

CAPILLARY ELECTROPHORETIC DETERMINATION OF SELENOCYANATE  
AND SELENIUM AND TELLURIUM OXYANIONS IN BACTERIAL CULTURES

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Master of Science

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by

Bala Krishna Pathem

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Bala Krishna Pathem

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APPROVED:

\_\_\_\_\_  
Dr. Thomas G. Chasteen  
Thesis Director

\_\_\_\_\_  
Dr. Darren L. Williams

\_\_\_\_\_  
Dr. Richard E. Norman

Approved:

\_\_\_\_\_  
Dr. Jaimie Hebert, Dean  
College of Arts and Sciences

## Abstract

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The purpose of this work was, in part, to develop a capillary electrophoretic method for the simultaneous estimation of selenium and tellurium anions in the presence of complex bacterial growth media. A simple capillary zone electrophoretic method for the determination of biospherically important oxyanions of selenium and tellurium and a biologically produced Se-containing anion,  $\text{SeCN}^-$ , was developed. The method uses direct UV absorption detection. Time course experiments with time slices as short as 6 min are possible. This method's detection limits and linear range compare well to other previously published methods involving complex biological matrices. The metalloid-containing anions examined were selenocyanate, selenite, selenate, tellurite and tellurate. This method was applied to live bacterial cultures of two different bacteria in two different growth media in time course experiments following the changes in metalloid-containing anion concentrations. The results show that this method is a useful means of following the biological processing of these analytes in bacterial cultures.

Experiments were also designed to detect the biological production of selenocyanate in Se-amended bacterial cultures using IC-ICP-MS. A selenium-resistant bacterium, 130404, when amended with 1.0 mM selenate biologically produced selenocyanate in replicate cultures grown to stationary phase. The amounts produced were statistically significantly different than the amounts of  $\text{SeCN}^-$  produced in sterile but warmed growth media. The headspace gases above growing cultures with or without metalloid amendments were analyzed using gas chromatography. Organo-selenium

compounds that were detected were methaneselenol, dimethyl selenide, dimethyl selenenyl sulfide, dimethyl diselenenyl sulfide, dimethyl diselenide. Two late eluting peaks were observed in the same cultures that were not characterized but can reasonably be inferred to be dimethyl diselenenyl sulfide and dimethyl triselenide.

Keywords: selenocyanate, capillary zone electrophoresis, tellurite, selenate, selenite

Approved:

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Dr. Thomas G. Chasteen,  
Thesis Director

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## Chapter 1

### Introduction

Selenium is a naturally occurring element widely distributed on the earth. The oxyanions of Se, selenate,  $\text{SeO}_4^{2-}$  and selenite,  $\text{SeO}_3^{2-}$  are the most common and mobile biospheric forms of this element, and, as a rule, both are quite harmful, with selenite normally more toxic than selenate to most organisms. Nutritional supplements containing inorganic selenium compounds such as sodium selenate and sodium selenite are used in the prevention of many nutritional deficiency diseases; however, selenium is toxic at levels only 25x the minimum daily requirement for a healthy level in human diet (Chasteen and Bentley, 2002; Chasteen and Bentley 2006).

Determination of selenium species in environmental samples has been studied extensively in recent years. In a review by Pyrzyńska (2002) concentrations of selenite and selenate in environmental samples of sea-water, ground water and fresh water have been reported and they range from pg/L to hundreds of ng/L in contaminated samples.

In contrast, tellurium is a relatively rare element. This chalcogen is seldom found in its elemental form,  $\text{Te}^0$ , but analogous to Se, biospherically mobile tellurium is usually found in its oxyanionic forms, tellurite ( $\text{TeO}_3^{2-}$ ) and tellurate ( $\text{TeO}_4^{2-}$ ), which are again, like selenium, quite toxic for most living organisms. The biospheric toxicity of tellurate is limited, however, by its poor solubility at normal environmental pH.

