ISOLATION AND INVESTIGATION OF ANTIMONY-RESISTANT BACTERIAL CULTURES

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ISOLATION AND INVESTIGATION OF ANTIMONY-RESISTANT BACTERIAL CULTURES

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ABSTRACT

Becer, Eser, Isolation and Investigation of Antimony-Resistant Bacterial Cultures. Master of Science (Chemistry), May, 1997, Sam Houston State University, Huntsville, Texas, 89 pp.

Purpose

The purpose of this study was to isolate and investigate the antimony-resistant bacterial cultures which had played a role in the biological methylation of antimony compounds in the soils and cultures examined.

Methods

Related experiments were carried out in order to investigate antimony-resistant bacterial cultures and also to try to enrich and isolate the ones which were responsible from the methylation of inorganic antimony compounds. After mixed bacteria cultures were amended with different antimony compounds (potassium antimonyl tartrate and potassium hexahydroxy antimonate) in various growth media (DMN, trypticase soy broth with nitrate), the ones which were resistant to antimony grew in anaerobic environment and produced trimethylstibine. Individual colonies were separated from the rest of the mixed colony by striking the solution that contained the bacterial colonies on agar plates that amended with potassium hexahydroxy antimonate; and then after they grew, they were picked off the plates and re-inoculated in sterile bacterial medium.

After the amendment of these monocultures with the specified antimony compounds, the headspace of the bacterial solutions were analyzed by gas chromatography coupled with fluorine-induced chemiluminescence detection. Bacterial growth was monitored by optical density.

In addition, theoretical treatment of retention time correlation experiments were designed and utilized for the determination of retention times of different organo-sulfur, and -selenium compounds of whose standard retention times could not be determined because of their commercial unavailability. Also this correlation method was employed to evaluate the standard retention times of organo-sulfur, and -antimony compounds when a different chromatographic temperature program was required in our analyses.

Findings

Trimethylstibine production (via biological methylation) from mixed bacterial cultures were identified for different soil samples (Swiss soil and Body Shop soil). Re-enrichment of these cultures was achieved; moreover, the separated colonies (from soil) also produced trimethylstibine when they were amended with antimony compounds. The lack of trimethylstibine production in the control groups (ones which were not amended with antimony) supported the conclusion that organo-antimony production by these organisms was a result of biological activity (biomethylation).

Mixed bacterial polycultures were isolated successfully; the individual isolated colonies were also found to be antimony-resistant. However, a solid conclusion could not be made on the antimony reducing capabilities of these isolated monocultures even though they were found to produce trimethylstibine since organo-antimony concentration which was produced by these isolated colonies was relatively low, and the trimethylstibine peak in the headspace analyses of these isolated cultures hardly rose above the level of 3 times signal to noise ration which is a benchmark in our laboratory for detection.

Retention time correlation technique was successfully employed to two major projects; in the Garlic experiment, 5 mixed organo-sulfur and -selenium compounds were identified. The practical use of this method allowed us to determine the retention times of

volatile and relatively reactive organo-sulfur and -selenium species whose standard were not available commercially.

Determination of retention times of organo-sulfur and -antimony compounds were also calculated with the same method (retention time correlation technique) when a different chromatographic temperature ramp was used in our analyses.

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First of all, I would like to thank a person who has always supported and encouraged me during my studies, who has always been there when I needed him, and who has given me a whole new perspective in my life and inspired me with his passion for science. It is this person whom I am proud to be a student of. Thank you Dr. Chasteen for being such a unique and almost perfect advisor to me. It's been my privilege to have been directed by you in this research. It could only have been better if you used a PC. Oh well, nobody is perfect. He not only has taught me a lot about my profession, but also has helped me to grasp the idea of cross-platform compatibility theorem for computers. But still, there will always be one thing that he could not change about me: my computer!

When someone looks at this thesis, he or she might think that I have a minor in biology, but I don't. I have learned everything there is to know (at least everything that I needed) from our research group's microbiologist, Dr. Verena Van Fleet-Stalder. I would like to thank her for all she has done for me for I could have never gone this far without her help and patience. Verena, I wish the best of everything for you.

To my friend and our associate biologist, Shiv Patel, thank you for your help and friendship. Best of luck in your graduate studies.

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I would especially like to express my appreciation to my sister and brother-in-law for their enormous and continuous support that they have shown to me (us).

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CHAPTER I

Introduction

The concept of biomethylation is mainly concerned with the biological conversion of inorganic species to organometallic products (e.g. mercury (II) $\longrightarrow$ methylmercury) or with conversion of partially methylated species to a fully methylated product (e.g., $Me_3Pb^+ \rightarrow Me_4Pb$). Figure 1 is a schematic representation of this process where M^+ inorganic species have been converted to organometallic products, $M(R)_n$, by the organism. Organisms that have been shown to accomplish this include plants, bacteria, fungi; and possibly animals although enteric organisms (bacteria) may be the source.

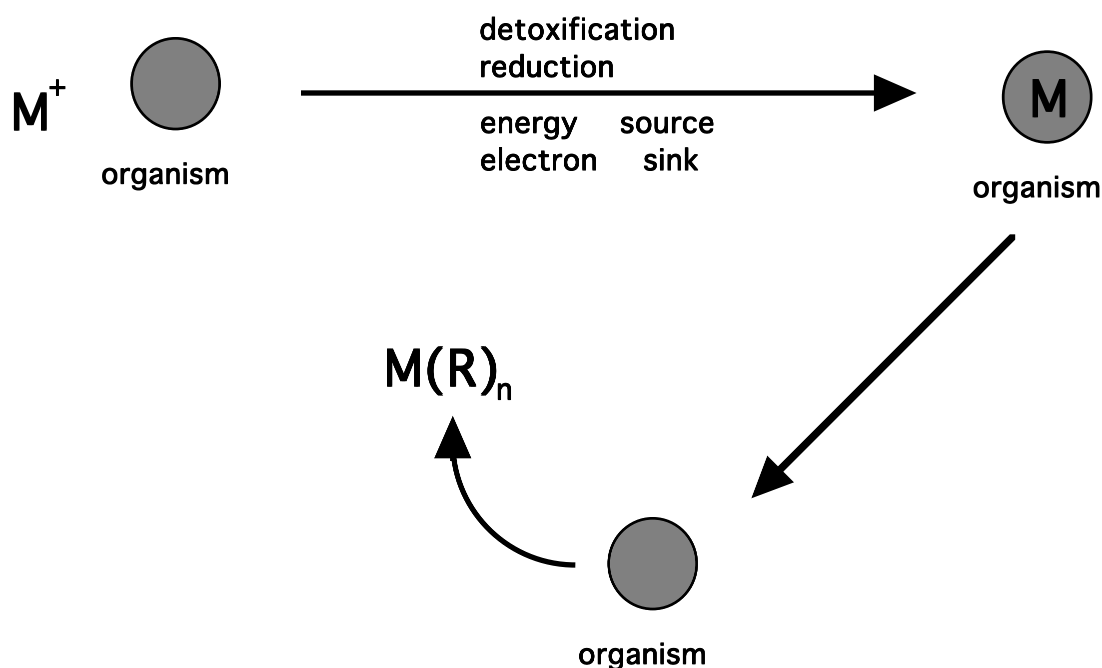

Figure 1. Representation of biomethylation process.

In general terms, methylated metals are more toxic than the ionic forms from which they derived, and this comes from higher solubility of these methylated species in lipid tissues resulting in longer residence times in the organism (e.g. the half life of

methylmercury in the human body is 70 days compared to 4-5 days for mercury(II) salts). Another measure of the possible relative bioavailability is the distribution coefficient between water and 1-octanol (Wasik, 1978); the higher the value for a compound, the more lipophilic that material. For the series $HgCl_2$, CH_3HgCl, $(CH_3)_2Hg$, the coefficients are 0.61, 2.54, and 191.3, respectively (Thayer, 1989). Such changes can contribute to differences in toxicity between inorganic compounds and their methylated derivatives of most elements and this has been also observed for alkyltin species (Craig, 1986; Thayer, 1984; Joseph and Wuertz, 1989) and alkyllead compounds (Yamamura, 1993). However, this can not be generalized for all inorganic and organic metals species. For instance, the relative toxicities of Sb(III) and Sb(V) are unknown; however, soluble salts of Sb have been shown to be toxic (Luckey, 1975) and organoselenium compounds are much less toxic than their oxyanionic precursors in biological methylation.

Transfer of methyl groups to a metal may occur by the help of a methyl transfer process within a cell of a living organism involving the role of an enzyme (i.e., enzymatically). Methylation may also occur if a metabolite of an organism has methylating properties, and this would constitute a non-enzymatic event. In stricter terms the methylation of mercury by organisms which use methionine synthetase to produce methionine from homocysteine is an enzymatic process and “biological”; while methylation by the algal metabolite iodomethane is non-enzymatic and “non-biological.” In fact both methylations are the products of living processes and allow the same result: a methylated metal product. In this thesis the word “biomethylation” will be used for all methylation processes initiated by microbes regardless of enzymatic or non-enzymatic formations since enzymatic activities are not the scope of this research (Becer, D., 1997)

The evidences concerning the idea of biological methylation (biomethylation) had started to shape up during the early 1810s after several cases of arsenic poisoning in Germany and England. The reason for these poisonings was the usage of wall papers

which contained copper hydrogen arsenite as a dye ingredient. Several poisoning cases had occurred at those times including some fatal ones.

The earliest and most obvious explanation about these arsenic poisonings was based on the absorption and inhalation of these arsenic containing pigments by persons living in the rooms when these pigments and particles detached from the wall paper by physical means. The presence of arsenic in the dust of such rooms had been demonstrated (Abel and Buttenberg, 1899; Huss, 1914), but arsenic poisoning had continued even though the arsenic containing wall papers were covered with new papers which did not contain arsenic at all (Fleck, 1872). This study proved the presence of some other factors contributing to the arsenic poisoning other than the inhalation of dust particles containing arsenic. These observations led to an intensive study and experiments which could be accepted as the first steps in the discovery of biomethylation.

Over the following years it was noticed that a garlic odor was usually present in the rooms where poisoning had occurred (Gmelin, 1839). Gmelin stated, in the light of this observation, that the garlic smell was due to the production of volatile arsenic compounds from the wall papers which were usually found to be damp and moldy. Further evidences were found by Fleck in 1872 when he exposed strips of paper coated with copper arsenite to moist air in flasks. Air was passed through these flasks and then into silver nitrate solution, giving a deposit which appeared to be silver. When this deposit was removed and mixed with ammonia, silver arsenite precipitated. Fleck was able to show that molds were able to produce this gas, but in those days he was unable to report the identity of this gas.

These individual and non-integrated studies did not bring out much reliable information until 1891 when Gosio began a systematic study of the whole process. He isolated various cultures that produced this gas which was then known as Gosio gas and concluded that the gas contained an alkylarsine, probably diethylarsine, $(C_2H_5)_2AsH$, although at the time this substance had not been prepared (Gosio, 1893a; 1893b; 1897; 1901). Gosio's work was continued by his assistant Biginelli who assigned the identity of

this gas as diethylarsine [trapped as $(CH_3CH_2)_2AsH•2HgCl_2$] (Biginelli, 1901). This structure was corrected to diethylarsine oxide by Klason in 1914 (Klason, 1914). But both of these compounds were synthesized by Wigren in 1924 and Gasio gas was proven to be something else since the synthesized compounds showed different behaviors towards solution of $HgCl_2$ in hydrochloric acid (Wigren, 1924). This was also confirmed by later experiments (Challenger et al., 1933). The Gasio gas discussion was concluded in May 1932 when Challenger and his coworkers conclusively identified the gas as trimethylarsine, $(CH_3)_3As$, (Challenger et al., 1933). For the experiments they followed the same procedures as Biginelli. They also synthesized trimethylarsine and compared its chemical characteristics with the Gasio gas.

In the following years Challenger's research group also provided experimental evidences for the biological methylation of selenium (Challenger and North, 1934), and tellurium (Bird and Challenger, 1939) by fungal cultures. Since then biomethylation of tin (Chau et al., 1981; Guard et al., 1981; Hallas et al., 1982; Gilmour et al., 1985), mercury (Yamada and Tonomura, 1971; Hamdy and Moyes, 1975; Pan-Hou and Imura, 1982), and lead (Wong et al.,1975; Schmidt and Huber, 1976) has been detected and confirmed; although lead biomethylation is still held to be controversial (Craig, 1996).

Evidence for the biomethylation of antimony was indirect until recent years (Dodd et al., 1996; Gürleyük, 1996). Previously methylstibonic $[(CH_3)SbO(OH)_2]$ and dimethylstibinic acids had been reported in marine and estuarine environments (Andreae et al., 1981) and in various natural water (Andreae et al., 1983; 1983) and biomethylation appeared to be their most likely source. Challenger found no evidence for biomethylation of antimony in most cases in his works with (aerobic) fungi, although $KSbO_3$ did give small quantities of an unidentified volatile Sb containing species (Barnard, 1947; Challenger, 1978). No traces of methylantimonials were found in phytoplankton (Kantin, 1983) or mollusk shells (Cullen et al., 1989), although methyarsenicals were found in both cases. A metabolic study on humans investigating Sb(V) oxy compounds showed no evidence for

biomethylation (Bailly et al., 1991). Recently two major findings had given very strong evidence for the biomethylation of antimony compounds. In 1996 Dodd and coworkers reported the first results to provide evidence for the presence of methylantimony compounds in biological systems (Dodd et al, 1996). Soon after Gürleyük reported the methylation of antimony compounds by some mixed bacterial cultures (Gürleyük, 1996; Gürleyük and Chasteen, 1997). These two findings are probably the most important and significant steps in biomethylation of antimony since 1913 when Knaffl-Lenz first reported the possibility of this process (Knaffl-Lenz, 1913).

In their work, Dodd and coworkers used a method based on semi-continuous hydride generation coupled on-line with gas chromatography and a mass spectrometric detector for the identification of the hydrides formed from antimony species in biological samples. Organoantimony compounds were identified in extracts of a freshwater plant, namely pondweed (*Potamogetan pectinatus*). These aquatic plants were collected from different lakes from a Canadian mining area, is known to have been influenced by gold-mine effluent containing various metals. At the same time, they also sampled from a relatively unpolluted lake which served as a reference. The total antimony concentrations in submergent aquatic plants that were collected from two selected lakes were found to be 68 and 48 ppm Sb, dry mass. Pondweed from the reference lake contained antimony at a much lower concentration (0.4 ppm). These workers concluded that these differences in Sb content was due to the uptake of antimony by pondweed in the selected lakes. Organoantimony compounds detected in the plant extracts were characterized on the basis of their GC retention times and characteristic ions formed in the mass spectrum. These researchers also managed to prevent the molecular rearrangements of antimony compounds during hydride generation which was a problem in earlier experiments (Dodd et al., 1992).

The most important problem in detection of trimethylstibine is its fast oxidation in gas phase. Parrish and Brinckman (1976) found that the gas phase rate constants for the oxidation of trimethylstibine and trimethylarsine by oxygen to be 10^3 and 10^{-6} $M^{-1}s^{-1}$

respectively. From these results it is apparent that the rapidity with which $(CH_3)_3Sb$ is oxidized would probably prevent hazardous concentrations from building up in well-aerated surroundings. Also trimethylstibine is much less volatile compared to trimethylarsine: the vapor pressures of trimethylstibine and trimethylarsine at 298 K are 103 and 322 torr respectively (Rosenbaum and Sandberg, 1940). These factors have been the major problem of the previous works in determining the biological production since the oxidation of trimethylstibine to, for instance, trimethylstibine oxide [$(CH_3)_3SbO$] may have been occurring before it could be detected. In order to solve this problem Barnard (1947) decreased the oxygen concentration in the aspiration of Sb amended microbial cultures by adding 8% N_2 to air. But low oxygen content altered the growth of the molds, and consequently he couldn’t find any volatile organoantimony compounds.

In order to prevent this oxidation process in our experiments we chose to examine anaerobic systems for the bacterial growth, and the identification method had to be chosen carefully in order to minimize the introduction of oxygen to trimethylstibine. These conditions were apparently achieved in Gürleyük’s experiments (Gürleyük, 1996; Gürleyük and Chasteen, 1997). Gürleyük was able to grow monocultures of bacteria and soil samples under anaerobic conditions and pursue the detection of trimethylstibine by determining the presence of this compound by headspace analysis using gas chromatography with fluorine-induced chemiluminescence detection (Chasteen et al., 1990). In this study single cultures of known metalloid-reducing and methylating bacteria as well as soil samples collected around Huntsville, Texas and an arsenic contaminated site in Switzerland (1581.7 ppb As) (Gürleyük, 1996) were examined by amending them with various antimony compounds to determine whether they exhibited antimony reducing/methylating ability.

As a continuation of this research, this thesis reports experiments that were carried out in order to repeat Gürleyük's results and also to try to isolate bacteria which are responsible from the methylation of inorganic antimony compounds. After mixed bacteria

cultures were poisoned with different antimony compounds in various growth media, the ones which were resistant to antimony had grown and produced trimethylstibine. Isolation and enrichment of those antimony-resistant bacteria were the main purpose of this research. These antimony-resistant bacteria were poisoned with different antimony compounds (potassium antimonyl tartrate trihydrate, potassium hexahydroxyantimonate) in various growth media. The bacteria were allowed to grow anaerobically in test tubes in the presence of these antimony compounds and then the headspaces were analyzed for reduced and methylated antimony compounds using a fluorine-induced chemiluminescence detector after separation by gas chromatography. The growth of the bacteria were monitored by optical density.

Another type of bacteria, phototrophic bacteria some of which had been shown to be successful in reducing selenium (Van Fleet-Stalder et al., 1997), were investigated by the same methods, and their resistance towards antimony compounds and their ability to reduce antimony were analyzed.

Antimony is a rare element. Its average concentration in indigenous rocks is 0.2 ppm, in shales 1.5 ppm, in limestone 0.3 ppm, in sandstone 0.05 ppm, and in soil 1 ppm (Boven, 1979). As a mineral, antimony may occur most commonly as stibnite (Sb_2S_3) (Gornitz, 1972) and it is present in the aquatic environment as a result of rock weathering, soil runoff and through effluents from mining and smelting. Typical concentrations of antimony in unpolluted waters are less then 1 ppb (mg L^{-1}) (Shigeru, 1994). However, because of the anthropogenic sources, concentrations can be substantially increased to the 100 ppb level (Andrea, et al. 1981). The US Environmental Protection Agency lists antimony and its compounds as priority pollutants.

Although headspace analysis with gas chromatography has been used as a powerful tool to identify the volatile compounds produced by a culture, it can not function as an absolute identifier if standard retention time for a compound is not known. In order to avoid the need for the preparation of various sulfur and selenium containing standards,

garlic headspace was analyzed by gas chromatography with fluorine-induced chemiluminescence detection, and retention times of some previously unidentified peaks were determined by calculating the retention time correlation with respect to previously determined literature data.

This thesis describes research that was carried out in order to determine the biomethylation and/or biological-reduction of inorganic antimony compounds by living organisms. Selected bacteria cultures were poisoned with different antimony compounds and their metabolization capability towards these compounds were determined by their population increase (optical density) and head space analyses (GC/fluorine-induced chemiluminescence detection). Our interest in biological processing of toxic metals and metalloids is serious because after they have been reduced and methylated, they are potentially available for redistribution to the environment in a manner different than that of their inorganic forms. Even though these metals and metalloids in their purely inorganic form are not mobile in the gas phase due to their high boiling points, the potential of the reduction and methylation of these compounds to form new organometallic products in the environment makes them available to spreading as lypophilic compounds. In such cases the important considerations are the general toxicity of these compounds at points other than that of intended application points. Therefore understanding the biochemistry of biomethylation may be an important step in solving some of today’s complicated environmental problems.

CHAPTER II

Part 1.
Experimental

1-1. Reagents

All chemicals used in this work were analytical reagent grade and used without further purification. Potassium antimonyl tartrate (PAT), potassium hexahydroxy antimonate (PHA), HPLC grade acetonitrile, potassium phosphate dibasic, potassium phosphate monobasic were purchased from Aldrich Chemical Company, Inc. (St. Louis, MO USA). Ammonium sulfate and glycerol were purchased from J.T. Baker Inc. (Philipsburg, NY USA). Trimethylstibine [$(CH_3)_3Sb$, TMSb] was ordered from Organometallics Inc. (East Hampstead, NH USA). Sodium citrate was obtained from EM Science (Gibbstown, NJ USA). Magnesium sulfate heptahydrate was obtained from Sigma Chemical Co. (St. Louis, MO USA). Potassium nitrate was purchased from Fisher Scientific (Pittsburg, PA USA). Triptic soy broth was obtained from Difco Laboratories (Detroit, MI USA). Nitrilotriacetic acid, aspartic acid, glutamic acid, potassium hydroxide, magnesium sulfate heptahydrate, calcium chloride dihydrate, iron(II)sulfate heptahydrate, succinic acid, potassium hydroxide, potassium phosphate monobasic, potassium phosphate, sodium chloride, ammonium sulfate, boric acid, manganese sulfate monohydrate, copper(II) nitrate trihydrate, zinc sulfate heptahydrate, sodium molybdate dihydrate, (ethylenedinitrilo)-tetraacetic acid disodium salt, nicotinamide (Vitamin PP), thiamine hydrochloride (Vitamin B1 hydrochloride), and biotin (Vitamin H) were ordered from VWR Scientific Products (Sugarland, TX USA)

1-2. Biological Media

Three different kinds of media, DMN, TSN and SMM, were used in this research. DMN medium was derived from DM1 medium, by adding 1.0 g/L potassium nitrate, which was prepared according to personal communications with Dr. Ray Fall at the University of Colorado (Boulder, CO USA).

