimization of GC–MS parameters

Removal of the GC guard column and reduction in diameter of the GC injector port inlet liner from 4 to 2 mm i.d. both resulted in peak sharpening, since the linear velocity of the analytes through the injector port was increased and analytes were introduced onto the column in a narrower band.

Decreasing the column temperature program (initial column temperature 40 °C to 35 °C; final temperature 100 °C at 10 °C min^{-1} to 50 °C at 5 °Cmin^{-1}) had no observed effect on the resolution of the four antimony species. Resolution of dimethylantimony and trimethylantimony species was achieved by cryogenic focusing of the antimony species desorbed from the SPME fibre before separation on the capillary column (Fig. 2). Decreasing the column temperature heating program from 5 °C min^{-1} to 0.1 °C min^{-1} after cryogenic focusing resulted in the resolution of all four volatile antimony species; stibine, monomethylantimony hydride, dimethylantimony hydride and trimethylantimony (Fig. 2). The identity of these species was confirmed by their mass spectra (see Fig. 3 for examples).

Optimized procedure

Extraction of volatile antimony species from culture headspace gases was performed at 25 °C for 20 min. The culture medium was agitated continuously using magnetic stirring. The gauge setting on the manual fibre holder was 1.8 during the extraction period. Once the extraction period was complete, the fibre was retracted and the gauge setting on the manual fibre holder was adjusted to 3.0. The fibre was immediately transferred to the GC injector port for analysis. Samples were desorbed and cryogenically focused by cooling the initial part of the column to −50 °C. Oven

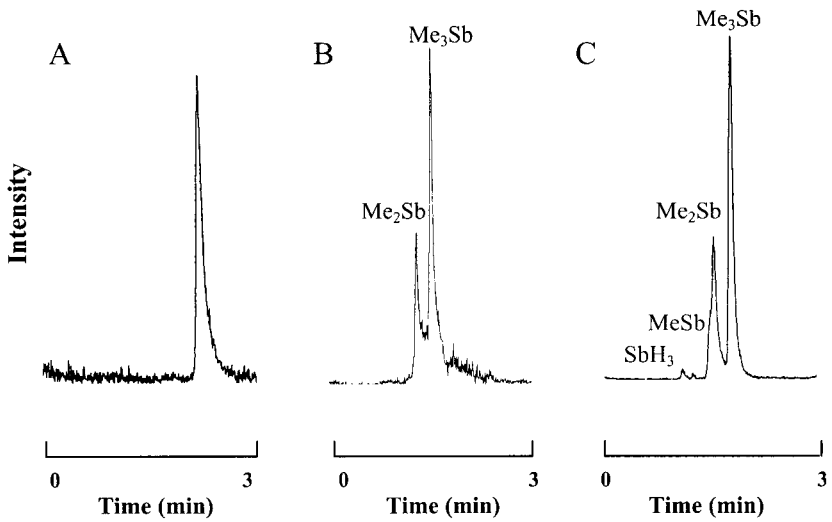

Figure 2. SPME–GC–MS chromatograms of volatile antimony species produced by sodium borohydride derivatization of trimethylantimony dichloride demonstrating the effect of initial column temperature and subsequent temperature ramp upon peak shape and resolution of all four volatile antimony species. The column was held at initial temperature for 2 min and then elevated to the final temperature at the ramp rate shown: (A) 35 °C, 50 °C; 5 °C min^{-1}; (B) 35 °C, 45 °C; 1.0 °C min^{-1}; (C) 30 °C, 36 °C; 0.1 °C min^{-1}. Resolution of dimethylantimony hydride and trimethylantimony (B), and stibine, monomethylantimony hydride, dimethylantimony hydride and trimethylantimony (C), was achieved by cryogenic focusing of the antimony species desorbed from the SPME fibre before separation on the GC column. Retention times of volatile antimony species were: stibine, 1.3 min; monomethylantimony hydride, 1.5 min; dimethylantimony hydride, 1.8 min; trimethylantimony, 2.1 min.