Another selenium anion, which has gained much interest recently, is selenocyanate ( $\text{SeCN}^-$ ). The only biospheric sources of  $\text{SeCN}^-$  discovered so far are those stemming from the wastes from oil refineries or industrial effluents, that is, ostensibly inorganic in nature. Chemically reducing environments in industrial settings or inorganic

reagents released into the environment react with Se and organics to abiologically produce  $\text{SeCN}^-$ . Organoselenium compounds containing - SeCN groups have been involved in a lot of chemopreventive studies. It has been found that they play a crucial role against cancers of the colon (Fiala et al., 1998), skin (Uddin et al., 2005) mammary gland, lung (Richie et al., 2006), and oral cavity (Guttenplan et al., 2004; Chinthalapally et al., 2001). Alkyl selenocyanates have been previously shown to possess powerful anticancer activity (Ganther and Lawrence, 1997; Ip, 1998).

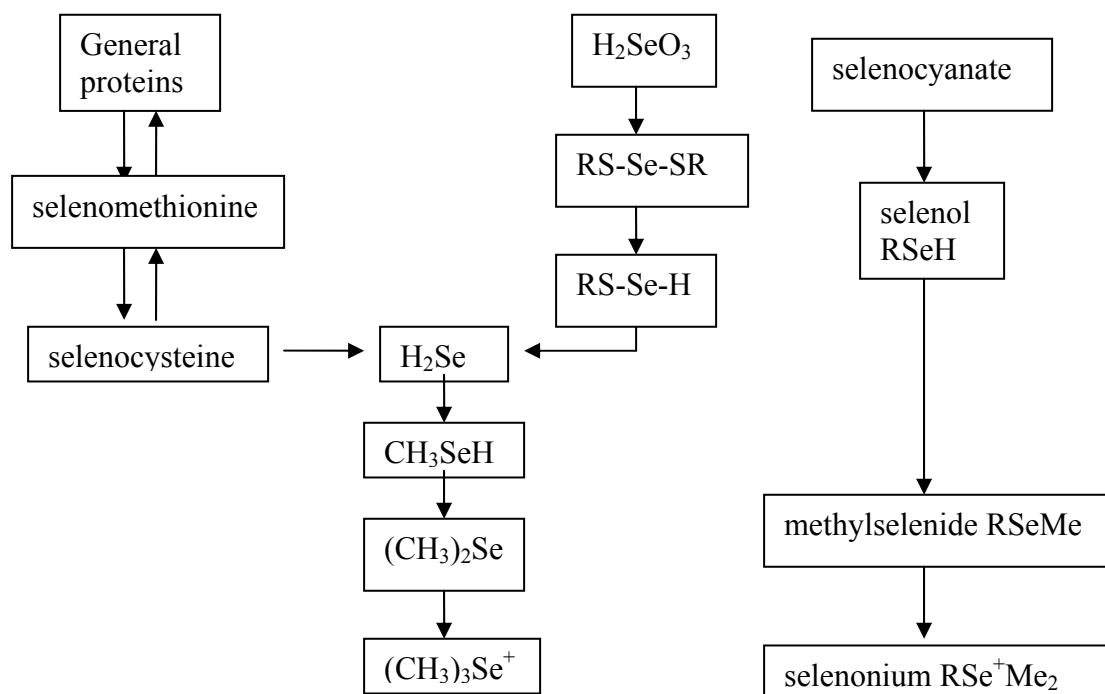
$\text{SeCN}^-$  has also been used in the synthesis of various aromatic and aliphatic diselenides. Terfort and Muller (2006) reported that products free of triselenides can be obtained by the use of selenocyanates. Ueda et al. (2005) proposed a mechanism in which they synthesized 4,5-dialkylsubstituted 2-imino-1,3-selenazolidine derivatives using potassium selenocyanate and evaluated the inhibitory activity against inducible nitric oxide synthase. Ostensibly selenocyanates are important in various synthetical routes of selenoorganic derivatives.

With that said, selenocyanates are indeed pollutants in waste waters. Previously published literature suggests that this is mostly due to oil refineries, power plants and in mining waste water (as a result of cyanide leaching of selenide ores). When crude oil is processed to remove sulfur, selenium too is removed in the form of hydrogen selenide. Much study has been done on the removal of  $\text{SeCN}^-$  from water by precipitating it using Cu, Ag, Au, Cd, Hg, Tl and Pb (Golub and Skopenko, 1965; Soderback, 1974). Removal of selenate by Fe(0) reduction to elemental selenium form has also been reported (Frankenberger, Jr. et al., 2005; Zhang and Frankenberger, 2006). Similar work on  $\text{SeCN}^-$

removal using Fe(0) was done by Meng et al. (2002). Sources of  $\text{SeCN}^-$  were mentioned to be the wastes from oil refineries or industrial effluents (Miekeley et al., 2005).