DMN medium: It was prepared by dissolving 7.0 g potassium phosphate dibasic, 3.0 g potassium phosphate monobasic, 1.0 g ammonium sulfate, 10.0 g 50% glycerol, 0.5 g. sodium citrate, 0.1 g. magnesium sulfate heptahydrate and 1.0 g potassium nitrate in 1.0 L deionized water and by adjusting the pH to 7.4 by adding 0.1 M sodium hydroxide.

TSN medium: (Trypticase Soy Broth with Nitrate) It was prepared by dissolving 30 g triptic soy broth and 1.0 g potassium nitrate in 1.0 L deionized water.

SMM medium: (Sistrom Minimal Medium) It was prepared by the mixing the following stock solutions (Sistrom, 1960).

Stock Solutions #1: (Salts and Amino Acids) Prepared by adding 10 g nitrilotriacetic acid, 2 g aspartic acid, 5 g glutamic acid, 11 g potassium hydroxide, 29.5 g magnesium sulfate heptahydrate, 3.3 g calcium chloride dihydrate and 100 mg iron(II) sulfate heptahydrate into 1000 mL deionized water and then autoclaving (see program below).

Stock Solution #2: (Carbon Source) Prepared by adding 236 g succinic acid and 224 g potassium hydroxide into 1000 mL deionized water.

Stock Solution #3: (Phosphate Buffer) Prepared by adding 133 g potassium phosphate monobasic, and 200 g potassium phosphate into 1000 mL deionized water and then autoclaving.

Stock Solution #4: (Sodium Chloride) Prepared by dissolving 100 g sodium chloride in 1000 mL deionized water and then autoclaving.

Stock Solution #5: (Nitrogen and Sulfur Source) Prepared by dissolving 100 g ammonium sulfate in 1000 mL deionized water and then autoclaving.

Stock Solution #6: (Trace Elements) Prepared by adding 2.8 g boric acid, 2.1 g manganese sulfate monohydrate, 40 mg copper(II) nitrate trihydrate, 240 mg zinc sulfate heptahydrate, 750 mg sodium molybdate dihydrate, and 6 g (ethylenedinitrilo)-tetraacetic acid disodium salt into 1000 mL deionized water and then autoclaving.

Stock Solution #7: (Vitamins) Prepared by adding 100 mg nicotinamide (Vitamin PP), 50 mg thiamine hydrochloride (Vitamin B1 hydrochloride), and 2 mg biotin (Vitamin H) into 100 mL water and then sterile filtering.

Recipe for SMM: SSM is prepared by adding 20 mL of stock solution #1, 10 mL of stock solution #2, 9 mL of stock solution #3, 4 mL of stock solution #4, 5 mL of stock solution #5, 1 mL of stock solution #6, and 2 mL of stock solution #7 into deionized water (final volume 1 L); pH of the solution is adjusted to 6.8 and then autoclaved.

Also in some experiments, soil extract was added to media in order to supply different kinds of minerals and that might be missing in the media recipes which were thought to be essential for bacterial growth. This concentrated extract was prepared by adding 50 mL of deionized water to 20 g of soil sample followed by sterilization (Tuttnauer USA Co. LTD., Ron Konkoma, NY USA), with solution temperature programming (temperature: 134 ^{o}C, pressure: 20 atm., sterilization time: 20 minutes). After sterilization, the water-soil mixture was filtered by suction filter and soil free extract was resterilized with the same sterilization program.

Part 2.
Microbiology

In this research both specific light depending bacterial cultures and polycultures from soil samples were investigated for their biomethylation and/or reduction activities towards specified antimony compounds. Specific bacteria cultures were all phototrophic species. A total of six different types were studied [A=*Rhodocyclus tenuis* (DSM 109^T); DSM 158=*Rhodobacter sphaeroides* 2.4.1 (DSM 158); C=*Rhodobacter capsulatus* (DSM 1710^T); D=*Rhodospirillium rubrum* S1 (DSM 467^T); E=*Rhodospirillum rubrum* G9 (DSM 468); F=*Rhodopseudomonas blastica* (DSM 2131^T)] (DSM=Deutsche Sammlung von Mikroorganismen, Gottigen, Federal Republic of Germany).

Two different soil samples were analyzed to find organisms that might methylate and reduce antimony compounds. Sample 1 (SS-Switzerland Soil) was collected from an abandoned arsenic polluted leather tannery in Switzerland and was supplied to our research group by Professor Reinhard Bachofen at the University of Zürich, Institüte Für Plant Biology and Microbiology, Zürich, Switzerland. Sample 2 (BSS-Body Shop Soil) was collected from the backyard of an auto repair shop named “Body Shop” in Huntsville, Texas by Hakan Gürleyük while he was working on his MS Degree in chemistry here. These soil samples were collected and stored in plastic sterile bags at room temperature.

Part 3.
Instrumentation

Gas chromatograph/fluorine-induced chemiluminescence detector (GC/SCD)

A Hewlett Packard (Norwalk, CT USA) 5890 Series II gas chromatograph, equipped with a Sievers 300 sulfur chemiluminescence detector (Sievers Instruments,

Boulder, CO USA) were used for all chromatographic analyses. A 30 m 0.32 mm i.d. capillary (Alltech Associates, Inc., Deerfield, IL USA) coated with 1 μm 5% phenyl and 95% methyl polysiloxane as the stationary phase was employed. The helium carrier gas (Bob Smith Gas Products and Conroe Welding) flow rate was 1 mL/min and the inlet head pressure was 11 psi. A split/splitless injector (275 ^{o}C) was used in the splitless mode; however, split injections (split ratio: 25.5) were performed in some cases where high organosulfur concentrations were observed. For the chromatographic separation, in-oven cryogenic trapping was applied by the cooling of the GC oven using liquid nitrogen.

Through a heated transfer line (150 ^{o}C), the capillary tube (320 micron fused silica) through which the GC column passes, was connected to a stainless steel reaction cell in the detector. In this instrument, molecular fluorine (F_2) was produced by a high power electrical discharge of sulfur hexafluoride (SF_6). A constant current, high voltage, and high frequency power supply was used to break the SF_6 molecule apart to form SF_4, F_2, and a very small amount of elemental sulfur. Reaction of molecular fluorine with organo-metals/metalloids resulted in chemiluminescence, which was detected by a red sensitive photomultiplier tube which was housed inside the PMT cooler. The PMT was cooled to below ambient temperature to reduce anode dark current resulting in a further reduction of noise. The reaction cell was kept under 1 torr (ca. 0.6 torr) by a two-stage, oil sealed, rotary vacuum pump (Sargent Welch Scientific Co., Skokie, IL USA). A chemical trap (NaOH and elemental carbon) was placed between the pump and the reaction cell to remove excess F_2, HF, and sulfur dust produced in the reaction cell. The following temperature program was used in most analyses -20 ^{o}C for 1 min initial time, 20 ^{o}C/min to 200 ^{o}C, then a final time of 1 min. A longer temperature program was used in some experiments to achieve a better chromatographic separation between organosulfur and organoantimony compounds. The resulting signals from the SCD were recorded by a Hewlett Packard 3396 Series II integrator.

Part 4.
Procedures

4-1. Experimental

4-1a. Phototrophic Bacteria: Preparation of preculture solutions and incubation procedures for all strains were performed similarly. Therefore, DMS 158 bacterial solution and its inoculation will be given as an example: To grow precultures, DMS 158 was inoculated into SMM medium which had been sterile-filtered previously. After the transfer of 10 mL of DMS 158 and 100 mL of SMM solution to a sterile bottle, the lid was closed and the bottle was shelved in a dark environment and left in the dark overnight in order to eliminate oxygen via nonphotosynthetic aerobic growth before being incubated in light. The bacteria were then allowed to grow anaerobically at room temperature in front of a light source separated by a measured distance for the optimal light intensity (10 Watt/m^2). This light source is required for the photosynthetic bacterial growth. After a week of growth, when the bacteria reached stationary growth phase, 30 mL of the culture was added to 300 mL of SMM medium and then individual 10 mL portions were transferred into sterile Hungate tubes (VWR Scientific). The tubes were then tightly capped with open-top screw caps sealed with Teflon® septa. Again, the cultures were left in the dark for overnight and then sterile filtered PHA and PAT stock solutions (5 mM, 1 mM respectively) were added into test tubes accordingly in order to give 0.01 mM antimony concentration in the final solution. Antimony was not added to a set of 10 bacteria-containing test tubes in order to serve as a control group; also another sterile control group was prepared without DMS 158 yet containing antimony. The overall phototrophic bacteria experiments included with the antimony concentrations are given in Table I.

Table I
Phototrophic Bacteria Experiment's Overview*

PHA (mM)				PAT (mM)			Phototrophic Bacteria Type					
0.01	0.1	1	5	0.01	0.1	1	DMS158	A	C	D	E	F
+							+					
	+						+					
		+					+					
				+			+					
					+		+					
						+	+					
		+						+				
		+							+			
			+							+		
		+									+	
			+									+
						+		+				
						+			+			
						+				+		
						+					+	
						+						+

* Controls which did not contain either antimony or bacteria are not shown in this table.

Headspace analysis of the phototrophic samples were performed after two weeks of incubation at room temperature. Throughout the phototrophic bacteria experiments, the same medium, SMM, was used, while different antimony concentrations (0.01 mM, 0.1 mM, 1 mM, 5 mM) were added to different bacterial strains. Bacterial growth was monitored by optical density during incubation using a Klett-Summerson Photoelectric colorimeter (Klett MFG. Co., NY USA), using a green filter (l_{max}=526 nm). Optical densities were calculated by,

$$D = \frac{Km \times 2}{1000} \qquad \text{(Equation1)}$$

where D is the optical density and Km is the Klett-Meter reading (Klett-Summerson Photoelectric Colorimeter, Clinical Manual).

4-1b. Soil Samples: These soil samples (BSS and SS) were inoculated into 2 different media (DMN and TSN) as described in Gürleyük's procedure (Gürleyük, 1996). Two tenths of a gram of soil samples were weighed and placed into sterile Hungate tubes with 10 mL of medium (DMN or TSN) amended with antimony stock solutions in order to get a specific antimony concentration (ranging from 0.01 mM to 7 mM). Also, control groups were prepared without any antimony addition. All equipment and media were sterilized by autoclave except antimony stock solutions. Because of the potential for decomposition of these chemicals at high temperatures, they were sterile-filtered using 0.2 mm disposable syringe filters (Uniflo-25, Schleicher & Schuell, Keene, NH USA). General overview of the BSS experiment and SS experiment is given in Table II and Table III respectively. Figure 2 represents a schematic of general inoculation and headspace analyses.

Table II

Body Shop Soil Experiment's Overview*

Samples	PHA	PAT	Soil (0.2 g.)
	0.01 mM	-	+
	-	0.01 mM	+
Control	-	-	+

*medium: DM-N

Table III

Swiss Soil Experiment's Overview*

Samples	DM-N	TSN	PHA	PAT
	+	-	0.067 mM	-
	+	-	0.667 mM	-
	+	-	6.667 mM	-
	+	-	-	0.067 mM
	+	-	-	0.667 mM
	+	-	-	6.667 mM
Control	+	-	-	-
	-	+	0.070 mM	-
	-	+	0.700 mM	-
	-	+	7.000 mM	-
	-	+	-	0.070 mM
	-	+	-	0.700 mM
	-	+	-	7.000 mM
Control	-	+	-	-

* 0.02 g. soil was added to each test tube.

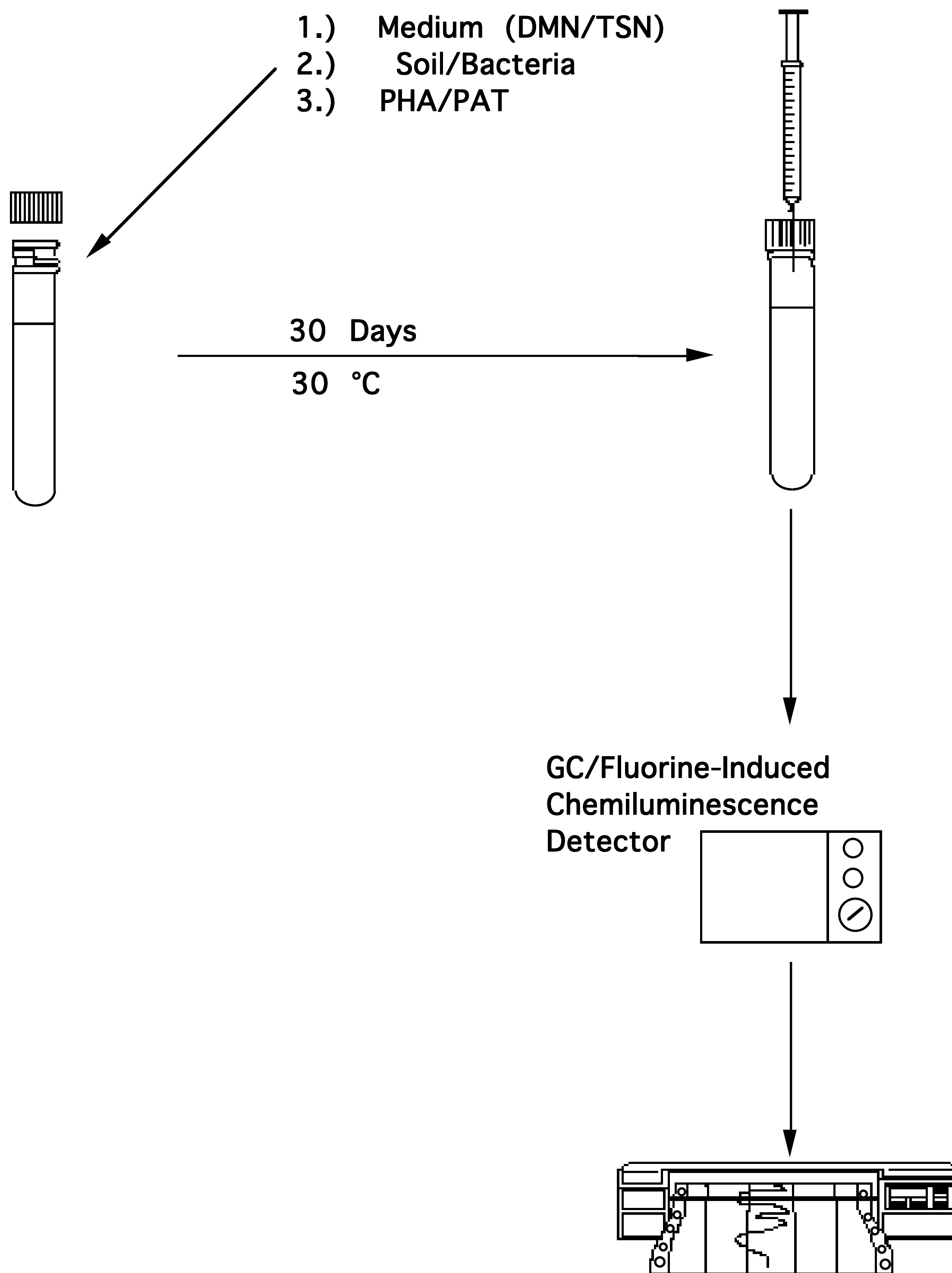

Figure 2. Schematic representation of general inoculation and headspace analyses.

4-1c. Extract Samples: Bacterial Extracts (I, II, and III) from soils were prepared in a manner identical to 4-1b by substituting the soil with bacterial solutions taken from cultures which were assumed to be active in the biomethylation of inorganic antimony species. Based on our preliminary results, 200 mL of solution from soil-containing test tubes were used as bacteria source (inocula) for this series of experiments. Also soil extract (Chapter II, section 1-2) was added to media (TSN) in order to fulfill the mineral needs of the bacteria cultures. General overview of the Extract experiments (I, II, and III) is given in Table IV, Table V, and Table VI respectively.

Table IV
Extract-I Experiment's Overview*

Samples	PHA	PAT	Bacteria	Extract
	0.01 mM	-	+	+
	0.10 mM	-	+	+
	1.00 mM	-	+	+
Control-1	1.00 mM	-	-	+
Control-2	1.00 mM	-	+	-
Control-3	-	-	+	+
	-	0.01 mM	+	+
	-	0.10 mM	+	+
	-	1.00 mM	+	+
Control-1	1.00 mM	-	-	+
Control-2	1.00 mM	-	+	-

*Bacteria source: BSS PHA #3, Control-3 was the common control, and TSN was the common medium for all extract experiments

Table V
Extract-II Experiment's Overview

Samples	PHA	PAT	Bacteria	Extract
	0.01 mM	-	+	+
	0.10 mM	-	+	+
Control-1	0.10 mM	-	-	+
Control-2	0.10 mM	-	+	-
	-	0.01 mM	+	+
	-	0.10 mM	+	+
Control-1	-	0.10 mM	-	+
Control-2	-	0.10 mM	+	-

*Bacteria source: BSS PHA #8

Table VI
Extract-III Experiment's Overview*

Samples	PHA	Bacteria	Extract
	0.1 mM	+	+
Control-1	0.1 mM	-	+
Control-2	0.1 mM	+	-

*Bacteria Source: Extract-I PHA 0.01 mM

4-1d. Bacteria Isolation: In order to isolate the bacteria cultures which were suspected of biomethylating and/or reducing antimony compounds, bacterial cultures were streaked out on agar plates. A 1.5 % (w/w) agar was added to the media to prepare solid

media for plates. Also, specific amounts of PHA and PAT were added to these plates in order to get 0.1 mM antimony concentration in these solid media. After isolated colonies started to grow, they were removed from the plate via sterile loop and taken and enriched in TSN medium for further investigation on their antimony biomethylation capabilities.

4-1e. Garlic Experiment: Throughout the bacterial headspace analyses, some of the chromatographic peaks could not be identified by their retention times due to the lack of our knowledge of the retention times of standard compounds which could not be purchased. Therefore a set of new experiments were performed to identify some less common organosulfur and organoselenium compounds without dealing with the standard retention time analyses procedure. In order to identify the retention times of the unknown peaks in our standard chromatographic temperature program, a regression line was calculated. The species were identified either by their standard retention times or by calculation of the retention time correlation by using the regression line equation. Fresh elephant garlic from a local grocery store was homogenized, saturated with sodium chloride in order to increase the ionic strength of the environment (another set was also prepared without NaCl addition), and capped in a 10 mL headspace vial. Each vial was heated to 30 ^{o}C for 24 hours. Headspace analyses were performed and retention times of the sulfur compounds were identified.

4-2. Headspace Analyses

All the headspace analyses were performed using 1 mL gas-tight syringes with push button valves, purchased from Alltech (Deerfield, IL USA). Headspace samples were taken from the test tube by piercing the Teflon septa by syringe. After the sample, whose volumes ranged from 0.1 mL to 1 mL depending on the organosulfur concentration, was taken, the valve was closed until the syringe was ready to be injected to split/splitless injector (275 ^{o}C) of the GC. The valve was opened just before the injection was performed;

the injection was made quickly, and the syringe was pulled out from the injector immediately to prevent inlet head pressure drops which might cause sample lost during injection. After a sample was injected, the syringe was cleaned using hot air in a syringe cleaning device which was composed of a flask and a hot plate. The device, which had been used in our research lab for six years, was modified. It utilized hot air warmed via air slowly passed through a flask on a hot plate which then passed through the syringe and the needle which were also heated gently by another hot plate. Even though an hour of cleaning was enough, in some cases the syringe had to be left in the cleaning device overnight to sweep out the high boiling compounds. Acetonitrile was also sometimes used to rinse the syringes before they were placed into the cleaning device since acetonitrile is not detected by the fluorine-induced chemiluminescence detector and that solvent dissolve organic species as well as high boiling compounds. To confirm a complete cleaning, the syringes were checked by injecting lab air, and the cleaning process was continued until no peaks were observed in subsequent syringe check chromatograms.

4-3. Chromatographic Retention Time Regression

4-3a. Retention time regression for garlic experiment: In 1994, organosulfur and organoselenium volatiles from garlic were identified (Cai et al., 1994) by using headspace gas chromatography with atomic emission detection. But since a different chromatographic temperature program with a similar chromatographic stationary phase (non polar, capillary) had been used in that study, a mathematical procedure was followed to establish a conversion method in order to apply the previously obtained data from that work to our temperature program. This was done in hopes of estimating the retention times of other less common organosulfur and organoselenium compounds which might appear in our samples but for which we do not have commercial standards. When the retention times of four common organosulfur and organoselenium compounds from our standard temperature program, whose retention times were obtained by injecting standards were

plotted, versus the retention times of the same compounds from the other work, a regression line with a regression coefficient of R=0.99818 was obtained. By plotting the retention times of the previously determined less common organosulfur and organoselenium compounds (from the Cai et al. work) to the regression line equation, a linear relationship between the retention times in these two different temperature programs was established and therefore, a new set of retention times were calculated regarding our temperature program. When these calculated retention times were compared with sample peaks from the garlic experiment retention times, none of them exceeded 1.5 percent relative error. Computation, percent error tables and regression line plot are given in Data and Results chapter.