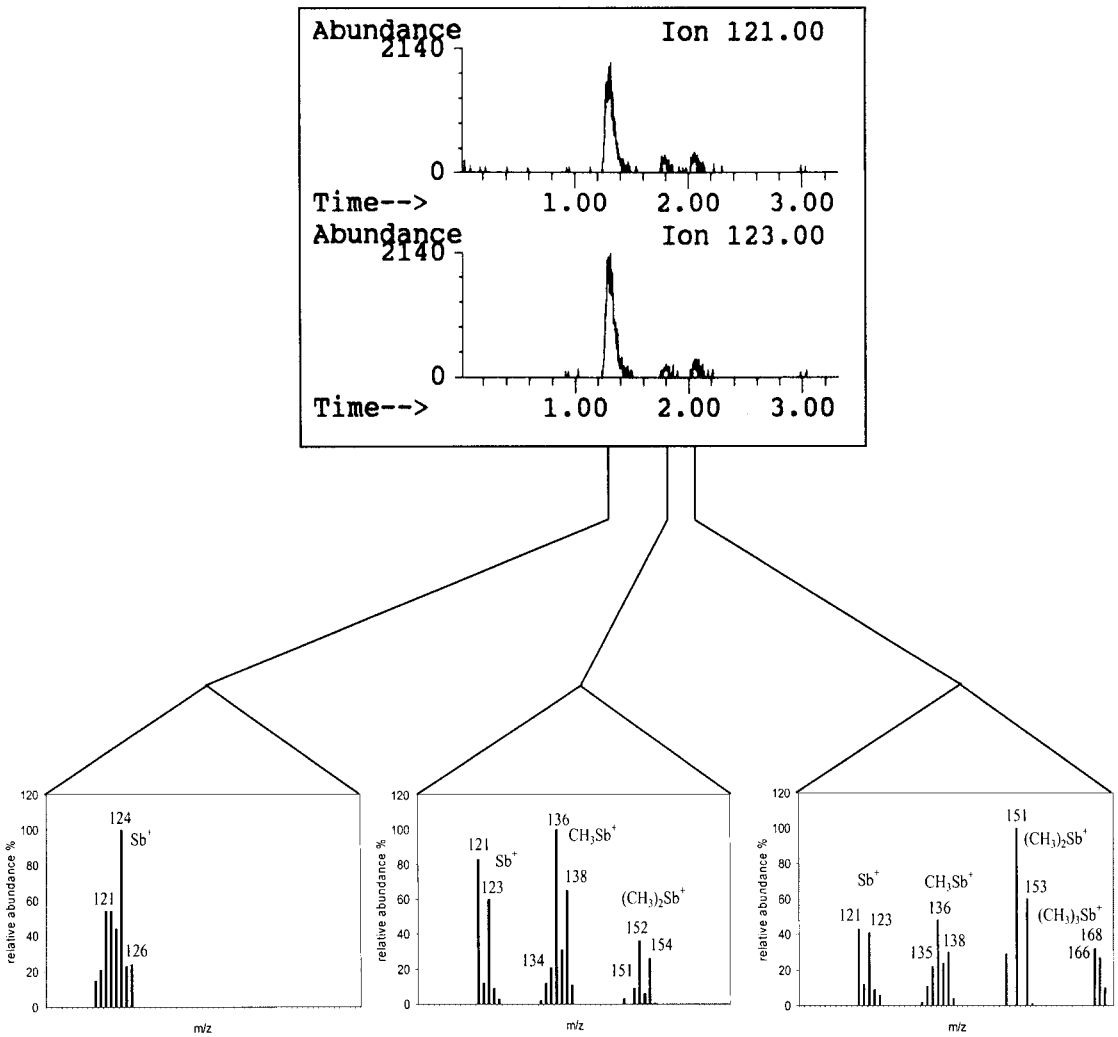

Figure 3. Mass/ion chromatogram and fragmentation patterns of (from left to right) stibine, dimethylantimony hydride and trimethylantimony obtained by SPME of the headspace of *C. humicolus* cultures supplied with PHHA.

temperature was programmed such that (oven) temperature was maintained at 30 °C for 2 min, and then ramped to 36 °C at 0.1 °C min^{-1} and held at this temperature for a further 2 min. After each sample was analysed the temperature of the oven was raised to 250 °C for 20 min to purge any materials retained on the column.