Some bacteria that exhibit resistance to these metalloids have developed the ability to reduce and methylate Se and Te oxyanions to volatile forms such as dimethyl chalcogenide (DMSe or DMTe), dimethyl dichalcogenide (DMDS<sub>e</sub> or DMDT<sub>e</sub>), as well as to produce the insoluble elemental forms of these metalloids:  $\text{Se}^0$  and  $\text{Te}^0$ .

Bioremediation of metalloid-amended cultures in laboratory settings using metalloid-resistant microorganisms such as *Pseudomonas fluorescens* K27 (Zhang and Chasteen, 1994; Hapuarachchi et al., 2004) and more recently genetically-modified *E. coli* with genes from a metalloid-resistant thermophile (Araya et al., 2004; Swearingen et al., 2006) has been reported. The production path of volatile selenium compounds observed in the headspace of selenite amended cultures agrees well with the following scheme, redrawn from Ganther and Lawrence (1997).



**Scheme 1. Scheme depicting the selenium metabolic pathways (Ganter and Lawrence, 1997).**

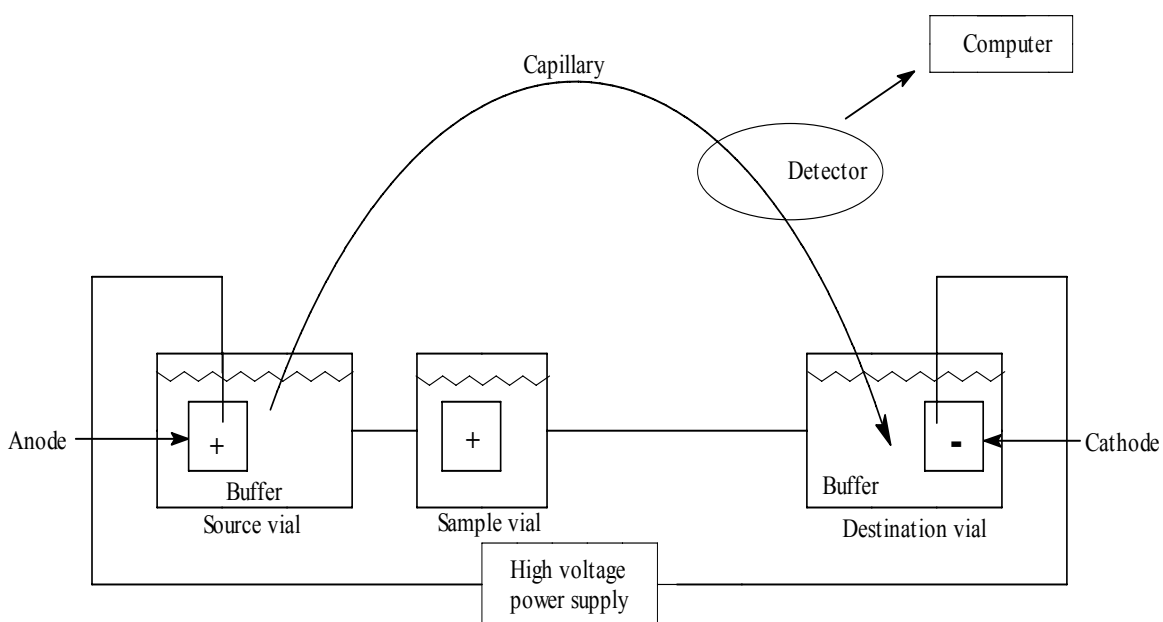
Bioaccumulation of selenium oxyanions and selenocyanate has also been reported in one study using selenium-resistant plants in a successful effort at phytoremediation (de Souza et al., 2002).