4-3b. Retention time regression for antimony experiments: In some headspace analyses, high concentrations of organosulfur production by bacteria cultures and relatively close chromatographic elution for organosulfur and organoantimony compounds forced a slower temperature ramp to be used for chromatographic separation of these compounds. However, by changing the temperature program, recalibration of the standard retention times of all organosulfur and organoantimony compounds was needed. Based on our previous experiences in obtaining regression equations for different temperature programs, a similar method was used for the retention time determination of these compounds for a slower temperature ramp (5 ^{o}C/min). By visually observing the sequence of the compounds in chromatography and comparing that to the order in the old program, new retention times were assigned for the slower temperature ramp. When these theoretical retention times for the slower ramp were plotted versus known retention times, again their consistency were proven in mathematical term with a regression coefficient of R=0.99767. The trimethylstibine retention time was determined by injecting standard sample (gaseous sample taken from closed-vial containing TMSb) and it was found to be exactly same with the estimated value.

CHAPTER III

Data And Results

Introduction

In this chapter, data and results from the experiments are given together, and each experiment is detailed under individual sections. Table VII lists the compounds, and their boiling points of the chemicals that had been identified in the headspace of the bacterial cultures (or soil samples). A similar table for garlic experiment is given in the Garlic Experiment section. The compounds in Table X were identified in the headspace of crushed garlic. The slower chromatographic temperature ramp, which was used in several different experiments, is discussed in the Theoretical Treatment of Retention Time Correlation Experiments section.

Table VII
Boiling points of the related compounds at 1 atm pressure

Formula	Compound	Boiling point*
CH_3SH	methanethiol	6 ^oC
$(CH_3)_2S$	dimethylsulfide	37.3 ^oC
$(CH_3)_3Sb$	trimethylstibine	80.6 ^oC
$(CH_3)_2S_2$	dimethyldisulfide	109.7 ^oC
$(CH3)_2S_3$	dimethyltrisulfide	170 ^oC (not in CRC)

* CRC Handbook, 56th edition, 1975

Body Shop Soil Experiment (BSS)

Body shop soil (BSS) samples were inoculated into DMN minimal medium by adding 0.2 gram of the soil sample into individual sterile test tubes (described in Chapter II, Table II). After 30 days of inoculation at 30 ^{o}C, headspace above the liquid was analyzed and following results were obtained:

In more than 71.5 percent of the samples, which were amended with potassium antimonyl tartrate (0.01 mM), trimethylstibine was found in the headspace (Figure 3, Figure 4). Slightly higher percentages of trimethylstibine (75 %) was achieved by the samples which were amended with 0.01 mM potassium hexahydroxy antimonate (Figure 5, Figure 6). Two out of 7 samples which were amended with potassium antimonyl tartrate, and 2 out of 8 samples which were amended with potassium hexahydroxy antimonate did not produce detectable amounts of trimethylstibine even though they were amended with the corresponding antimony compounds [detection limit for trimethylstibine was calculated to be 15.2 pg/mL of 2.2 ppbv in a 1 mL gas injection (Gürleyük, 1996)]. But investigation of cultures that produced these chromatograms indicates a very low “bacterial-activity” in these systems (Figure 7). This could be easily concluded by comparing their organosulfur production with the rest of the chromatograms. Since these soil samples were not sterilized, this low activity could be due to the lack of antimony-resistant bacteria in these soil samples which could be explained by the inhomogeneity of the soil. This suggests that some parts of the soil did not contain antimony-resistant bacteria which

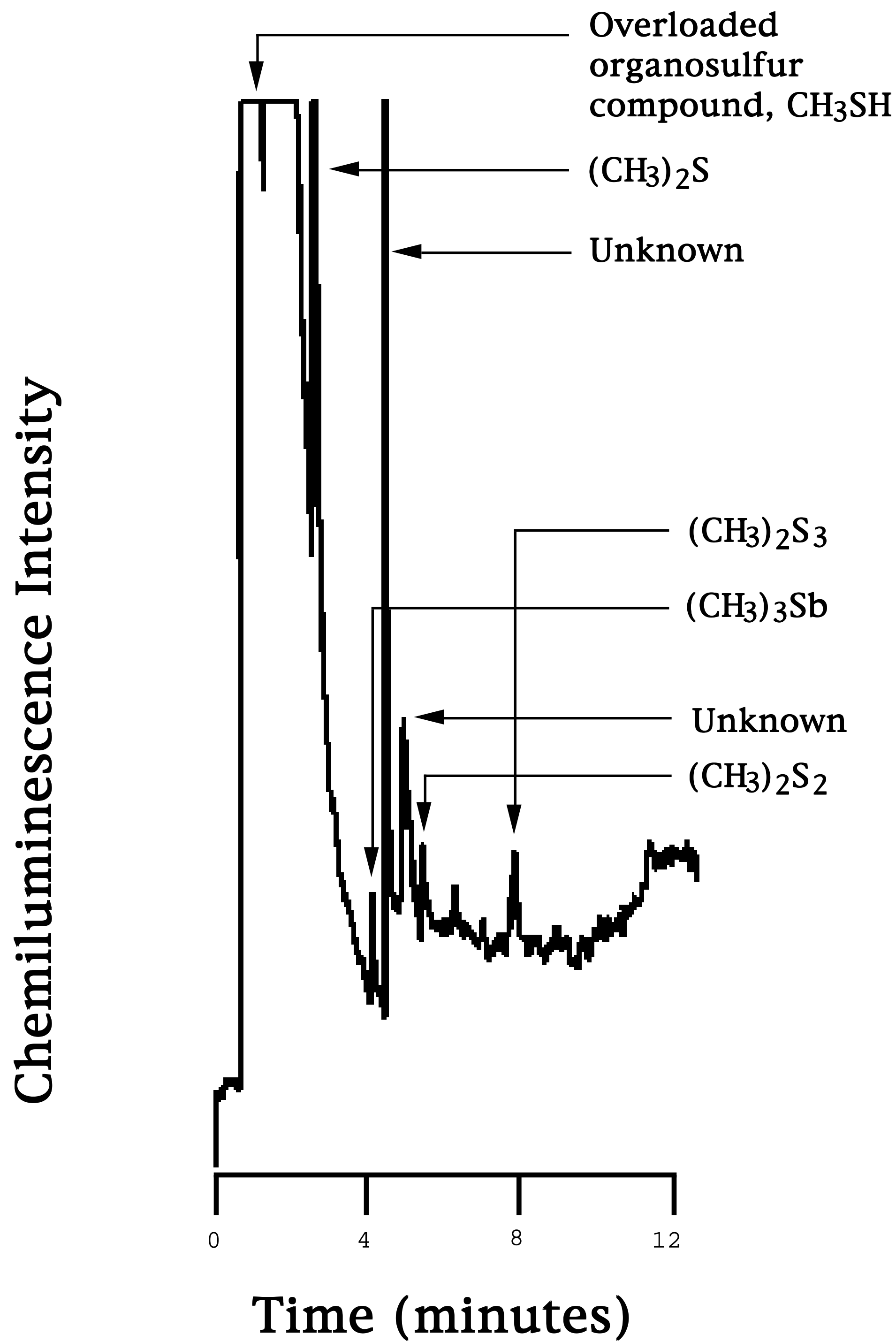

Figure 3. The chromatogram of the headspace of BSS soil amended with 0.01 mM potassium antimonyl tartrate, 1 month after inoculation. (I)

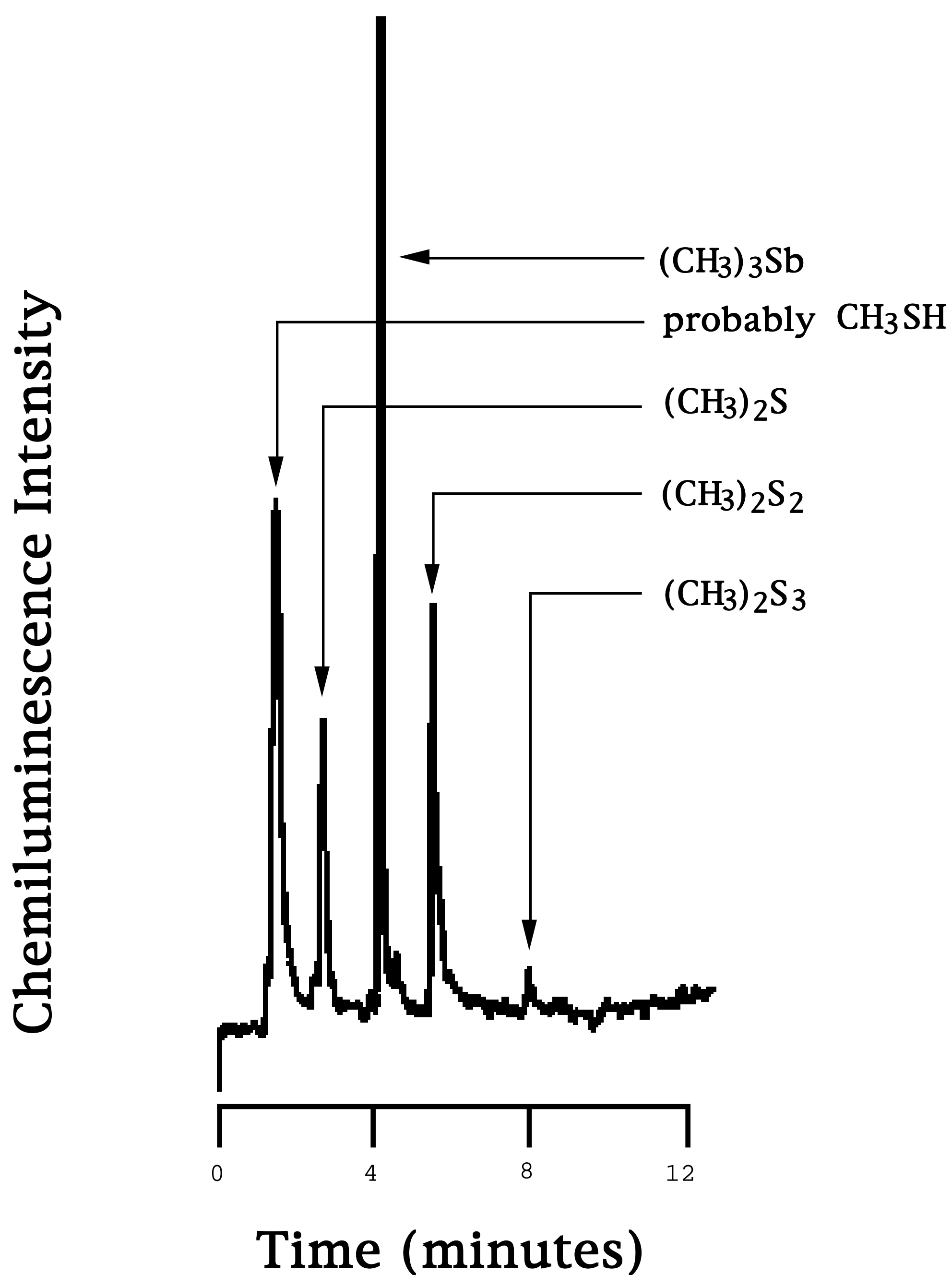

Figure 4. The chromatogram of the headspace of BSS soil amended with 0.01 mM potassium antimonyl tartrate, 1 month after inoculation. (II)

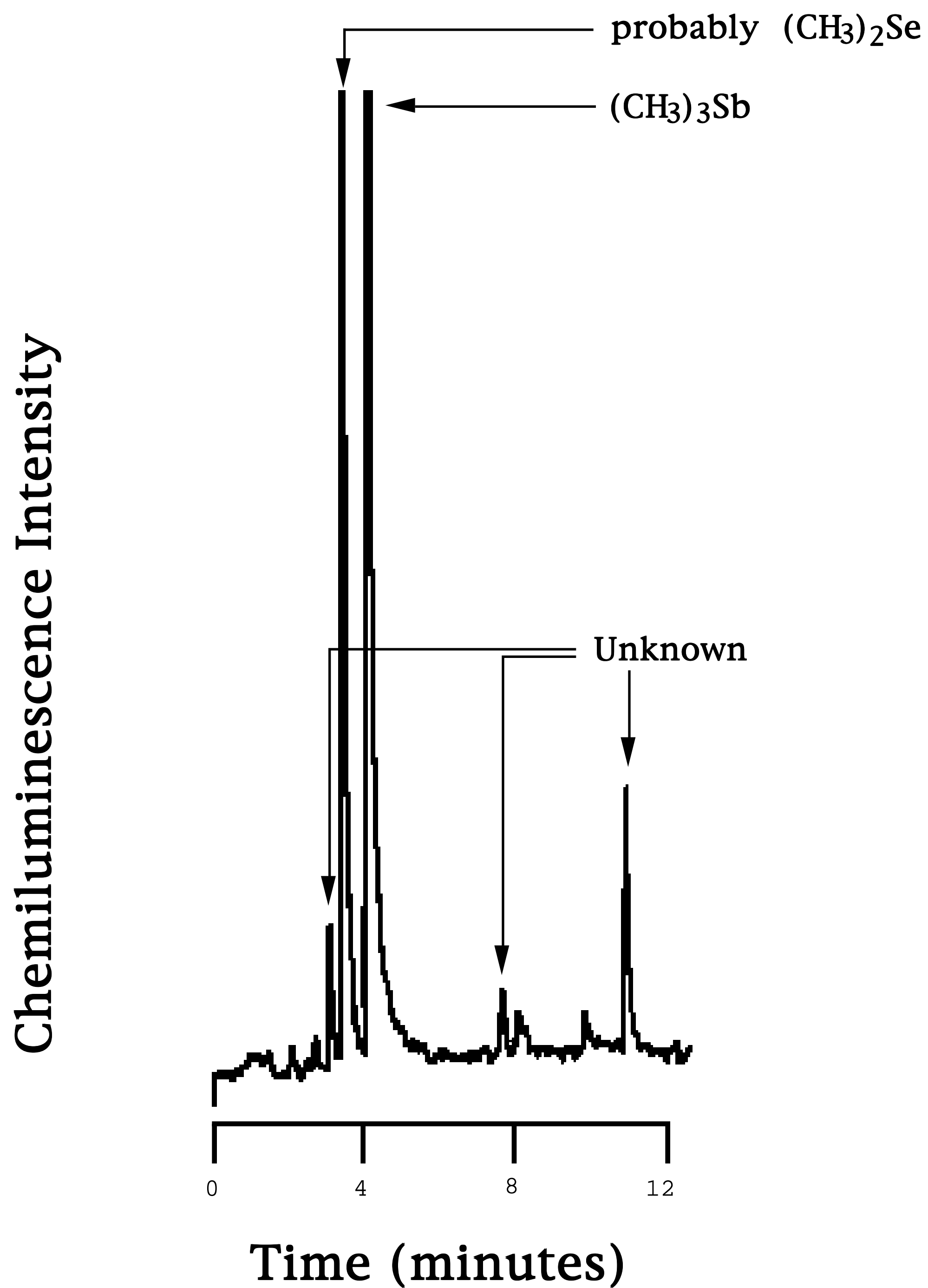

Figure 5. The chromatogram of the headspace of BSS soil amended with 0.01 mM potassium hexahydroxy antimonate, 1 month after inoculation. (I)

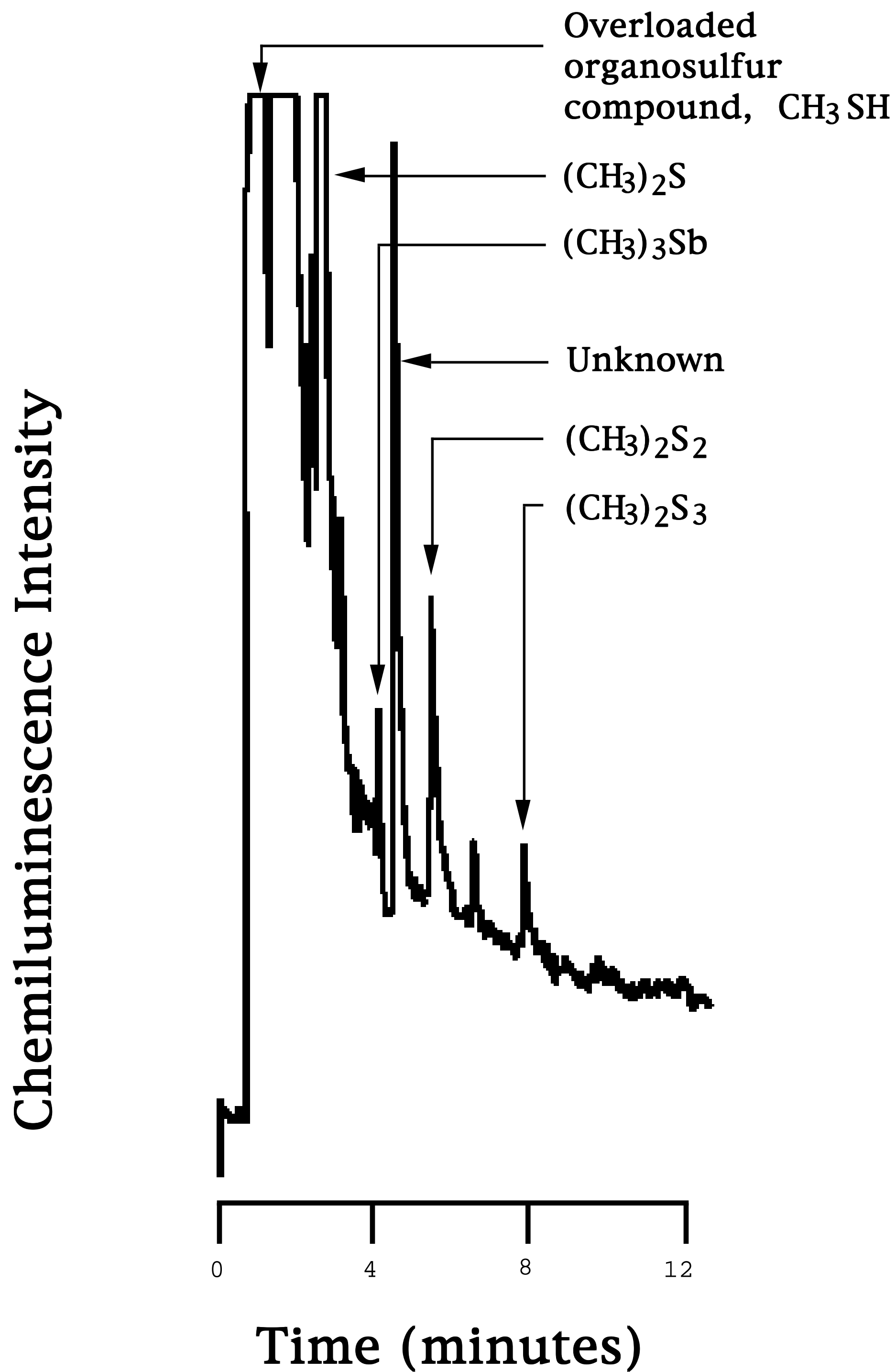

Figure 6. The chromatogram of the headspace of BSS soil amended with 0.01 mM potassium hexahydroxy antimonate, 1 month after inoculation.(II)

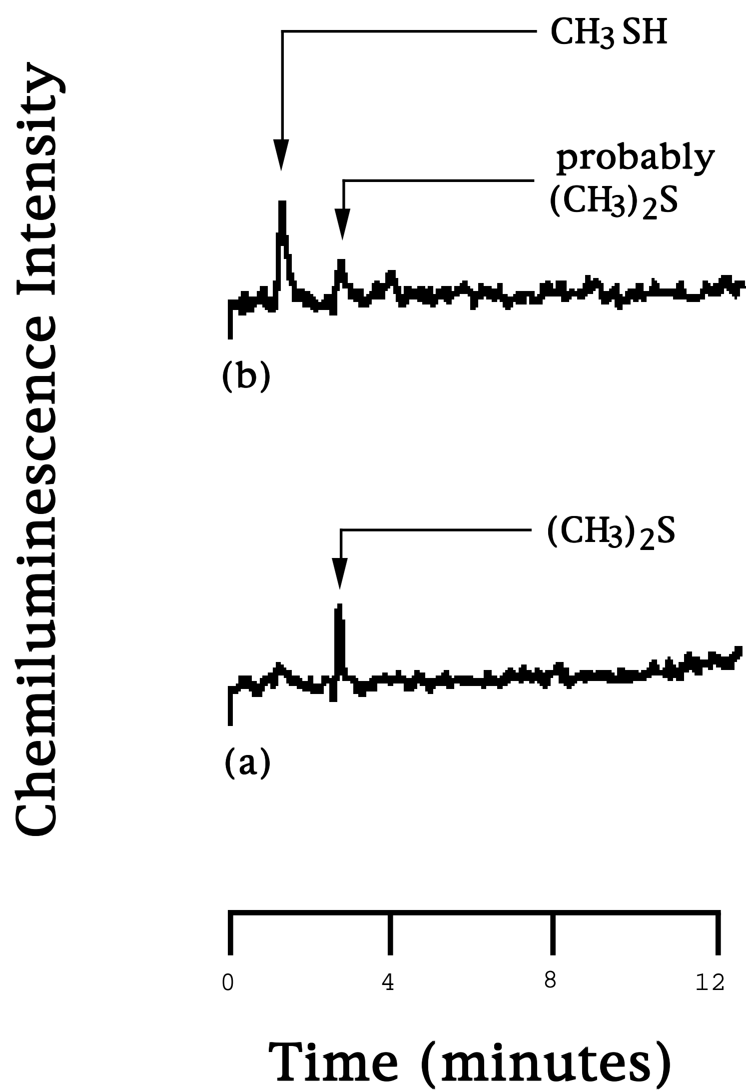

Figure 7. The chromatograms of the headspace analyses of BSS soil amended with (a) 0.01 mM potassium antimonyl tartrate; (b) 0.01 mM potassium hexahydroxy antimonate, 1 month after inoculation.

we were interested. While working with the soil samples, this was the major problem since the soil samples, collected from the environment, were not ideally homogeneous even though they were mixed completely before being placed into the test tubes.