A linear calibration was obtained for trimethylantimony dichloride loading and total amount of volatile antimony species detected by GC–MS ($y = 4.898x - 1.8339$, $R^2 = 0.978$). Volatile antimony species could be reliably detected at a loading of 0.8 μg Sb l^{-1} trimethylantimony dichloride. Quantification of volatile antimony species in culture headspace gases was made by correlating the peak area of standards with the amount of volatile antimony derivatized from trimethylantimony dichloride (based on measurement of antimony, by ICP-MS in reaction mixtures pre- and post-derivatization of trimethylantimony dichloride). Our results indicated that around 50% (RSD 6.8%) of the trimethylantimony dichloride is derivatized to volatile antimony species by sodium borohydride under these reaction conditions. Hence, for standards: with a 4.5 ml aqueous phase and a 0.5 ml headspace, 1.1 μg Sb l^{-1} (5.0 ng total aqueous loading) trimethylantimony dichloride standard in the aqueous phase was assumed to form 2.5 ng of volatile antimony species (stibine 0.05 μg Sb l^{-1}, monomethylantimony 0.03 μg Sb l^{-1}, dimethylantimony 1.43 μg Sb l^{-1}, trimethylantimony 3.49 μg Sb l^{-1}). The ratio of volatile antimony species was observed to be constant over the concentration range of trimethylantimony dichloride studied. The concentration of volatile antimony species detected in culture headspace gases was volume corrected and expressed in terms of amount of antimony per gram dry weight biomass.

SPME–GC–MS analysis of volatile antimony species produced by *C. humicolus*

Analysis of the headspace gases from antimony(V)-amended

Table 1. SPME–GC–MS analysis of volatile antimony species present in headspace gases from *C. humicolus* incubations

Metal substrate	Antimony species (ng g^{-1} dry biomass)[a]			
	SbH_3	MeSb	Me_2Sb	Me_3Sb
PAT [Sb(III)]	nd[b]	nd	nd	22.1 ± 1.5
PHHA [Sb(V)]	110.6 ± 8.4	nd	5.1 ± 0.3	11.1 ± 0.8

[a] Mean ± standard deviation, $n = 3$ replicate culture incubations.
[b] nd = not detected (<0.4 ng g^{-1} dry biomass), inorganic antimony substrate was supplied to incubations at 50 mg Sb l^{-1}.

cultures after 6 days aerobic and 18 days anaerobic incubation showed the presence of stibine, dimethylantimony hydride and trimethylantimony (Fig. 3). Stibine was the predominant antimony species detected (Table 1). In contrast, trimethylantimony was the sole volatile antimony species detected in the headspace of cultures amended with antimony(III) (Table 1). No volatile antimony species were detected in any of the control incubations in which *C. humicolus* was absent, indicating that the volatile antimony species detected arose as a result of biological activity.

Direct GC–AAS analysis of headspace gases (transferred under helium flow to a liquid-nitrogen-cooled column) from *C. humicolus* cultures (tenfold biomass-concentrated) supplied with PAT confirmed the detection of trimethylantimony as sole volatile antimony species in culture incubations supplied with antimony(III) substrate (Fig. 4). Up to 22.7 ng Sb g^{-1} dry biomass of trimethylantimony was observed, which is compatible with the SPME–GC–MS measurement of 22.1 ng Sb g^{-1} dry biomass for similar incubations (Table 1). This corresponds to a volatilization efficiency of 0.001% of the inorganic antimony substrate supplied. No volatile antimony species were detected in controls without *C. humicolus* or antimony substrate, confirming the biogenic nature of the trimethylantimony formation and volatilization. These data are the first demonstration of antimony biomethylation from an inorganic antimony substrate by this organism, and demonstrates stibine formation by a eukaryotic organism. Although Andrewes *et al.*[17] described the detection of trace concentrations (picogram) of stibine, monomethylantimony and dimethylantimony during incubation of *S. brevicaulis* with PAT and isotopically enriched (98.7%) ^{123}PHHA, the detection of stibine and monomethylantimony hydride in *S. brevicaulis* headspace gases was intermittent and not reproduced in replicate incubations.