**Analytical methods for detection of compounds of interest**

Various instrumental techniques including capillary electrophoresis (CE), high performance liquid chromatography – inductively coupled plasma – mass spectrometry (HPLC-ICP-MS), and ion chromatography - inductively coupled plasma - mass spectrometry (IC-ICP-MS) have been used by others for the speciation of selenium/tellurium-containing compounds (Casiot et al., 2002; Vogt and Werner, 1994; Schlegel et al., 1996; Albert et al., 1995; Gilon and Potin-Gautier, 1996; Kannamkumarath et al., 2002; Kahakachchi et al., 2004; Caruso and B'Hymer, 2006). For instance, Walker et al. (1996) reported an analytical method for the estimation of selenate and selenite using CE, but had difficulty separating nitrate and sulfate/selenate at higher concentrations. In a short communication Dzierzgowska et al. (2003) have reported an analytical method in which they could separate nitrate and sulfate peaks by using pyromellitic acid (PMA) in the buffer. But the concentration of nitrate was only 50 nmol l<sup>-1</sup> in their method. Speciation of inorganic selenium has also been done by using CE-ICP-AES (Deng et al., 2006). Uden et al. (2004) have published a review on the selective detection and identification of selenium containing compounds and the recent developments in the analytical techniques.

Selenocyanate has been found (at oil refineries in Brazil) in water that was in contact with raw oil before processing (Miekeley et al., 2005). The authors used IC-ICP-MS for its estimation with a method offering a detection limit of about 2 ppb.

CE is an excellent analytical tool for the estimation of anions. Electrophoresis is defined as ‘the differential movement or migration of ions by attraction or repulsion in an electric field’ (Background theory and principles of capillary electrophoresis, <http://www.rsc.org/pdf/books/capelectrosc.pdf>, (accessed Apr, 5, 2007)). A simple schematic of a typical CE instrument is illustrated in Figure 1.



**Figure 1. Schematic of a CE instrument.**

As can be seen from the figure above, a capillary (typically 25 to 100  $\mu\text{m}$  in internal diameter) is placed in separate buffer vials. Each vial contains an electrode connected to a high voltage supply. Capillaries are usually 25 to 100 cm long. Separation is then achieved by applying a voltage across the electrodes. The capillaries are made up of fused-silica, with an internal surface  $\text{pK}_a$  of approximately 1.5. Capillary tubing has a

high dielectric strength, giving it ample isolation for the high voltages (typically up to 30 kV). The buffer pH controls the degree of ionization of the walls of these capillaries. Fused-silica capillaries are coated with a protective polymer. A portion of this polymer of about 4 mm is scraped off (capillary window) so as to allow UV light to pass through, thereby allowing the analysis of different analytes passing through this detection window. The capillary windows have low background fluorescence. This property makes CE ideal for fluorescent detection methods, including laser induced fluorescence.

Different types of capillaries are used depending upon the type of molecules that are being separated. Untreated, neutral and amine-treated capillaries are typically used. Neutral-surface capillaries are designed such that one can coat the exposed silanol groups of the capillary walls. This feature makes it possible to reduce electroosmotic flow (EOF). For working with buffers at high pH or to induce EOF, amine capillaries can be used. They are highly effective in the separation of basic proteins or basic drugs.

The surface of the capillary contains negatively-charged functional groups. Attracted to these negatively charged silanoate groups, the positively charged cations of the buffer solution will form two inner layers of cations called the diffuse double layer on the capillary wall. The first layer is called the fixed layer and the second, the mobile layer. Because of this mobile layer not only do the cations in the capillary traverse towards the cathode (negative electrode), they pull along with them the anions and the neutral molecules, thus creating an electroosmotic flow.

In order to analyze anions using CE, the flow has to be reversed so that the anions can travel towards the anode (positive electrode). This can be done by using cationic surfactants. These reverse the charge on the walls of the capillary, and by having the

cathode at the injection end, it is possible to have the anions travel fastest, then the neutral species and then the cations.

EOF can be controlled by various parameters during separation. Table 1 shows the results of such variations. In this research, the change in separation by varying all these parameters was studied during the method development stage.

**Table 1. Change in EOF by changing the parameters in CE.**

| # | Parameter            | Result  |
|---|----------------------|---|
| 1 | Electric field       | Proportional change in EOF  |
| 2 | Buffer pH            | EOF decreased at low pH, increased at high pH.                      |
| 3 | Buffer concentration | Decreases EOF when increased  |
| 4 | Temperature          | Changes viscosity   |
| 5 | Organic modifier     | Changes viscosity (usually decreases EOF)                           |
| 6 | Surfactant           | Adsorbs to capillary wall via hydrophobic and/or ionic interactions |

By using the modifiers shown in Table 2, EOF can be controlled and modified to achieve the best separation. Cationic surfactants have been used in this research work to reverse the charge of the capillary wall.