In the control groups, which did not contain any added antimony compounds, no trimethylstibine was found for most of the cases (Figure 8). But for some control samples, which contained only the soil and the culture medium, trimethylstibine was found (Figure 9). Similar results had been observed in Gürleyük's experiments (Gürleyük, 1996) while he was investigating the same soil samples for antimony biomethylation activity. He concluded the production of trimethylstibine from the control groups to be the natural occurrence of antimony in the soil. He analyzed the specific soil samples using inductively coupled plasma spectroscopy and high concentrations of environmental antimony (@ 468.6 ppb) were found in 2 out of 4 soil samples analyzed.

Optical densities of the bacterial liquids could not be taken because of the soil in the test tubes, but still the bacterial growth was examined by shaking the test tubes gently without disturbing the soil, and looking the turbulence in the liquid which would suggest bacterial growth.

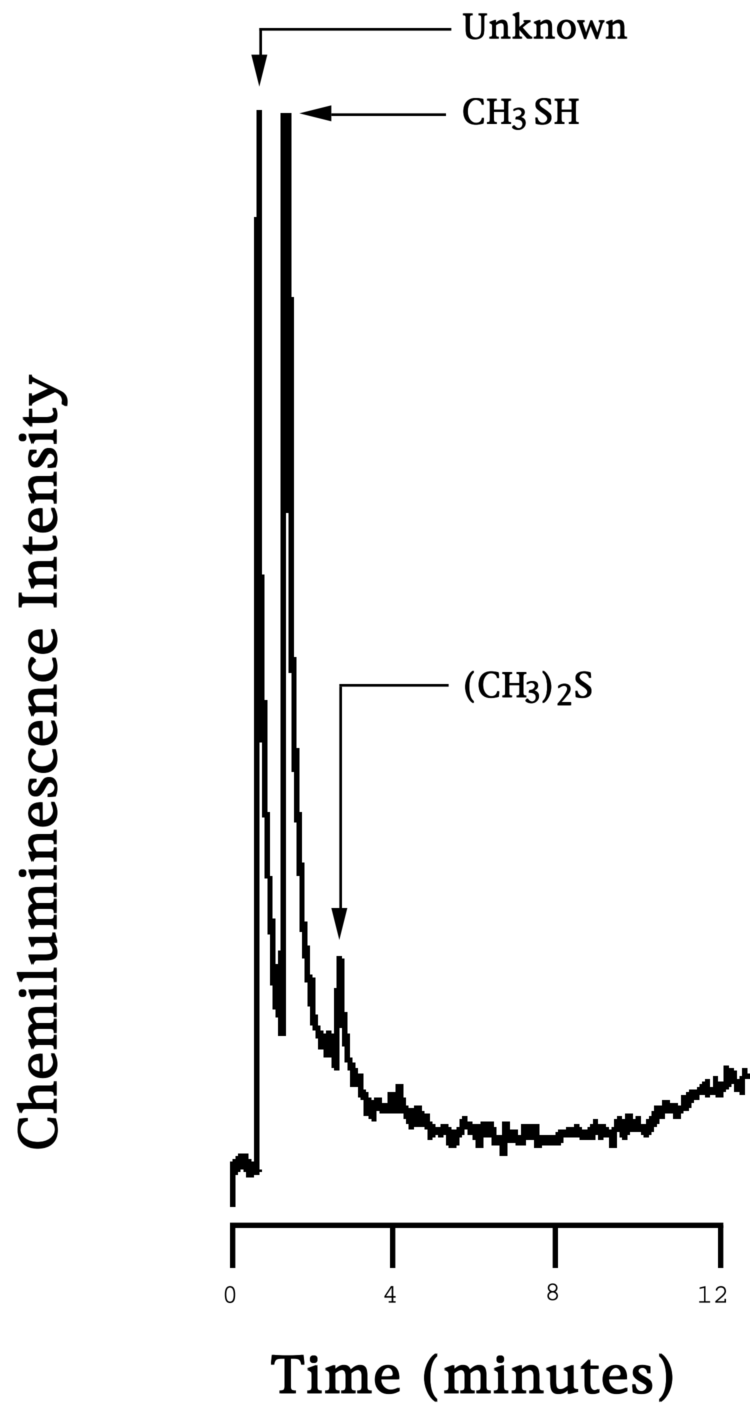

Figure 8. The chromatogram of the headspace of BSS soil, 1 month after inoculation (no antimony added).

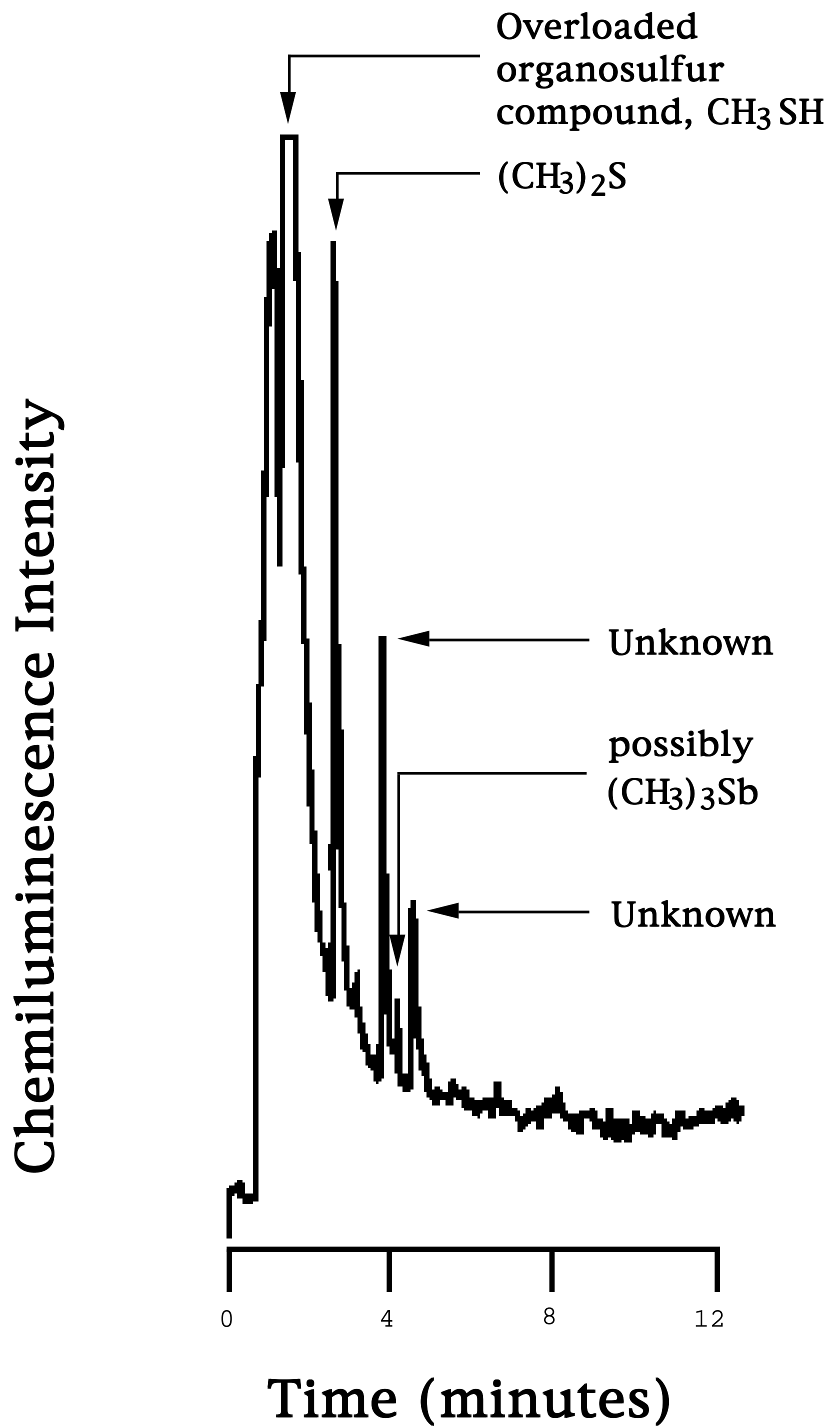

Figure 9. The chromatogram of the headspace of BSS soil, 1 month after inoculation (no antimony added).

Swiss Soil Experiment

Another experiment was performed with a soil sample which was collected from an arsenic contaminated tannery site in Switzerland. Two different media (TSN and DMN) were used for the bacterial growth (described in Chapter II, Table III). In some cases high organosulfur production by the bacterial cultures lead to a split chromatographic injection to be used in headspace analyses. Figure 10 shows a typical overloaded case in a sample which was poisoned with 7 mM potassium hexahydroxy antimonate in TSN. At least these high organosulfur productions proved the presence of antimony-resistant bacteria in our soil samples. Otherwise the bacteria would have been killed or their growth severely suppressed and little or no organosulfur would be detected.

For the Swiss soil experiments, when the potassium hexahydroxy antimonate concentration (in TSN) was decreased to 0.0667 mM, trimethylstibine was found (Figure 11) in the headspace. Consecutive diluted injections, for a better chromatographic separation, also contained trimethylstibine (Figure 12, Figure 13), and no trimethylstibine was found in the control groups (Figure 14) which means that the production of trimethylstibine was the result of biological activity (biomethylation) of the organisms.

However, the same bacterial activity could not be observed for the samples which were amended with potassium antimonyl tartrate in TSN. For high potassium antimonyl tartrate concentrations, bacterial activities seemed to be attenuated (Figure 15). When the potassium antimonyl tartrate concentration was decreased, however,

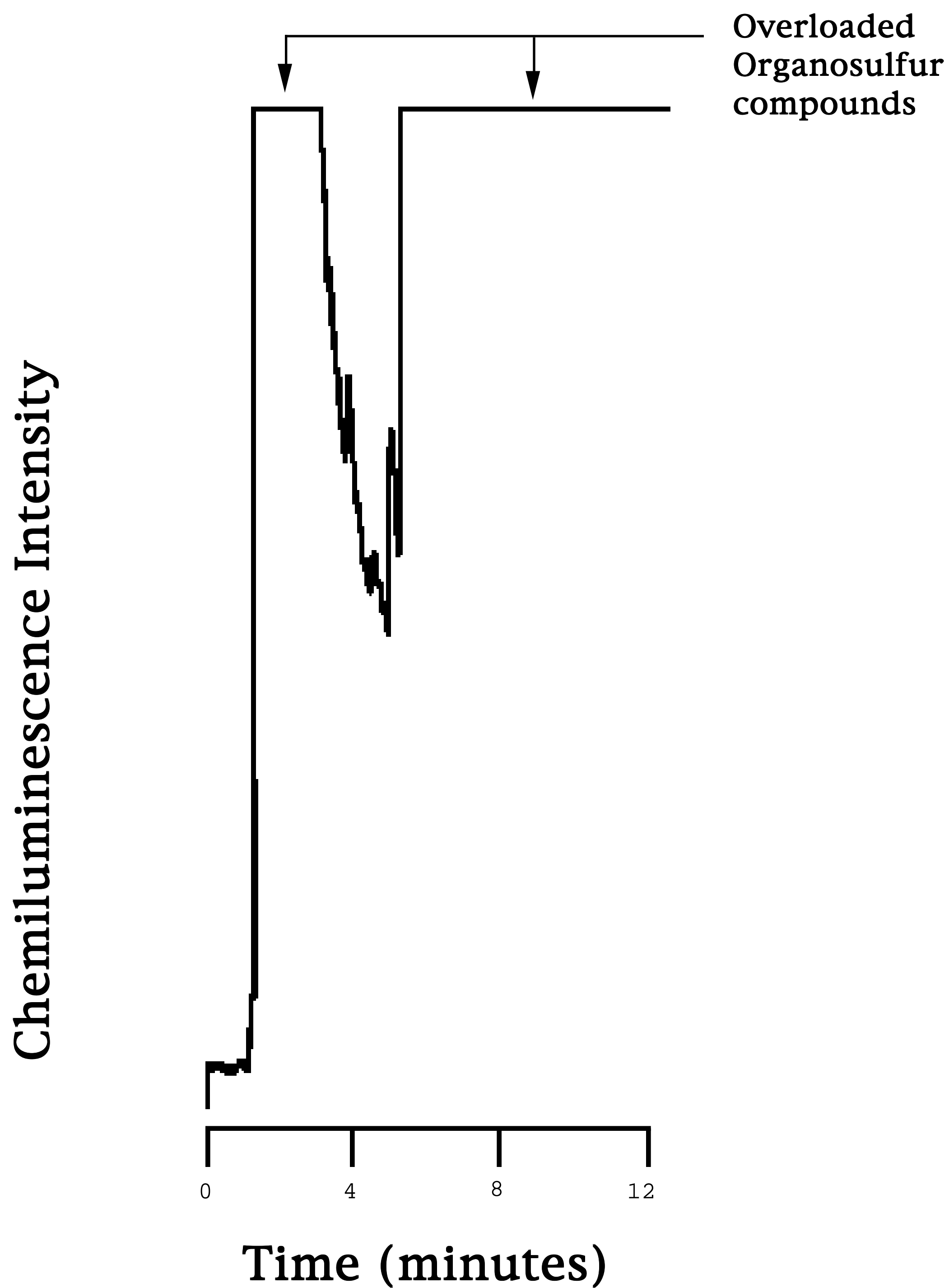

Figure 10. The highly overloaded chromatogram of the headspace of SS soil in TSN amended with 7 mM potassium hexahydroxy antimonate. The headspace (1 mL) was analyzed 1 month after amendment.

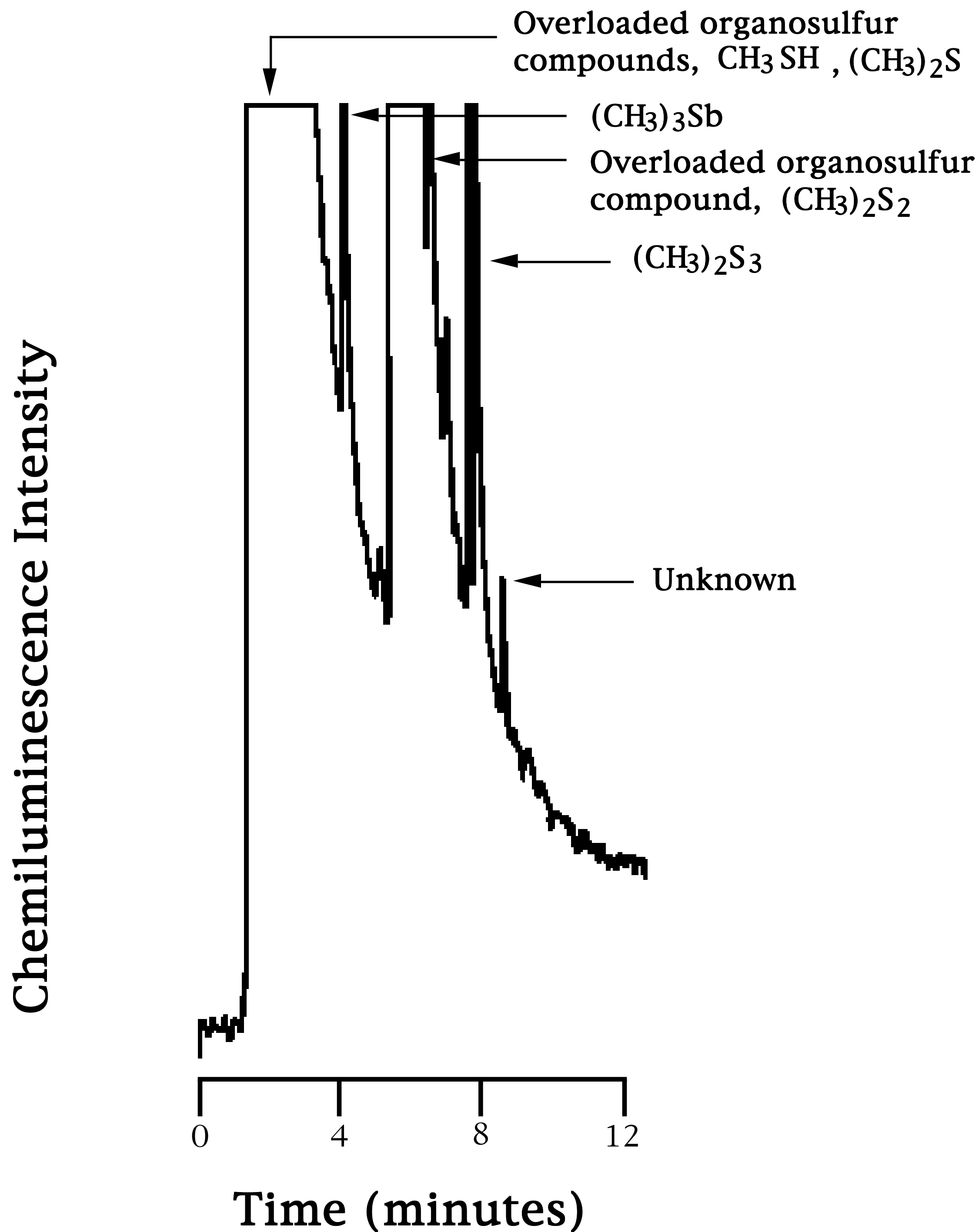

Figure 11. The chromatogram of the headspace of SS soil in TSN amended with 0.0667 mM potassium hexahydroxy antimonate. The headspace (0.1 mL) was analyzed 1 month after amendment.

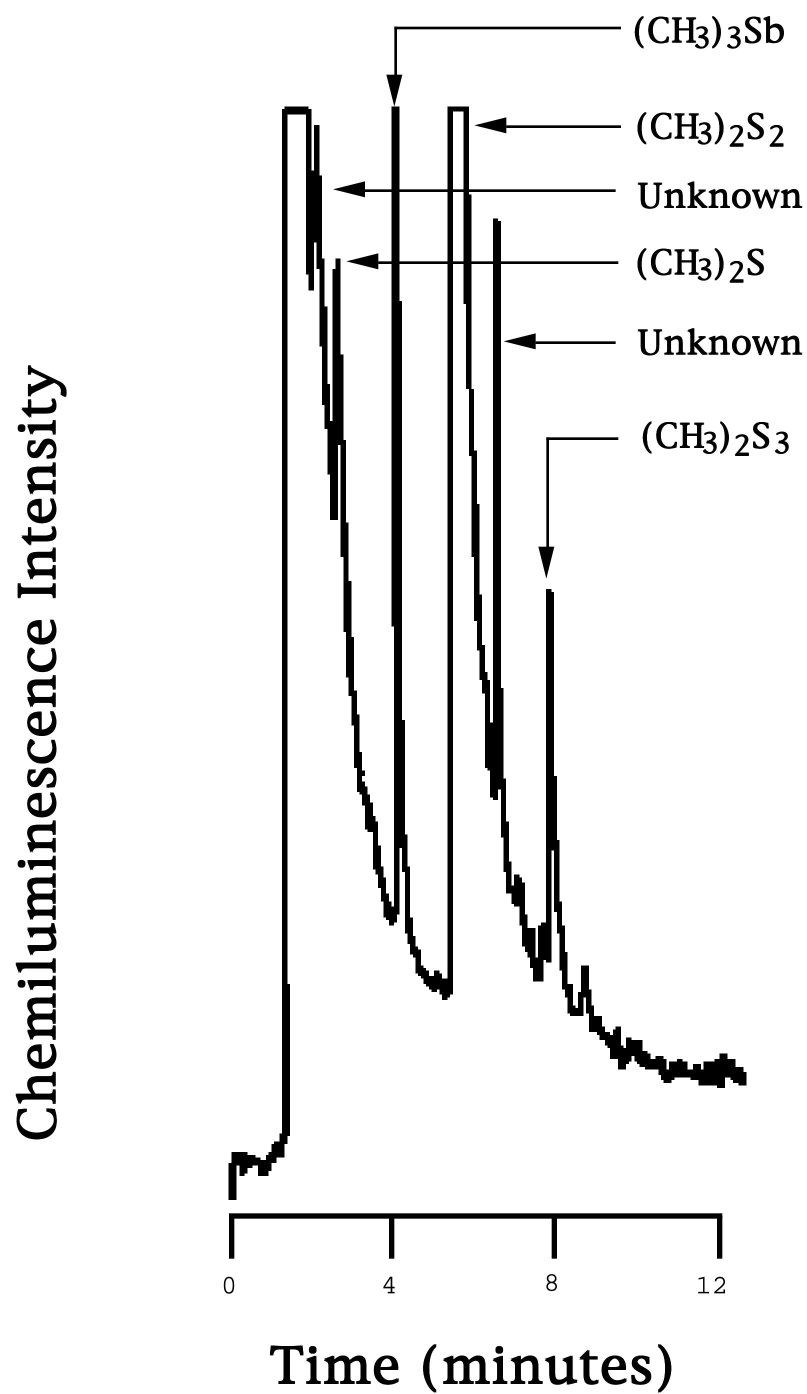

Figure 12. The chromatogram of the headspace of SS soil in TSN amended with 0.0667 mM potassium hexahydroxy antimonate. The headspace (0.025 mL) was analyzed 1 month after amendment.