Prokaryotic volatilization of antimony has recently been demonstrated Michalke *et al.*[15] These authors reported the detection of nanogram quantities of stibine in culture headspace gases of *M. formicium* cultures supplied with antimony(III) substrate. Monomethylantimony hydride, dimethylantimony hydride and trimethylantimony were also shown to be present in culture headspace gases. By contrast, in the work reported on here for the eukaryote *C. humicolus*, stibine was produced from antimony(V) substrate but not from antimony(III) substrate. Initial oxidation of antimony(III) substrate to an antimony(V) form by *M. formicium* would account for this apparent difference in antimony substrate requirement between these organisms. Prokaryotic bio-oxidation of antimony [(III) to (V) transformation] and of the closely related Group 15 element arsenic have been reported under aerobic cultivation conditions;[26–29] energy derived from the oxidation of antimony trioxide by *Stibiobacter senarmontii* has been shown to be coupled to biosynthesis.[29] However, antimony(III) to (V) transition by *M. formicium* is most unlikely to have occurred within the highly anaerobic and negative redox potential environments required for cultivation of the methanogenic Archaea.

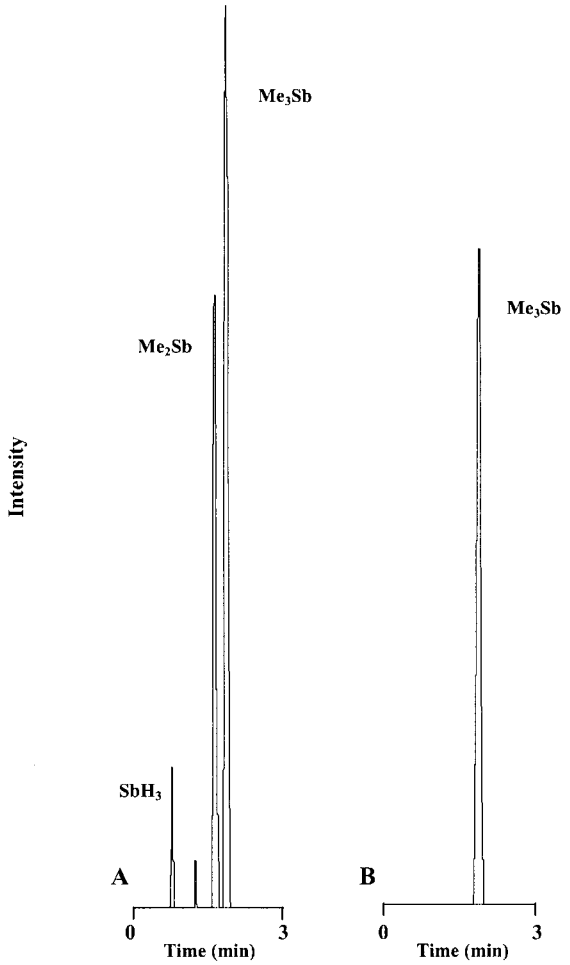

Figure 4. Typical GC–AAS chromatogram of volatile antimony compounds produced by *C. humicolus* from inorganic antimony substrate. (A) Chromatogram of volatile antimony (standards) produced by derivatization of trimethylantimony dichloride. (B) Headspace analysis of *C. humicolus* culture incubation supplied with PAT.

Andrewes *et al.*[17] reported the detection of small quantities (*ca* 20 pg) of stibine from aerobic cultures of *S. brevicaulis* supplemented with antimony(III). The ability of this fungus to oxidize antimony(III) to antimony(V) has also been reported, although supporting data were not provided.[17] It is possible, therefore, that this eukaryote initially oxidized some of its antimony(III) substrate to antimony(V), which then served as biotransformation substrate for stibine formation.

Our work on the fungus *C. humicolus* indicates that, for this eukaryotic organism at least, stibine formation occurs from antimony(V) but not from antimony(III) substrate. It is possible that this holds for other fungi (eukaryotes); there is nothing in the literature to suggest otherwise. Based on the present report and the current literature on microbial stibine formation,[15,17] we propose that two mechanisms of microbial formation of stibine from inorganic antimomy exist: one requiring antimony(V) substrate present in certain eukaryotic microrganisms, the other requiring antimony(III) substrate present in certain prokaryotic microrganisms.

The research reported on here shows that SPME can successfully be applied to the compound-specific determination of microgram per litre concentrations of volatile antimony species in environmental samples, and indicates that headspace SPME extraction has significant potential in the speciation and analysis of organometallic compounds in environmental samples.

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