**Table 2. Modifiers and their effects.**

| #  | Parameter            | Result   |
|----|----------------------|--|
| 1. | Inorganic salts      | Protein conformational changes                     |
| 2. | Organic solvents     | Solubilizer, modify electroosmotic flow            |
| 3. | Urea                 | Solubilize proteins and denature oligonucleotides  |
| 4. | Sulfonic acids       | Ion pairing agents, hydrophobic interaction agents |
| 5. | Cationic surfactants | Charge reversal of capillary wall                  |

CE offers a broad linear range and lower limits of detection compared to most other chromatographic techniques like HPLC. By using a photo diode array (PDA) detector, one can sample over a range of interest and chose different wavelengths for different ions present in the same sample mixture.

### **Chromatography**

Volatile organo-selenium/tellurium compounds such as dimethyl selenide (DMSe), dimethyl diselenide (DMDS<sub>e</sub>), dimethyl telluride (DMTe), and dimethyl ditelluride (DMDT<sub>e</sub>) can be determined using gas chromatography (GC) equipped with a fluorine-induced chemiluminescence detector (Van Fleet-Stalder and Chasteen, 1998; Basnayake et al., 2001). For the identification of unknown compounds, a GC equipped with a mass spectrometer (GC-MS) can be used provided that the concentration of volatiles in the headspace is very high. In cases where the headspace concentration is low, a gas syringe can be replaced by an extraction technique called solid phase micro extraction (SPME) (Swearingen et al., 2006). In this technique, analytes are allowed to adsorb to the thin film of a stationary phase by exposure for a period of time (typically

10-30 minutes). Equilibrium is then attained between the stationary phase and the analytes. This equilibrium depends on the affinity of the analytes towards the stationary phase. The fiber can then be directly inserted into the GC injector. The analytes are then thermally desorbed in the GC injector and the chromatography begins.

Depending upon the analytes of interest various SPME fibers can be selected. Different kinds of fibers including polydimethylsiloxane (PDMS), divinylbenzene (DVB), and Carboxen<sup>TM</sup> are commercially available. Table 3 shows the most widely used commercially available SPME fibers.

**Table 3. Commercially available SPME fibers for selective adsorption.**

| # | Solid phase / Thickness                | Compounds  |
|---|--|--|
| 1 | PDMS / 100 µm                          | Low molecular weight / Semivolatiles             |
| 2 | PDMS / 30 or 7 µm                      | Nonpolar high molecular weight / Semivolatiles   |
| 3 | Polyacrylate / 85 µm                   | Very polar from polar samples                    |
| 4 | PDMS/DVB / 65 µm                       | Volatile polars                                  |
| 5 | DVB/Carboxen<br>on PDMS 50 µm or 30 µm | C <sub>3</sub> – C <sub>20</sub> Chain molecules |

### Isolation and identification of bacteria

Four different species of bacteria were studied in this research work. They are 130404, *Aeromonas* spp., *Bacillus* spp. and *Escherichia coli* 1VH. All these species are anaerobes. The strains were isolated and were provided by the researchers at the University of Santiago, Chile. 130404 was isolated from mining waste. *Aeromonas* spp. was isolated from a stagnant water sample (from a jar) found in their research lab. A

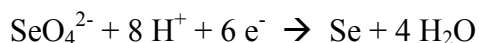
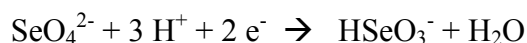
metalloid-resistant, facultative anaerobe, *Pseudomonas fluorescens* K27 that we have worked with in Dr. Chasteen's research lab for a long time is routinely stored on selenite-amended agar plates in a 4° C refrigerator, harvested, and replated periodically. When cultivating a culture of this microbe for metalloid amendment experiments, a different organism on a K27 plate that had a much faster specific growth rate and did not require nitrate for fast growth was found. Work with monocultures, isolated via streaking and selection of individual colonies from selenite-amended agar plates, yielded a facultative, metalloid-resistant, Gram positive bacterium that has been characterized as *Bacillus* spp. as determined by light microscopy. Bacterial cultures of *Escherichia coli* K-12 carrying the recombinant plasmid p1VH were used as described elsewhere (Araya et al., 2004). Briefly, genes contained in a 3.8-kb chromosomal DNA fragment from the tellurite-resistant, thermotolerant microorganism *Geobacillus stearothermophilus* V, were transferred to the Te-sensitive microbe, *E. coli* K-12, using plasmid pSP72 as cloning vector (Promega, Madison, WI, USA). Expression of these genes conferred a metalloid resistance 25 times that observed for the uncloned organism. The 1VH-encoded protein products are about 80% similar to the SUMT methyltransferase and the BtuR protein of *Bacillus megaterium*, and to the UbiE methyltransferase of *Bacillus anthracis* A2012.