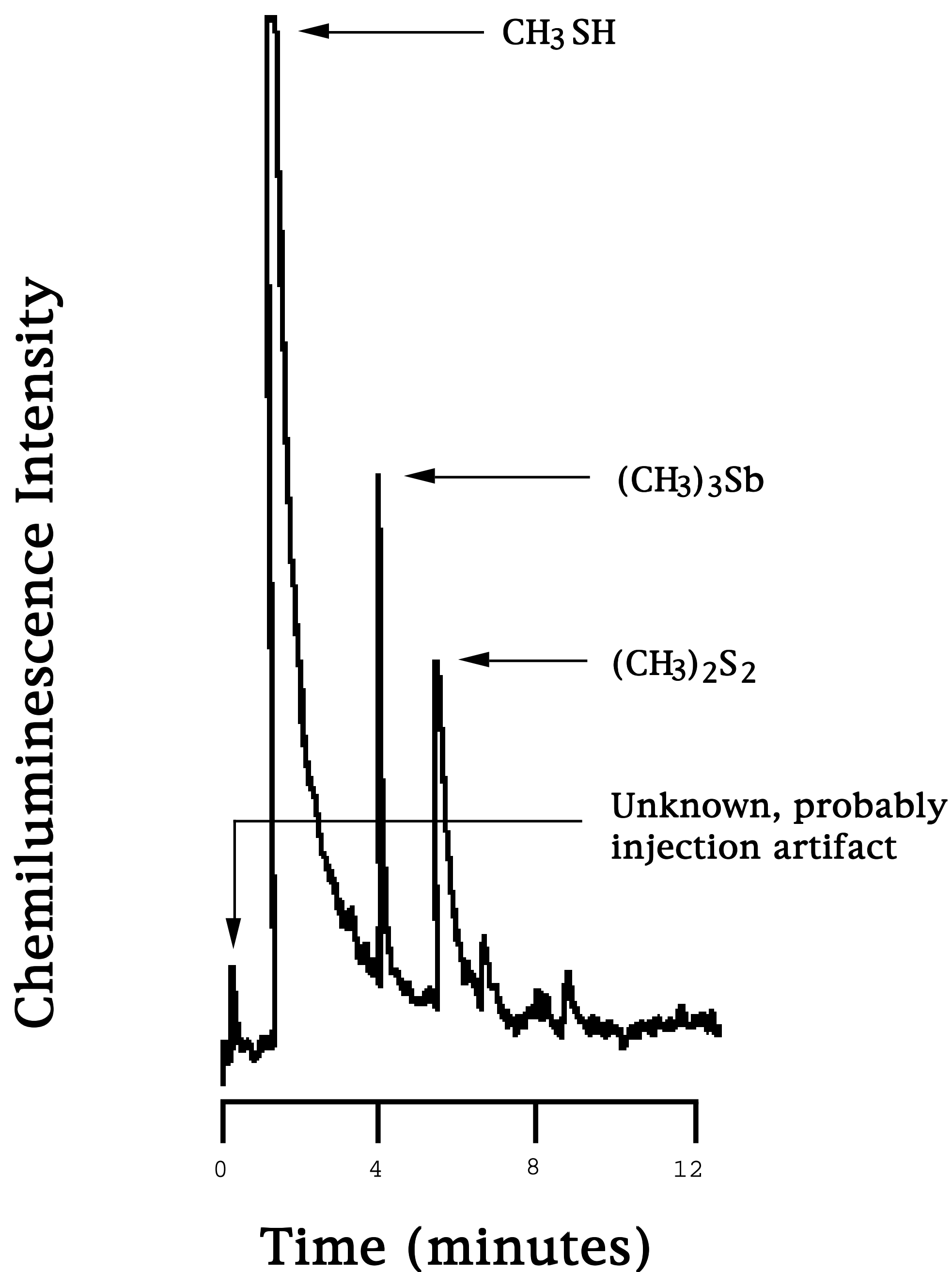

Figure 13. The chromatogram of the headspace of SS soil in TSN amended with 0.0667 mM potassium hexahydroxy antimonate. The headspace (0.2 mL, split injection; split ratio: 1/25.5) was analyzed 1 month after amendment.

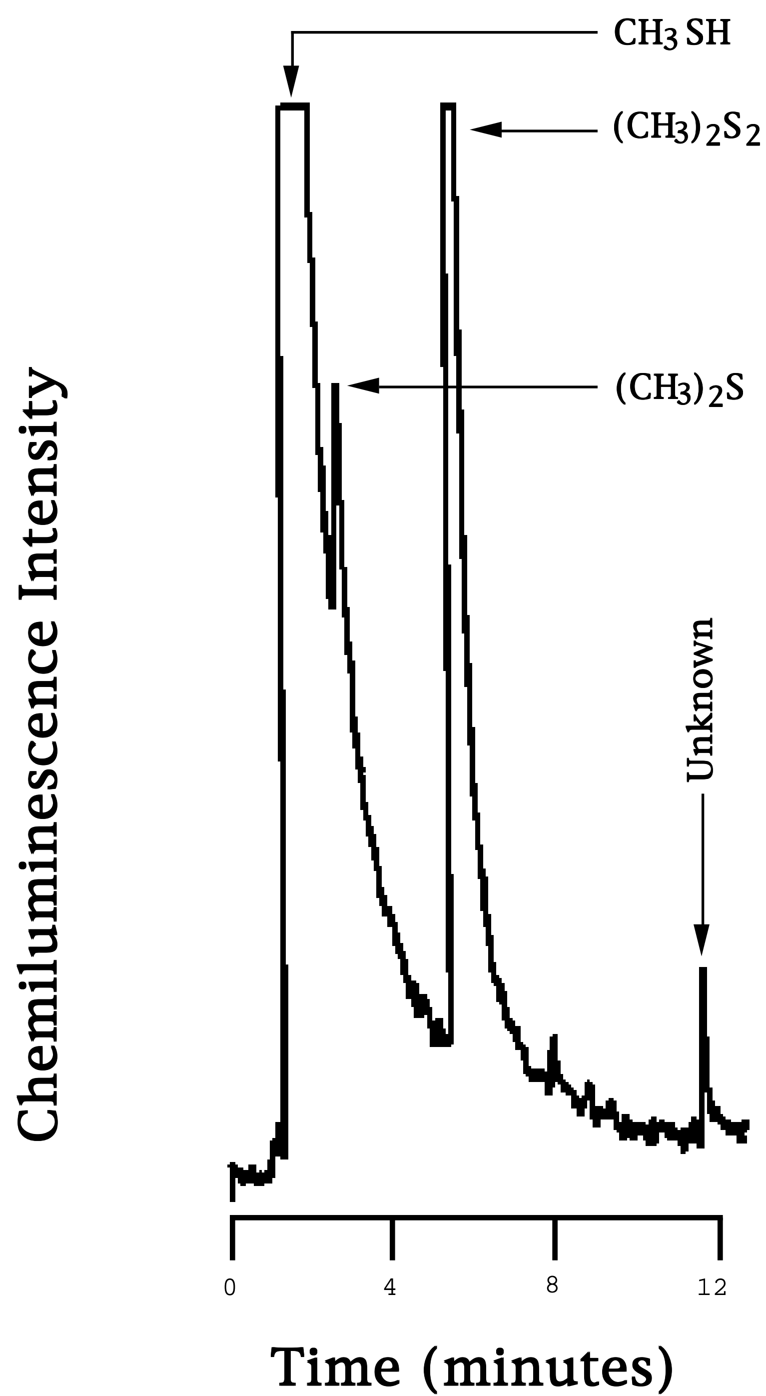

Figure 14. The chromatogram of the headspace of SS soil in TSN (no antimony added). The headspace (0.2 mL, split injection; split ratio: 1/25.5) was analyzed 1 month after amendment.

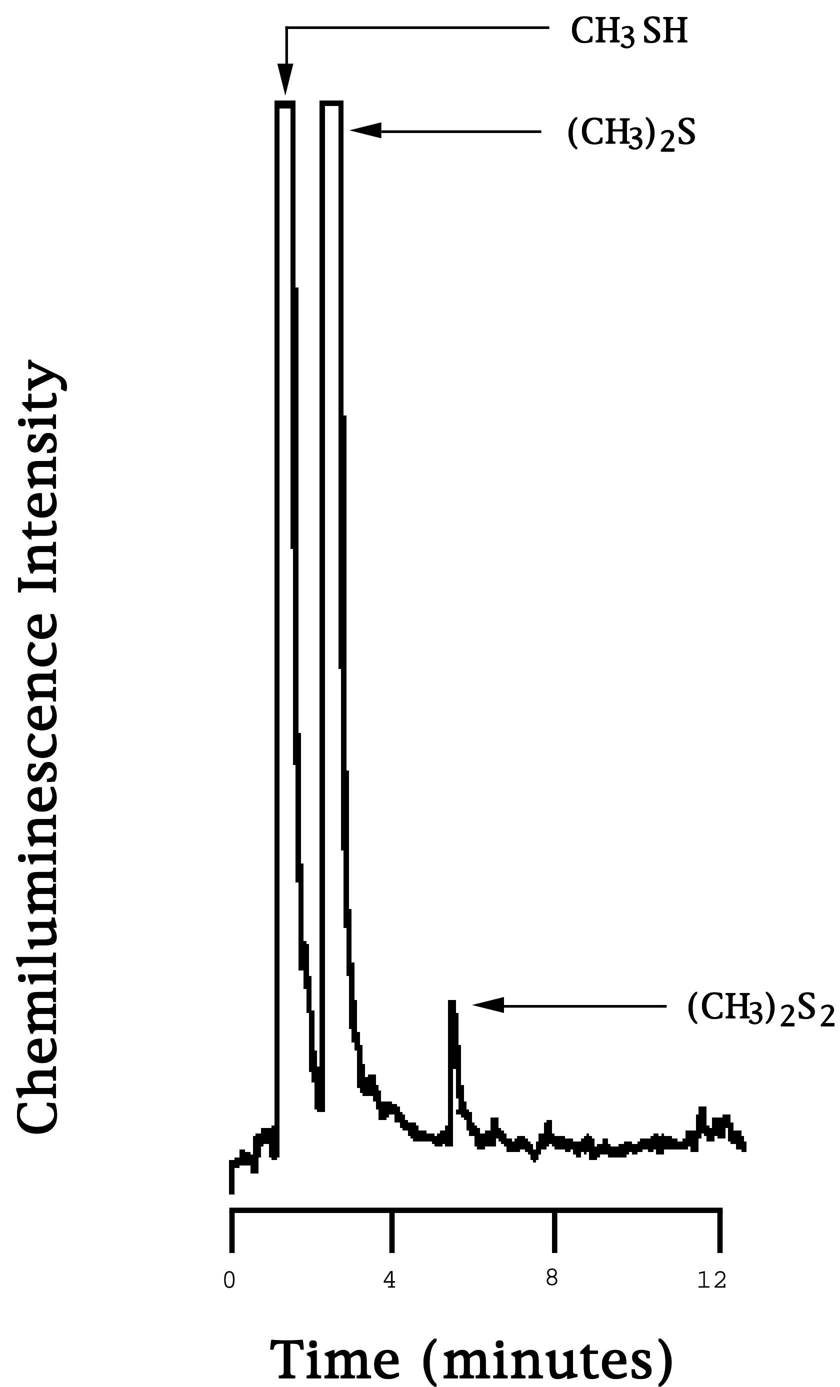

Figure 15. The chromatogram of the headspace of SS soil in TSN amended with 7 mM potassium antimonyl tartrate. The headspace (1 mL) was analyzed 1 month after amendment.

relatively high organosulfur production was observed, but still no trimethylstibine was found in the headspace samples.

This same trend was also observed for the samples that were antimony poisoned in DMN (minimal medium). Even though organosulfur production of the samples amended with 6.67 mM potassium hexahydroxy antimonate in DMN was smaller (Figure 16) than the ones in TSN, trimethylstibine was still found for lower (0.067m M) potassium hexahydroxy antimonate concentrations (Figure 17).

As observed in TSN, no trimethylstibine was found in samples which were amended with potassium antimonyl tartrate, and for the control groups in DMN medium.

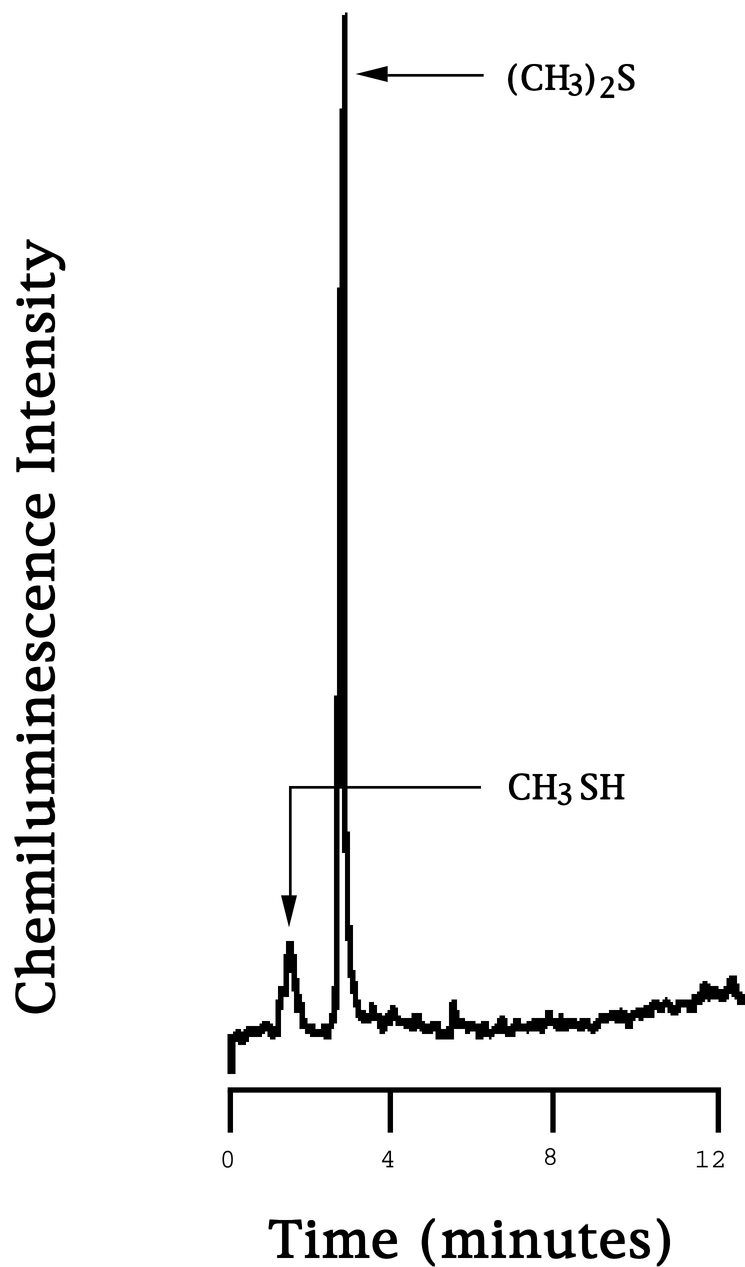

Figure 16. The chromatogram of the headspace of SS soil in DMN amended with 6.67 mM potassium hexahydroxy antimonate. The headspace was analyzed 1 month after amendment.

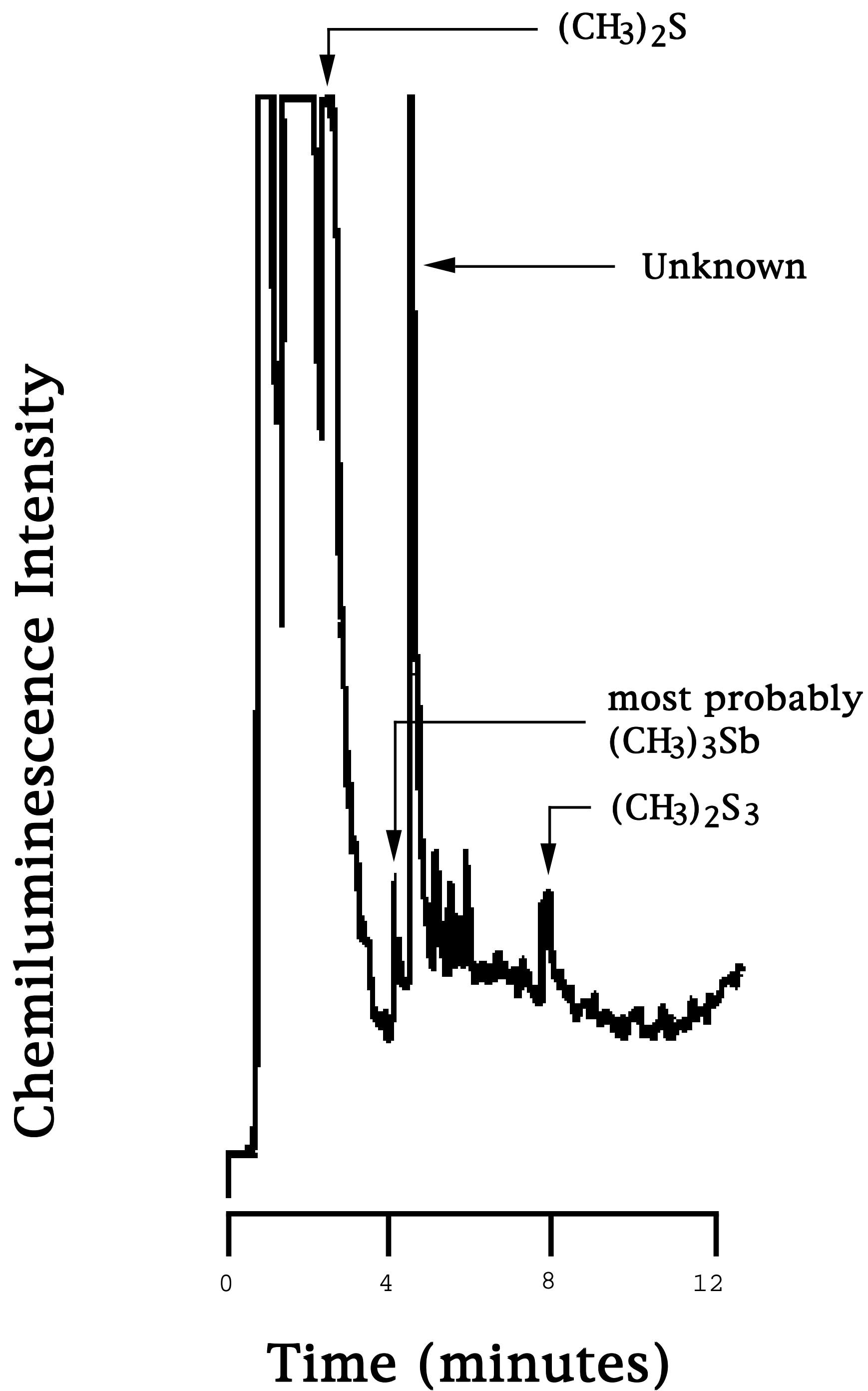

Figure 17. The chromatogram of the headspace of SS soil in DMN amended with 0.0667 mM potassium hexahydroxy antimonate. The headspace was analyzed 1 month after amendment.

Soil Extract (I, II, and III) Experiments

After trimethylstibine was found in soil samples (Figure 5), extract experiments were designed as the first step in the isolation of the bacterial cultures from soil samples which were responsible from the biological methylation of inorganic antimony compounds. Liquid samples taken from the soil experiments (BSS, and SS) were used as bacterial sources for extract experiments (for Extract III experiment, the bacterial source was the liquid sample taken from Extract I experiment). All extract experiments were performed in TSN medium as described in Chapter II (Table IV, Table V, Table VI).

For Extract I experiment, the bacterial source was the Body Shop Soil experiment's liquid sample which had been amended with 0.01 mM potassium hexahydroxy antimonate (Figure 5) and which had produced trimethylstibine. One tenth millimolar potassium hexahydroxy antimonate amendment for Extract I also produced trimethylstibine successfully for both duplicates (Figure 18, Figure 19). Presence of trimethylstibine in the samples was even more clearly indicated by a slower chromatographic temperature ramp (5 ^{o}C/min) which gave a better chromatographic separation between organosulfur and organoantimony compounds (Figure 20, Figure 21). No trimethylstibine was found in the samples which were amended with potassium antimonyl tartrate and in the control sets of Extract I experiment.

For Extract II experiment, the bacterial source was the Body Shop Soil experiments liquid sample which had been amended with 0.01 mM potassium hexahydroxy antimonate (different test tube

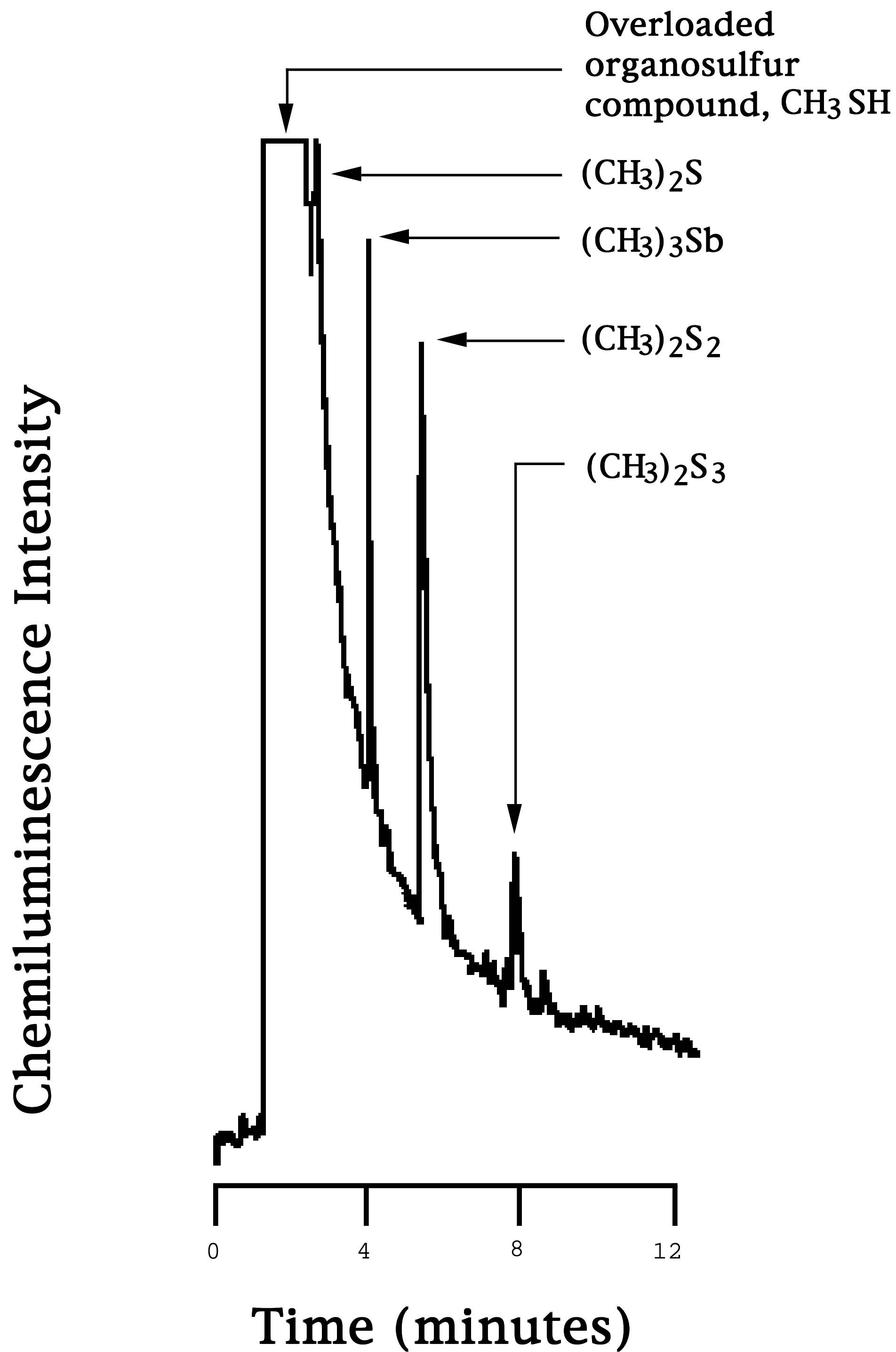

Figure 18. The chromatogram of the headspace of Extract I sample amended with 0.1 mM potassium hexahydroxy antimonate. The headspace (0.5 mL split injection) was analyzed about 3 weeks after inoculation.

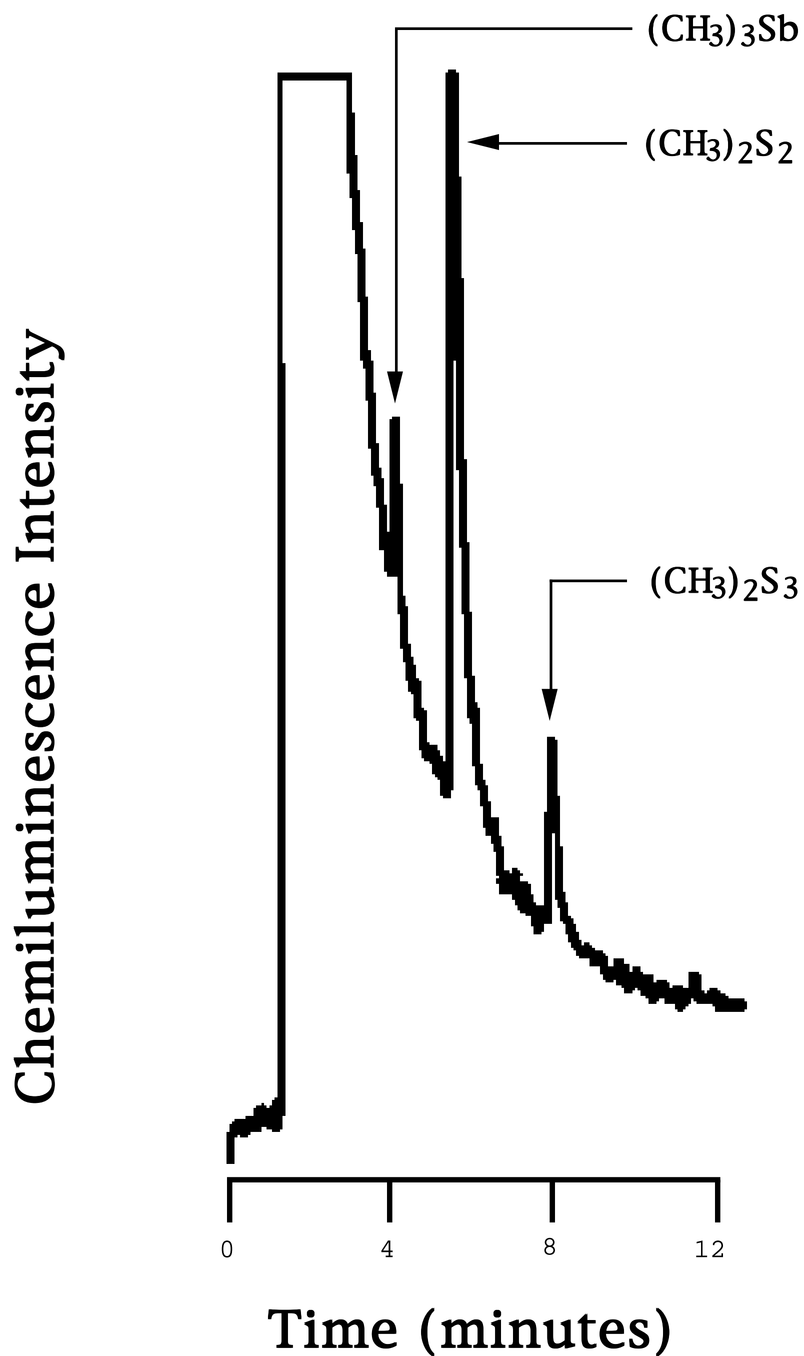

Figure 19. The chromatogram of the headspace of Extract I sample amended with 0.1 mM potassium hexahydroxy antimonate (duplicate). The headspace (0.5 mL. split injection) was analyzed about 3 weeks after inoculation.

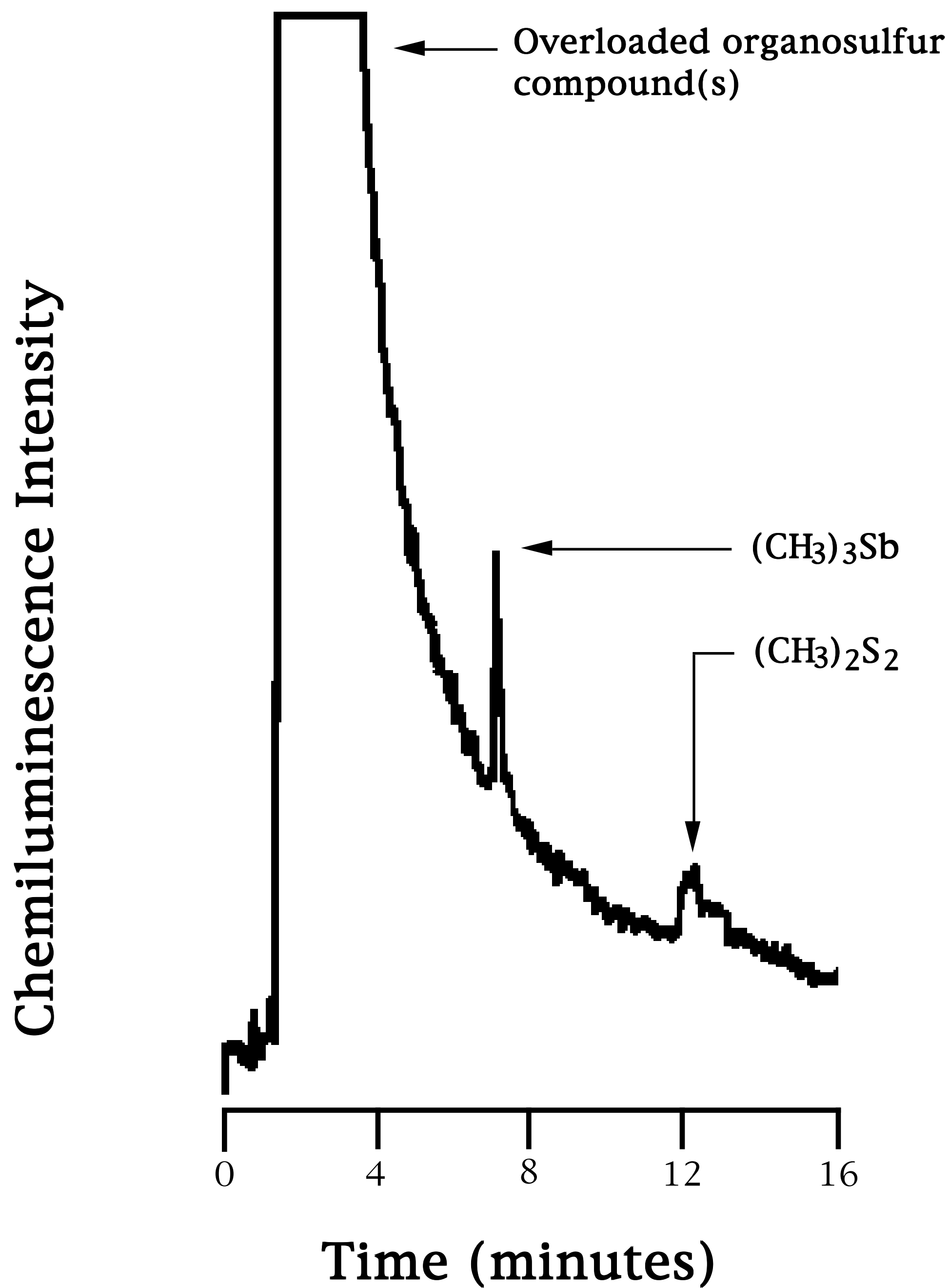

Figure 20. The chromatogram of the headspace of Extract I sample amended with 0.1 mM potassium hexahydroxy antimonate (5 ^{o}C/min temperature ramp). The headspace (1 mL split injection) was analyzed about 3 weeks after inoculation.

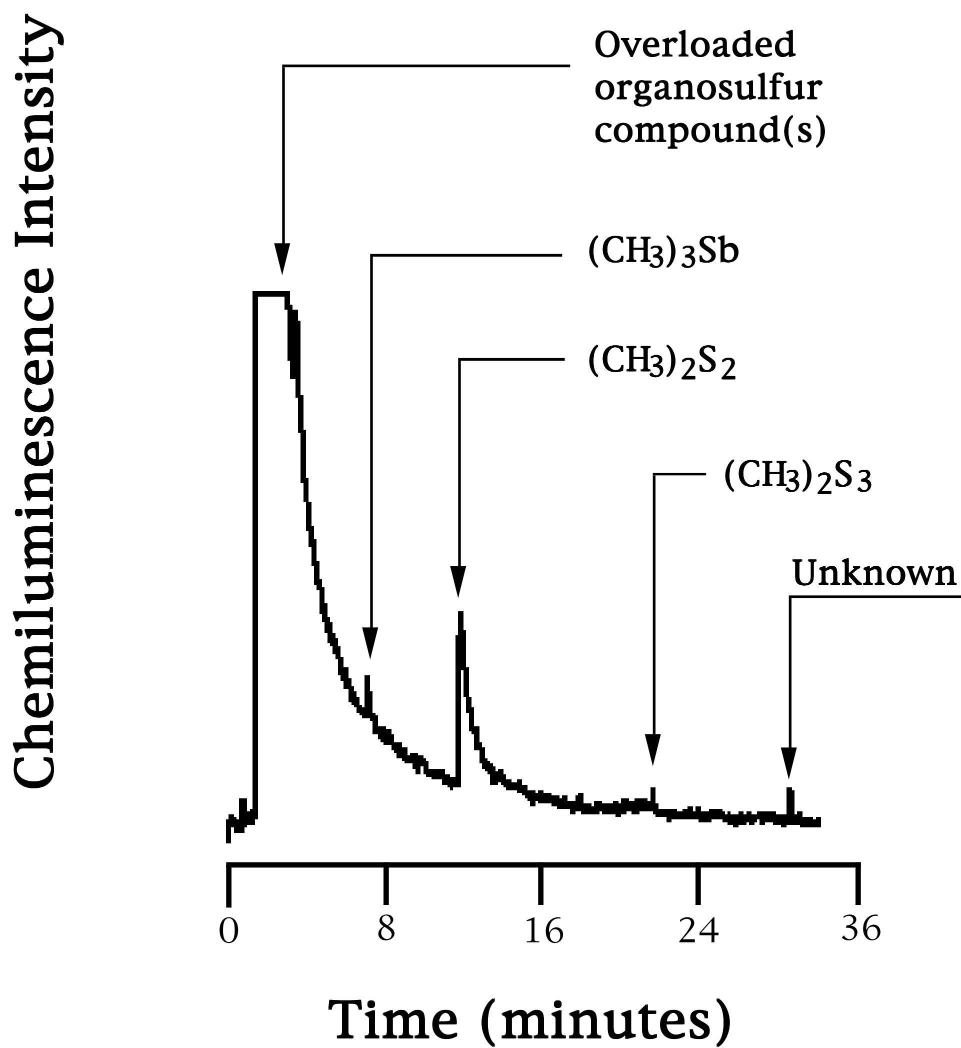

Figure 21. The chromatogram of the headspace of Extract I sample (duplicate) amended with 0.1 mM potassium hexahydroxy antimonate (5 ^{o}C/min temperature ramp). The headspace (0.5 mL split injection) was analyzed about 3 weeks after inoculation.

than the one used for Extract I experiment). In this case, even though trimethylstibine was observed in the original source, no trimethylstibine was found for both the samples and the controls of these (Extract II) experiments.

For Extract III experiment, the bacteria source (liquid sample) was taken from the Extract I experiment sample which had been amended with 0.01 mM potassium hexahydroxy antimonate and which had produced trimethylstibine. Again this second re-enrichment process gave positive results and trimethylstibine was found in **4 out of 4 samples**. (Figures 22, Figure 23). Two control sets were prepared; the first one which did not contained bacteria but did have potassium hexahydroxy antimonate added, did not produce trimethylstibine, as expected; the second control set which had everything but the extract solution (described in Chapter II, part 1-2) did produce trimethylstibine, which failed the "soil extract dependency assumption" of the bacterial cultures in our experiments.

One more time, a slower temperature ramp was found to be very useful for chromatographic separation and identification of the organoantimony compounds in our research, since antimony presence in the Extract III samples could not be identified with the normal temperature ramp (20 ^{o}C/min), which has been used for several years in our research lab for chromatographic separations of different organic metal/metalloid compounds.

After the positive results of Extract III experiment, the next step in the isolation of the bacterial cultures was performed by streaking out the liquid samples (from Extract III) on agar plates as described in Chapter II, section 4-1d. The isolated colonies, then,

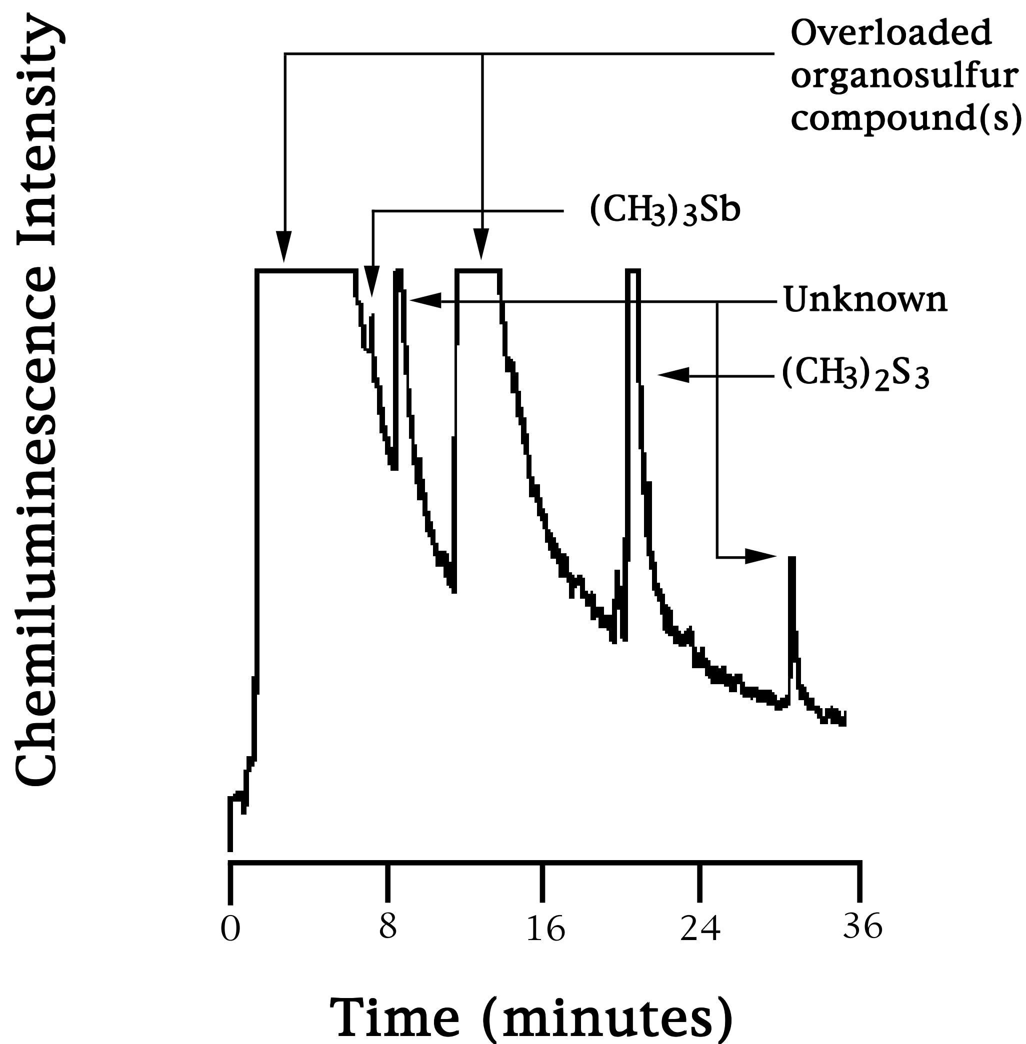

Figure 22. The chromatogram of the headspace of Extract III sample (I) amended with 0.01 mM potassium hexahydroxy antimonate (5 ^{o}C/min temperature ramp). The headspace (0.2 mL) was analyzed about 3 weeks after inoculation.