### Relevance of this research

Various analytical techniques including fluorescence spectroscopy, AAS, CE-ICP-MS, HPLC-ICP-MS, IC-ICP-MS have been used before for the estimation of selenium and tellurium species. These instrumental techniques are costly and complex. The need to develop a fast and easy analytical method for the identification of oxyanions of selenium and tellurium in bacterial cultures amended with selenite and tellurite was addressed in this research work. Experiments described here were designed to achieve that goal using capillary electrophoresis.

Others who have developed time-course analytical methods for metalloid oxyanions have determined the relative order of oxyanion reduction in selenium-amended cultures. For instance, Walker et al. (1996) found that selenate was reduced to selenite and subsequently to elemental selenium, although this last step was much slower than the initial reaction.

These bacteria have electron transport chains that can operate with exogenous electron acceptors other than oxygen (such as nitrate and selenate). For example selenate can act as the terminal acceptor in bacteria and is reduced to  $\text{HSeO}_3^-$  or to elemental selenium.



The CE method described here allowed us to follow tellurite and/or tellurate in tellurium-amended *E. coli* cultures of genetically-engineered clones.

The capability of analyzing five different anions using the CE method within a period of five minutes makes this method an excellent tool in the field of environmental

and agricultural analysis. In this thesis, the method development of CE for the estimation of the anions of interest and then the application of the method using different bacterial species grown in different bacterial growth media is shown. Bacterial cultures were amended with 0.2 mM or 0.05 mM selenite or tellurite and time-course analysis was performed to observe the fate of the oxyanions by sampling at regular intervals and subsequent analysis by CE.

Experiments were also designed to follow the production of gases in the headspace above the bacterial cultures with or without metalloid amendments. Multiple test tubes of the bacterial cultures were prepared to follow the time-course production of gases in the headspace. The gases were then extracted using SPME and injected into the GC. Analysis was also carried out using GC-MS in an effort to identify the unknown compounds found in the headspace of 130404 cultures amended with 1.0 mM selenate.

## Chapter 2

### Experimental

#### Part 1: Anaerobic Bacterial Growth

##### *Reagents*

Chemical reagents were obtained from the following sources. Sodium selenate, sodium selenite, sodium tellurate, sodium tellurite, and sodium hydroxide from Aldrich (Milwaukee, WI, USA); tryptic soy broth (TSB) and yeast extract from Difco (Detroit, MI, USA); HPLC grade water, potassium selenocyanate from Acros organics (New Jersey, USA); bacto<sup>TM</sup> tryptone (pancreatic digest of casein) from Becton Dickinson and Company (Sparks, MD, USA); sodium chloride, and potassium hydroxide from EM Science (Gibbstown, NJ, USA).

##### *Bacterial cultures and growth media*

Bacterial cultures of a genetically-modified clone of *E. coli* K-12 (1VH) were grown anaerobically at 37 °C in LB (Lauria-Bertani) medium for 24 h as described elsewhere (Araya et al., 2004). LB medium was prepared as follows. Ten grams of pancreatic digest of casein, 5.0 g of NaCl, and 5.0 g of yeast extract were added to distilled/deionized water and the volume was brought to 1.0 L and mixed thoroughly. The pH was brought to  $7.0 \pm 0.2$  using 1.0 M NaOH. The cultures were then amended with sodium tellurite to yield a final concentration of 0.05 mM in the growing cultures.

A metalloid-resistant, Gram positive, *Bacillus* sp. was cultured in the following manner. Precultures of *Bacillus* sp. were grown in tryptic soy broth (TSB) medium adjusted to pH 7.0 using 1.0 M NaOH at 30 °C under anaerobic conditions for 24 hrs. The cultures were then amended with sodium selenate to yield a final concentration of

1.0 mM in the growing cultures. Replicate test tubes, containing 10 mL of growth medium amended with a known concentration of a metalloid-containing chemical species, were prepared and inoculated with a 1:10 v/v inoculum from a metalloid-free bacterial preculture in stationary phase, capped, and incubated at the appropriate temperature.