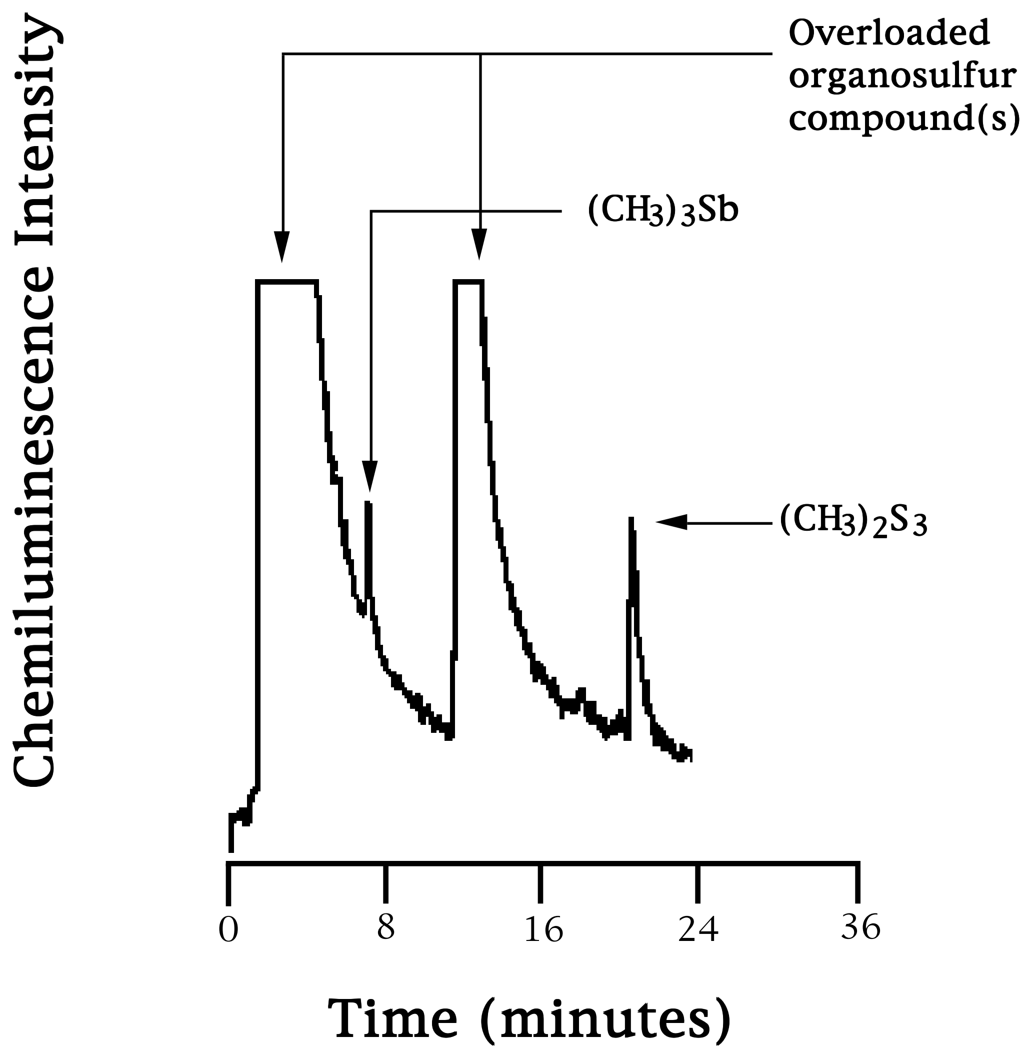

Figure 23. The chromatogram of the headspace of Extract III sample (II) amended with 0.1 mM potassium hexahydroxy antimonate (5 ^{o}C/min temperature ramp). The headspace (0.05 mL) was analyzed about 3 weeks after inoculation.

were amended with 0.01 mM and 0.1 mM antimony compounds (PHA, and PAT) in TSN medium. These **isolated mono cultures** were found to be resistant (as measured by growth curves; Figure 24) to potassium hexahydroxy antimonate and potassium antimonyl tartrate. However, trimethylstibine production of these isolated cultures could not be confirmed since the total organo-sulfur and -antimony production of these isolated cultures were lower than the polycultures (non-isolated). As a result of this decrease, the trimethylstibine peaks in the headspace analyses of these cultures stayed barely above a signal to noise ratio of 3 (Figure 25).

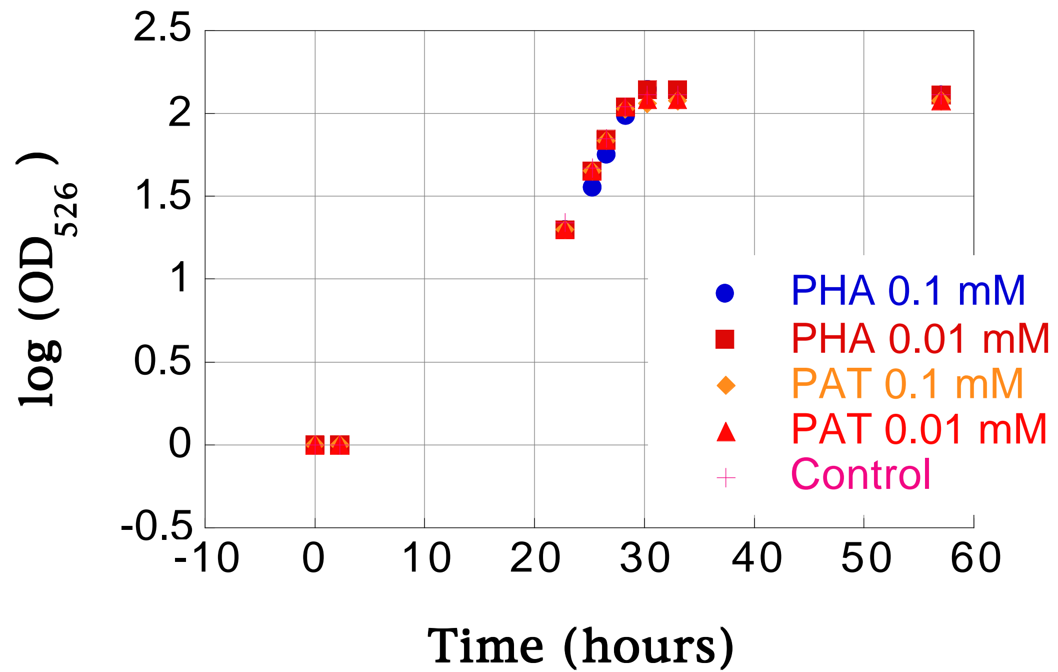

Figure 24. Growth curve of isolated monoculture (from Extract III) amended with antimony compounds in TSN.

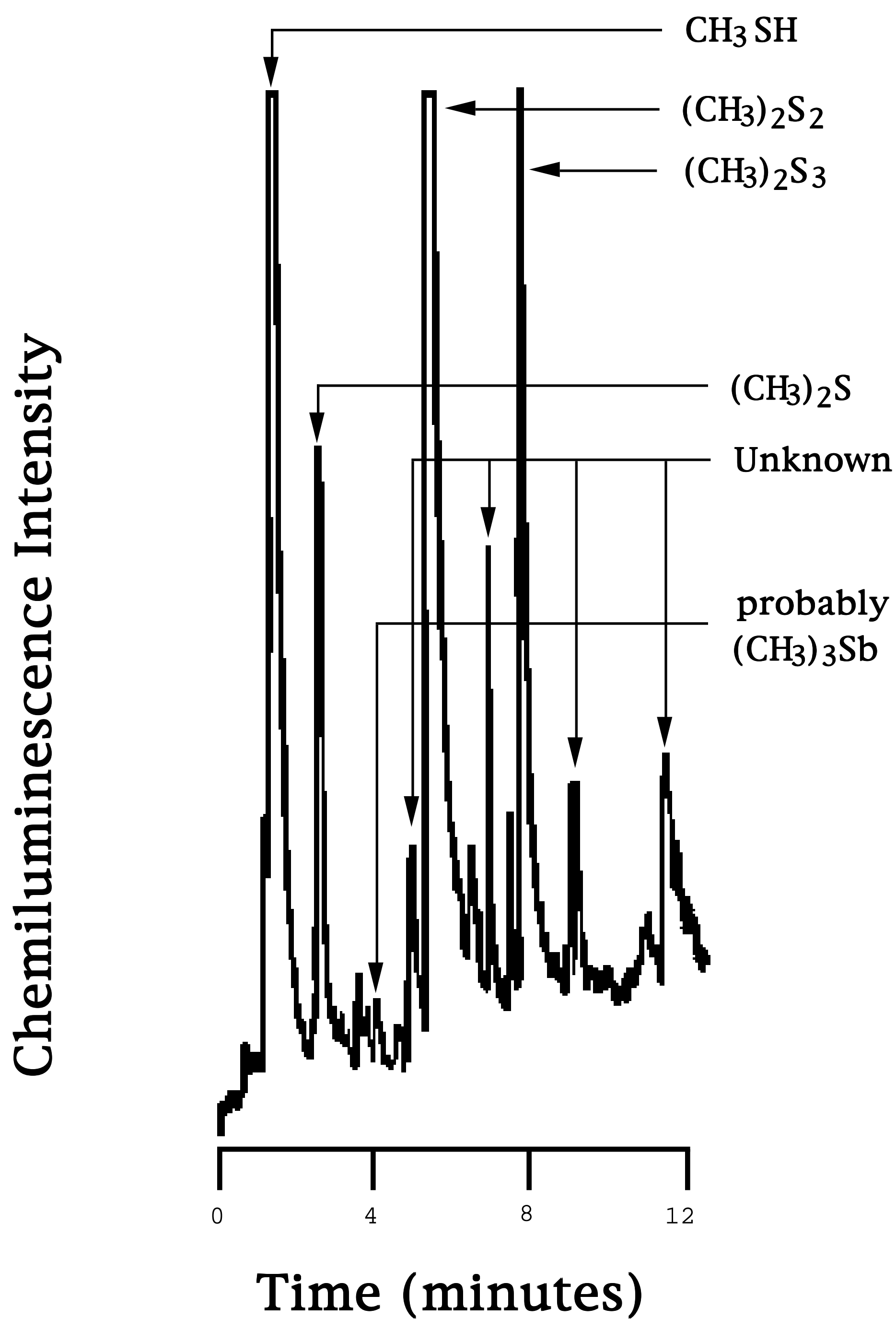

Figure 25. The chromatogram of the headspace of isolated monoculture (from Extract III experiment) amended with 0.01 mM potassium hexahydroxy antimonate. The headspace (1 mL) was analyzed about 3 weeks after inoculation.

Phototrophic Bacteria Experiments

None of the phototrophic bacteria types produced trimethylstibine even though they were found to be resistant (as measured by growth curves) to potassium hexahydroxy antimonate concentrations up to 5 mM and potassium antimonyl tartrate concentrations up to 1 mM. Their growth was monitored both from optical density readings (Figure 26, Figure 27, Figure 28) and from methylated sulfur production by headspace analyses. Overall results for all types of phototrophic bacteria is given in Table VIII.

Table VIII
Phototrophic Bacteria Results

Type	Chemical		Observations
DMS 158	PAT, PHA	0.01 mM	no effect on growth when compared to control.
	PAT, PHA	0.1 mM	no effect on growth when compared to control and 0.01 mM samples.
	PHA	1 mM	no effect on growth when compared to control and 0.01 mM samples.
	PAT	1 mM	growth was depressed; they started growing a week after amendment.
A	PHA	1 mM	no effect on growth
	PAT	1 mM	growth after 2 months
C/E	PHA	1 mM	no effect on growth
	PAT	1 mM	no growth
D	PHA	5 mM	no effect on growth
	PAT	1 mM	no growth
F	PHA	5 mM	no effect on growth.
	PAT	1 mM	growth after 2 months

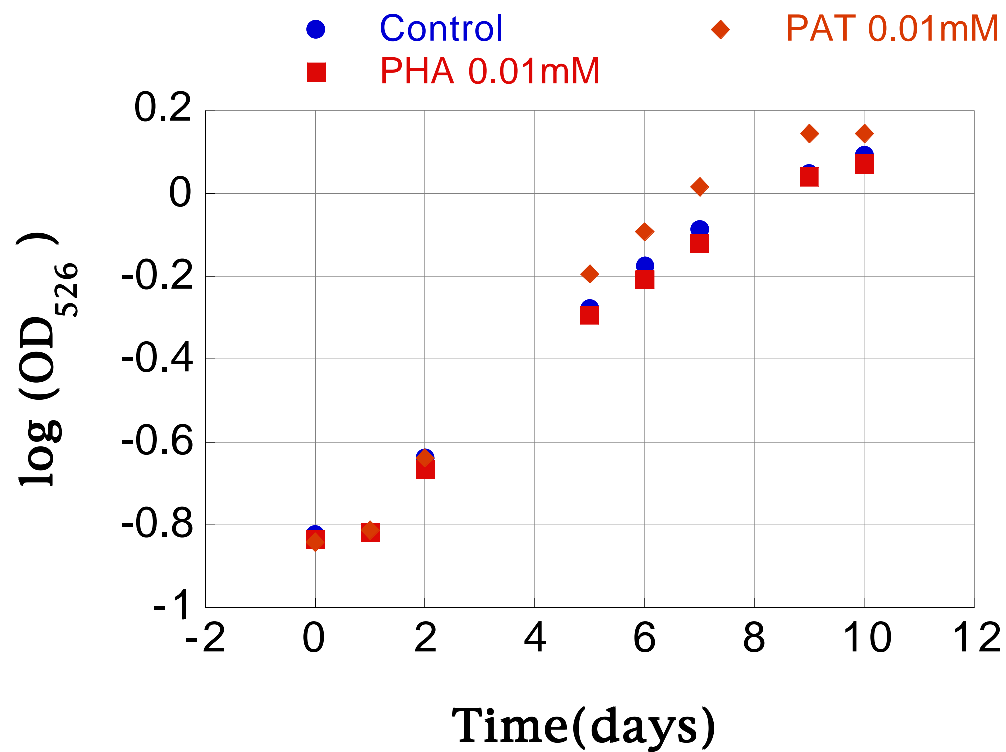

Figure 26. Typical growth curve of DMS 158 amended with 0.01 mM antimony compounds (PHA, PAT) in Sistrom minimal medium (SMM).

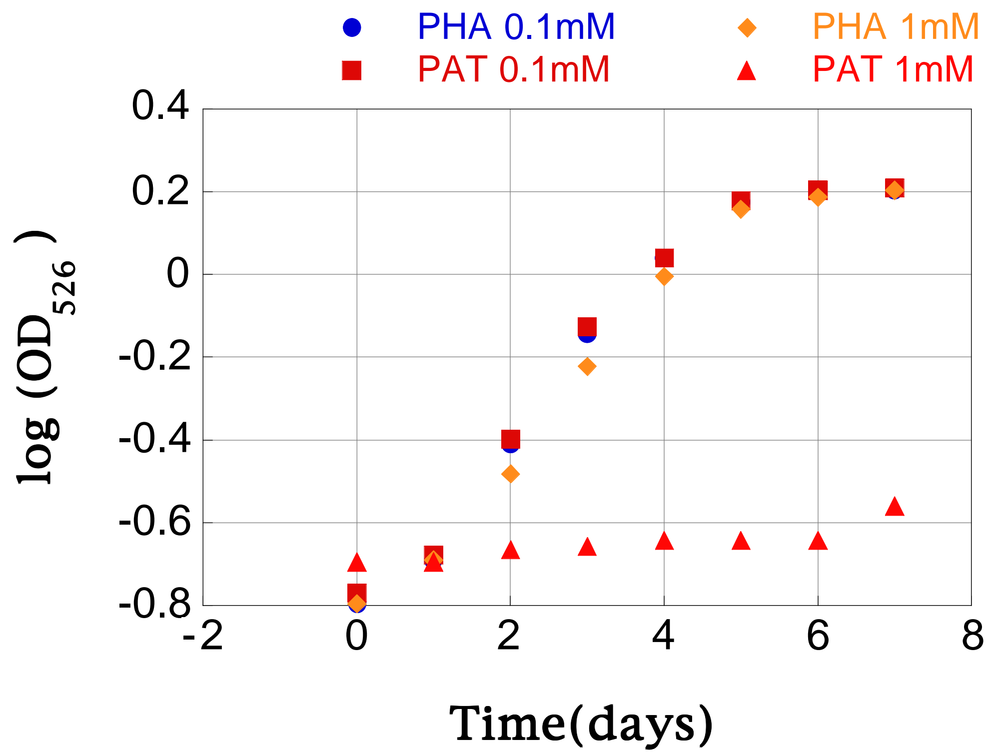

Figure 27. Typical growth curve of DMS 158 amended with 0.1 mM, and 1 mM antimony compounds (PHA, PAT) in Sistrom minimal medium (SMM).

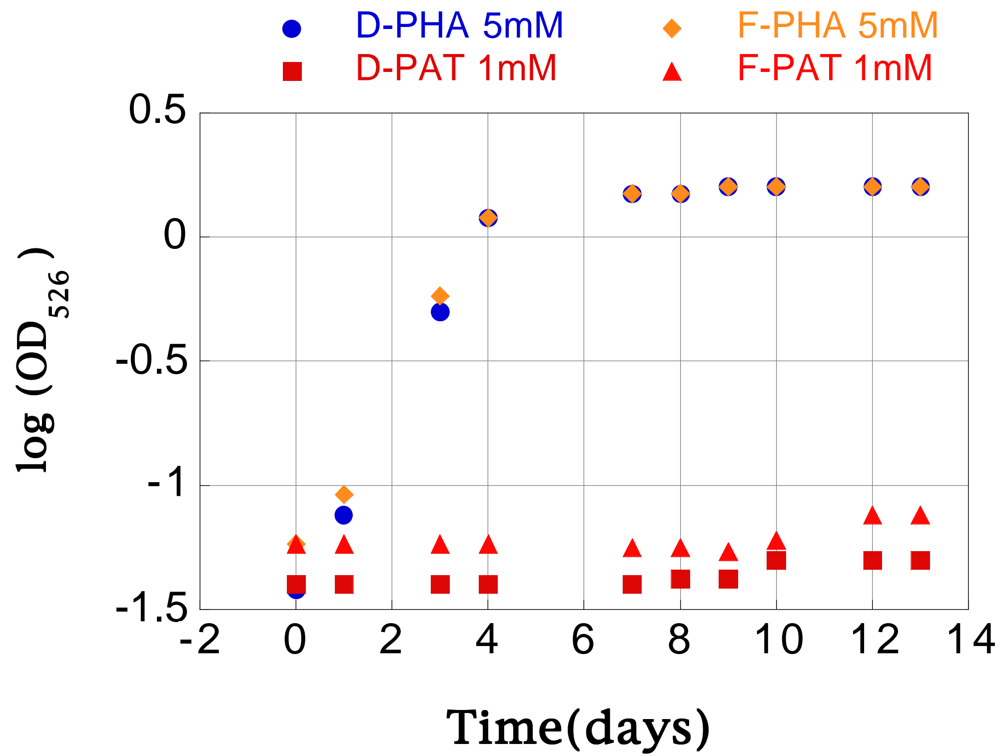

Figure 28. Typical growth curve of phototrophic bacteria (type D and F), amended with antimony compounds (PHA, PAT) in Sistrom minimal medium (SMM).

Garlic Experiment

Data and results section of this experiment is composed of 3 parts and is discussed accordingly:

i. Retention times regression line calculation

ii. Chromatograms from headspace analyses

iii. Identified mixed organo sulfur and selenium compounds (Table X), and conclusion

i. Retention times regression line calculation

Four common mixed organo-sulfur and -selenium compounds retention times were picked both from our previously obtained data and Cai et al.'s research (Table IX).

Table IX
Retention Times of organosulfur and organoselenium compounds

Compound	Our retention times (min)	Cai et al. (min)
$(CH_3)_2S_2$	5.6	8.16
$(CH_3)_2SeS$	6.5	11
$(CH_3)_2Se_2$	7.1	13.2
$(CH_3)2S_3$	8	16.8

When Cai and coworkers' retention times versus our retention times were plotted (Figure 29) a regression line with a regression coefficient of R=0.99818 was obtained which suggested a linear relationship between our retention times and theirs. The equation of the regression line was calculated to be Y=-12.217 + 3.6039 X.

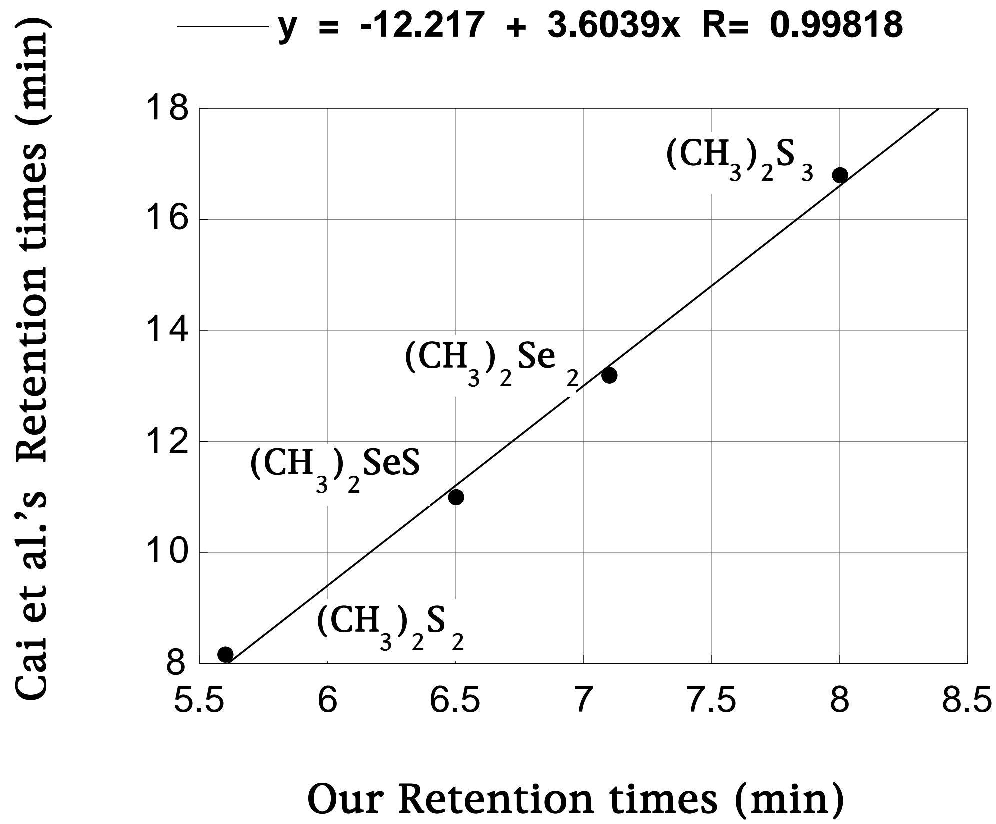

Figure 29. Regression line of the 4 common mixed organo-sulfur and -selenium compound's retention times.

ii. Chromatograms from headspace analyses.

Headspace analyses of garlic samples were performed as described in Chapter II, section 4-1e. (Figure 30, Figure 31)

iii. Identified mixed organo sulfur and selenium compounds, and conclusion.

Cai et al.'s retention times for all organosulfur and organoselenium compounds were substituted as Y in regression line equation (Y=-12.217 + 3.6039 X) and our calculated retention times were calculated. The percent error between our calculated retention times and our real retention times (standard retention times) were evaluated and none of them exceeded 0.95 percent error. After chromatographic separation of garlic headspace, we had identified a total of 9 mixed organo sulfur and selenium species, RS_nSeR' (n=0,2, R, R'= methyl, allyl, propylene). From these 9 compounds, 5 new mixed organo-sulfur and-selenium compounds retention times were determined besides those previously known. Table X shows the complete comparison data with relative error. Garlic Experiment-I was an experiment in which NaCl was added to crushed garlic in order to increase the ionic strength of the environment. NaCl was not added to Garlic Experiment-II. No conclusion could be derived from the addition of NaCl because of the high organo-sulfur and -selenium production for both cases; all headspace analyses had to be performed with split injection (split ratio: 1/25.5) mode because of this high production.

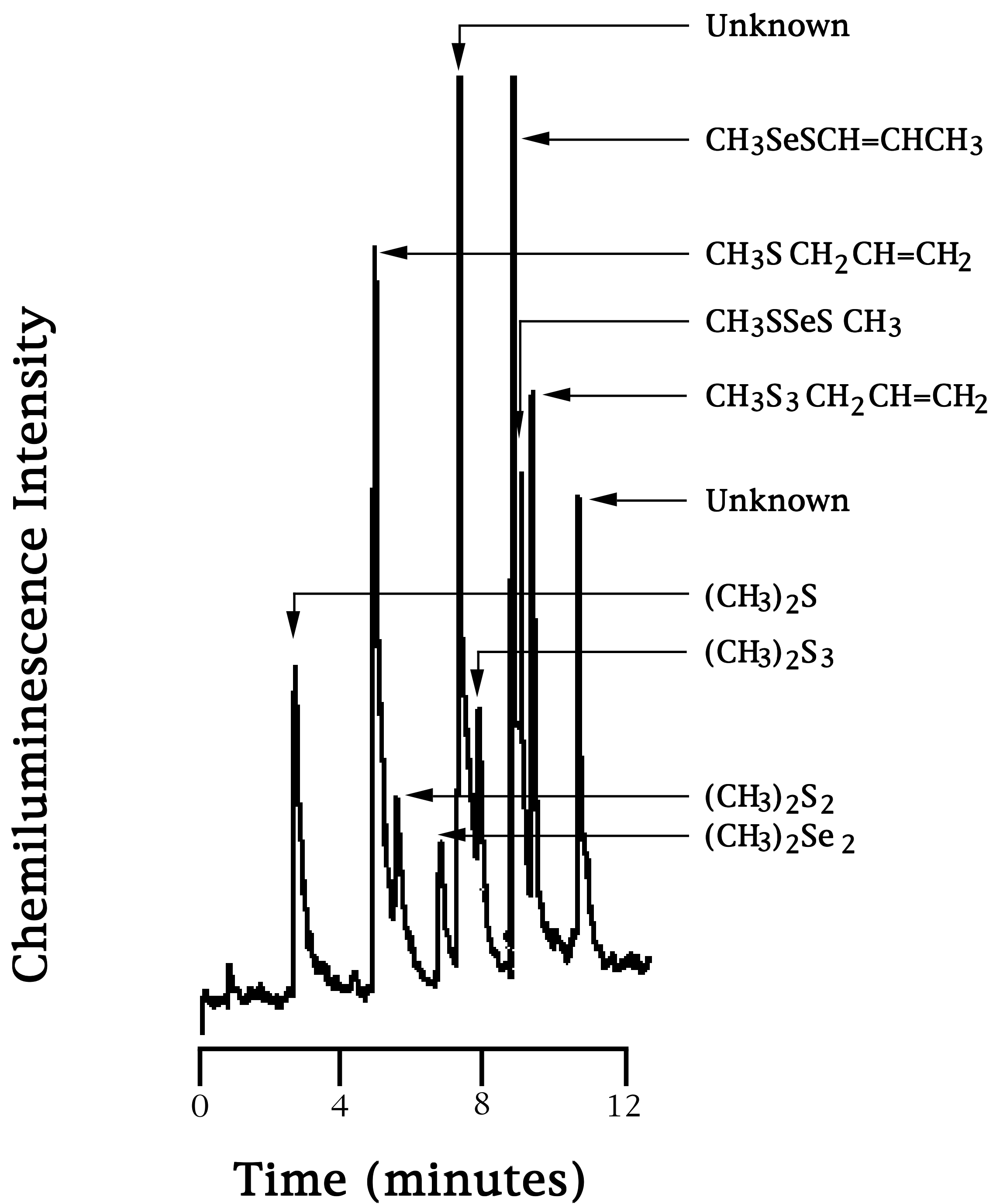

Figure 30. The chromatogram of garlic headspace (0.1 mL, split injection), 24 hours after preparation (no NaCl added).

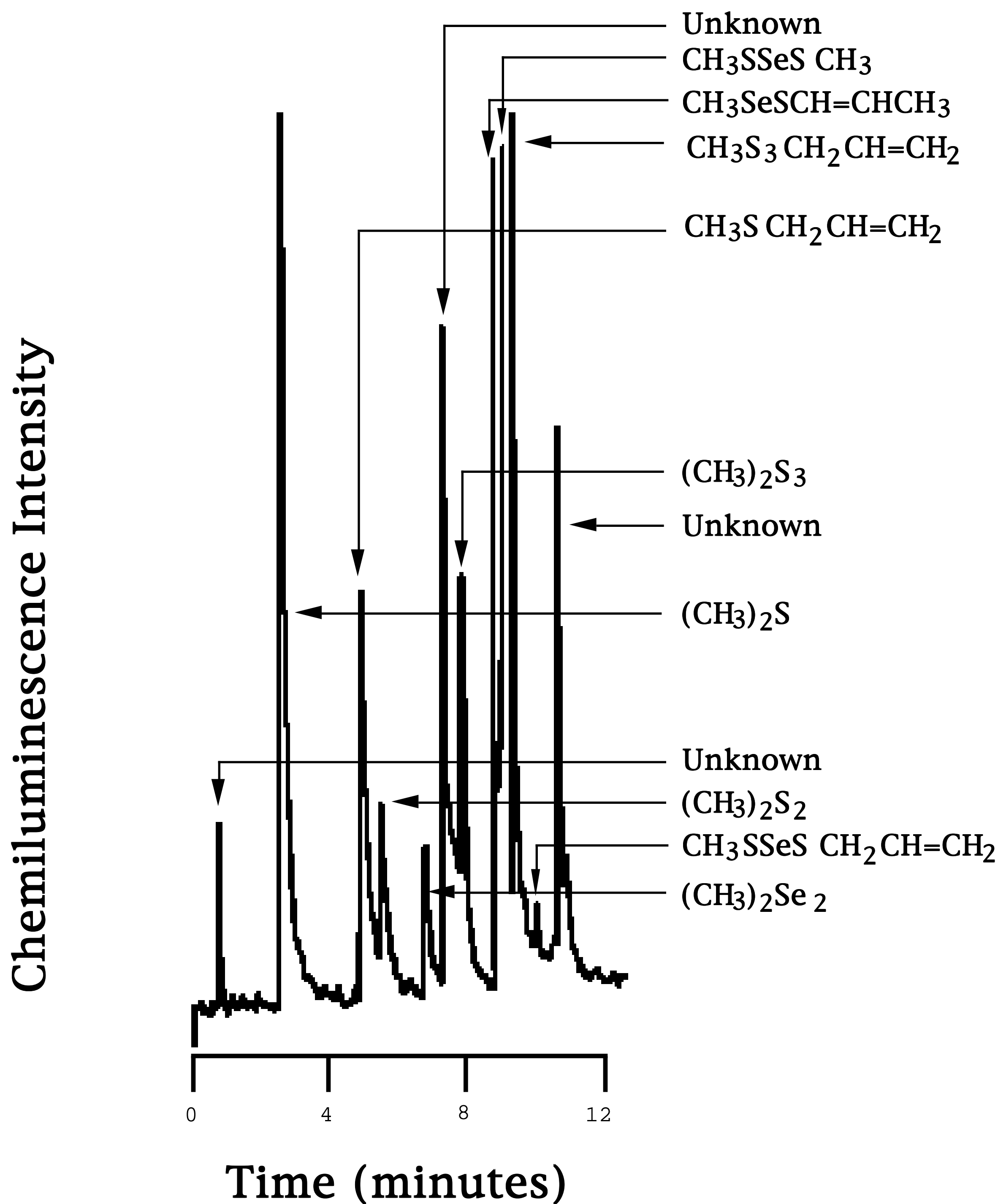

Figure 31. The chromatogram of garlic (saturated with NaCl) headspace (0.5 mL, split injection), 24 hours after preparation.

Table X

Complete comparison table for garlic experiment.

Cai et al.Õs References

Compound	Retention Times (min)	Our Calculated Retention times (min)	Our Real Retention times	% error	Our Garlic Exp. I (NaCl)	% error	Our Garlic Exp. II	% error
$CH_3SC_3H_5$	6	5.05			5.06	0.26	5.01	0.76
$CH_3SeC_3H_5$	7.6	5.49						
$CH_3S_2CH_3$	8.16	5.65	5.6	0.95	5.64	0.19	5.65	0.03
$C_3H_5SC_3H_5$	10.4	6.27						
$CH_3SeSCH3$	11	6.44	6.5	0.89				
CH_3Se_2CH3	13.2	7.05	7.1	0.67	6.94	1.54	6.94	1.52
$CH_3S_2C_3H_5$	14	7.27						
$CH_3SeSC_3H_5$	16.6	7.99						
$CH_3S_3CH_3$	16.8	8.05	8	0.64	8.04	0.08	8.05	0.04
$C_3H_5S_2C_3H_5$	19	8.66						
$CH_3SeSCH=CHCH_3$	20	8.94			9.04	1.15	9.04	1.18
$CH_3SSeSCH_3$	20.8	9.16			9.23	0.82	9.25	0.96
$CH_3S_3C_3H_5$	22	9.49			9.60	1.11	9.61	1.25
$CH_3SSeSC_3H_5$	24.8	10.27			10.27	0.05	*	
$C_3H_5S_3C_3H_5$	26.2	10.66						

* under detection limits; C_3H_5 = Allyl

Theoretical Treatment of Retention Time Correlation Experiment

In some headspace analyses a different chromatographic temperature ramp was used. In order to avoid the recalibration of the standard retention times, a regression line was established by plotting two different retention times from two different temperature ramps (described in chapter 2, section 4-3b), and a regression coefficient of R=0.99767 was calculated (Figure 32).

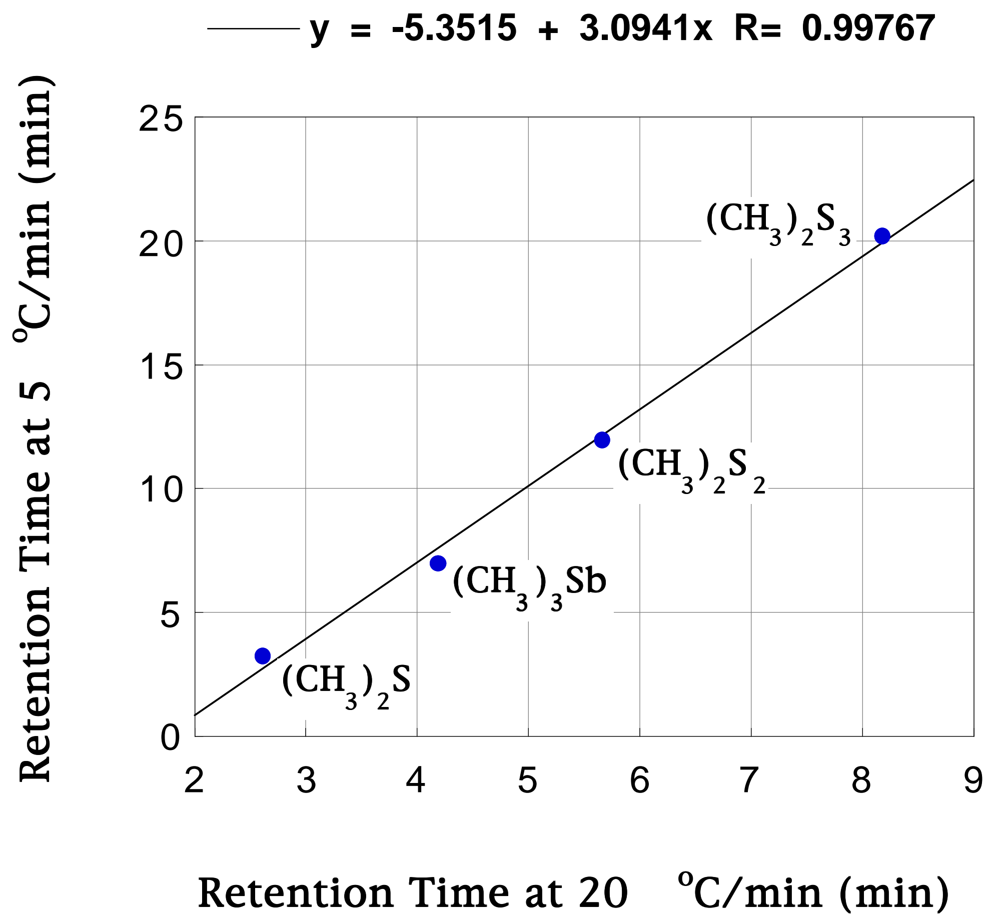

Figure 32. Regression lines of the retention times between 5 °C/min and 20 °C/min temperature ramps.

Chapter IV

Conclusions

Biomethylation of metals and metalloids has been studied for more than a century and has been demonstrated for some species. However, evidence for the biomethylation of antimony was very weak until the last couple of years when a Canadian research group reported the biological production of trimethylstibine in the extracts of submergent aquatic plant (Dodd et al, 1996). Soon after, biological methylation of antimony compounds by some mixed bacteria cultures were identified (Gürleyük, 1996; Gürleyük and Chasteen, 1997) in our research group. Also detailed in that work was the production of trimethylstibine by a live monoculture of *Pseudomonas fluorescens* that had been amended with trimethyl-dibromoantimony. Although this process was ultimately biological that is, sterile controls produced no trimethylstibine-the presence of three methyl groups in the antimony amendment reagent, trimethyldibromoantimony-holds out the possibility that reduction processes were carried out by reduced compounds, formed by bacterial growth, such a sulfides. This, however still meets our practical definition of biomethylation as stated in the introduction to this thesis.

The isolation of these bacteria cultures has never been tried or succeeded by any researcher up to now (Dodd et al, 1996). Following this idea, we reproduce Gürleyük's previous results and then, constructed a series of experiments in order to **isolate** the bacteria cultures which had played a role in the biological methylation of antimony compounds in soils whose headspace analyses had yielded detection of trimethylstibine.

We successfully identified the biological methylation of antimony from different soil samples, Body Shop Soil and Swiss Soil, amended with potassium antimonyl tartrate and potassium hexahydroxy antimonate in different bacterial media (DMN and trypticase soy broth with nitrate). The headspace analyses that most clearly detailed this success can

be seen in Figure 4 and Figure 5. Presence of trimethylstibine can clearly be seen in these chromatograms by the peak at 4.19 minutes which appears after dimethylsulfide (BP 37.3 ^{o}C) but before dimethyldisulfide (BP 109.7 ^{o}C). The success rate of trimethylstibine production in cultures amended with potassium hexahydroxy antimonate was slightly higher than for potassium antimonyl tartrate for Body Shop soil samples. All this evidence supports the conclusion that trimethylstibine production by the organisms that were present in these soil samples amended with PAT and PHA was a result of biological activity (biomethylation).

One of these soil samples, Swiss Soil, which had been collected from an abandoned arsenic-contaminated tannery site in Switzerland had been investigated for its antimony biomethylation activity before (Gürleyük, 1996), but the experiments did not give a positive result and organoantimony production by this soil sample could not be identified. In the experiments described here, however, we identified trimethylstibine production from a completely inorganic antimony salt, potassium hexahydroxy antimonate, and proved the biological methylation activity of the antimony-resistant bacteria in this soil sample. The experimental evidence for this is most clearly depicted in Figure 12. Again, trimethylstibine production can clearly be confirmed based on the presence of the 4.19 minute peak in these fluorine-induced chemiluminescence chromatograms.

The complex nature of soil matrices and their nutrients' affect on bacterial growth led us to try soil enrichment experiments in which soil components were added to bacterial inocula in tripticase soy broth with nitrate. Also in these experiments (Extract experiments), soil samples which had been used as bacterial source in previous experiments (BSS and Swiss Soil) were substituted by liquid samples, which were taken from the solutions of the previously mentioned soil experiments that had been found to be capable of biomethylating antimony compounds. By this way elimination of soil from bacterial cultures and environment was achieved and bacterial growth was monitored by optical density readings. These experiments gave positive results (except one, Extract II), and antimony-resistant

bacteria cultures were isolated from soil samples. These cultures were found to be capable of converting potassium hexahydroxy antimonate to trimethylstibine by biological means. These trimethylstibine peaks can be clearly seen in Figures 18 and 23.

Bacterial enrichment efforts were followed by isolation of antimony-resistant bacteria cultures from polycultures which had originated from trimethylstibine producing soils. This process was performed after two consecutive successful re-enrichment processes (Extract I and Extract III experiments). This isolation process involved agar plates streaking in which individual bacterial colonies- that grew after a bacteria-containing culture medium was thinly spread on agar medium amended with potassium hexahydroxy antimonate-were individually picked off the plate with a sterile loop and inoculated in sterile medium.

Again, these newly isolated bacteria cultures were found to be antimony-resistant as shown by growth curve experiments detailed in Figure 24. But even though these monocultures were antimony-resistant, the biological methylation by these species could not be convincingly confirmed since the total methylation capacity decreased due to the bacterial isolation. That is, the monocultures isolated from trimethylstibine-producing polycultures, while still antimony-resistant, did not produce large amounts of trimethylstibine. Therefore production of trimethylstibine in these monocultures, as measured by our chromatographic method, yielded a trimethylstibine peak that hardly rose above the level of 3 times signal to noise ratio (Figure 25), the defined benchmark detection in our laboratory. However, we had isolated a total of 4 individual colonies from the previously mentioned agar plates, and detailed investigation focused only on one of them (one out of four). Therefore, we can not conclude that the result of this isolation process is failure before the other 3 colonies are also investigated. Possible future experiments can deal with the other 3 isolated monocultures and examine their antimony biomethylating ability. It is known that growth and possible activities of these bacterial colonies depend on many different factors and variables, in which one of them might have been unsatisfied

during the enrichment of the investigated colony. Therefore investigation of the other three colonies must be performed before a conclusion on the isolation process can be drawn.

In other experiments detailed above, different phototrophic bacteria types had been shown to be unsuccessful in biological methylation of antimony compounds. However, they were found to be antimony-resistant up to 1 mM for potassium antimonyl tartrate and 5 mM for potassium hexahydroxy antimonate. The higher toxicity of potassium antimonyl tartrate was clearly indicated with bacterial growth curves of these phototrophic bacteria types as shown in Figure 27 and Figure 28.

Theoretical treatment of retention time correlation experiments were found to be very successful and employed in two different experimental procedures: in the Garlic Experiment, 5 mixed organo-sulfur and -selenium compounds, which are very reactive and have been seen in the headspace analyses of several different experiments in our research group, were identified by this method (Figure 31). The practical use of this experimental procedure is to allow us to identify the presence (in bacterial headspace) of relative, volatile organo-sulfur, -selenium, or mixed sulfur/selenium species whose standards are not available commercially. The compounds newly identified are listed in Table X.

Retention time correlation techniques were also used to evaluate the standard retention times of organosulfur and organoantimony compounds when a different chromatographic temperature program was required in our analyses. By this method production of trimethylstibine was detected in some experiments whereas trimethylstibine production could not be determined by the original faster chromatographic temperature program.

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APPENDIX

Chemical Abstract Service Registry Numbers

Compound Name	CAS Registry Number
Acetonitrile	75-05-8
Ammonium sulfate	7783-20-2
Aspartic acid	56-84-8
Boric acid	10043-35-3
Calcium chloride dihydrate	10035-04-8
(ethylenedinitrilo)-tetraacetic acid disodium salt	60-00-4
Iron(II) sulfate heptahydrate	7782-63-0
Glutamic acid	56-86-0
Glycerol	56-81-5
Magnesium sulfate heptahydrate	10034-99-8
Manganese sulfate	7785-87-7
Nicotinamide	98-92-0
Nitrilotriacetic acid	139-13-9
Potassium antimonyl tartrate	28300-74-5
Potassium hexahydroxo antimonate(V)	12208-13-8
Potassium hydroxide	1310-58-3
Potassium phosphate dibasic	7758-11-4
Potassium phosphate monobasic	7778-77-0
Sodium chloride	7647-14-5
Sodium citrate	6132-04-3

Sodium molybdate dihydrate	10102-40-6
Succinic acid	110-15-6
Thiamine	67-03-8
Trimethylstibine	594-10-5
Zinc sulfate	7446-20-0

VITA

Eser Becer was born in Ankara, Turkey on July 24, 1972. He graduated from O. Dost High School in 1990. The same year he entered Bogaziçi University in Istanbul, Turkey. In 1995 he graduated with a Bachelor of Science degree in chemistry, and pursued his higher education at Sam Houston State University as a graduate student in the Chemistry Department. He graduated with a Master of Science degree in chemistry in May 1997.