The 130404 strain was isolated from mining waste by researchers at the University of Santiago, Chile as follows (Pradenas, G.A., personal communication). Thirty mg of mining waste was mixed with 10 mL of LB broth in a sterile tube; the tube was incubated at 37 °C with constant shaking at 100 to 120 RPM overnight; 100 µL of this was inoculated to an LB agar plate containing 100 µg/mL of K<sub>2</sub>TeO<sub>3</sub>, then the inoculated plate was incubated at 37 °C for 48 hrs. Bacterial colonies observed were plated in LB agar, and then the pure bacterium was tested for tellurite resistance.

*Aeromonas* spp. strain was isolated by the following procedure. A sample of 100 µL of stagnant water (found in a jar at the University of Santiago, Chile) was diluted 100 times with LB broth; 100 µL of the diluted stagnant water was inoculated to a LB agar plate containing 100 µg/mL of K<sub>2</sub>TeO<sub>3</sub>, then the inoculated plate was incubated at 37 °C for 48 hrs; bacterial colonies observed were plated in LB agar, and then the pure bacteria was tested for tellurite resistance. The genus of this bacterium was assigned by following the classical procedure (Prescott et al., 2002) for identification of gram negative bacteria (Perdenas, G.A., personal communication).

Bacterial precultures of 130404 were grown anaerobically at 37 °C in LB medium at a pH of 7.0 (adjusted with 1.0 M NaOH) for 24 h as described above. The solution was then distributed into tubes/flasks. These tubes/flasks were then autoclaved for 25 min at

15 psi pressure and 121 °C. The solutions were then taken out and allowed to cool. For inoculation, a single colony of bacteria taken from a storage plate was added to the tube/flask using a sterile loop.

Fresh cultures were then prepared in a similar way from the precultures. The cultures were then amended with sodium selenate to yield a final concentration of 1.0 mM in the growing cultures. Replicate test tubes, for the growth curve study and IC-ICP-MS study, containing 10 mL of growth medium amended with a known concentration of sodium selenate or potassium selenocyanate, were prepared and inoculated with a 1:10 v/v inoculum from a metalloid-free bacterial preculture in stationary phase, capped, and incubated at the appropriate temperature. Stock solutions of 10 mM sodium selenate and 10 mM potassium selenocyanate were prepared in HPLC grade water and sterile filtered.

Precultures of a partially identified organism dubbed *Aeromonas* spp. were prepared in the same manner as was followed for strain 130404. Fresh cultures with metalloid amendments were prepared from precultures that were 24 h old.

## **Part 2: Analysis of liquid samples using CE**

### *Reagents*

The reagents required for CE analysis were purchased from the following sources. Potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) from Difco, HPLC grade water, tetradecyl trimethyl ammonium bromide (TTAB) from Acros Organics, and potassium hydroxide from EM Science.

### *Apparatus*

CE experiments were carried out using a P/ACE MDQ system (Beckman Coulter, Fullerton, CA, USA), configured with an auto sampler, capillary and sample tray temperature controls. Fused silica capillaries were purchased from Beckman Coulter, (Fullerton, CA, USA); dimensions were 57 cm total length/50 cm effective length, 75  $\mu\text{m}$  I.D., 375  $\mu\text{m}$  O.D.

### *CE calibration*

External calibration and determination of figures of merit of the instrument were carried out as follows.

**Precision:** The precision of the instrument was measured by injecting 1.0 mM standard solution six times. The %RSD of the migration time was calculated. A limit of %RSD not more than 0.1% was desired, though the results have never crossed 0.05% throughout the research work.

**Calibration curve:** A five point calibration curve using peak integration was created over a range of 0.10 mM to 1.0 mM standard. The target  $R^2$  value was 0.999 or better. Solutions were prepared from a 10.0 mM stock solution of the standard of interest. A solution of 1.0 mM concentration was prepared by 1 in 10 dilution using HPLC grade water. Serial dilutions were made using the same solvent.

### *Sampling and CE conditions*

The CE's fused silica capillary was initially conditioned with 1.0 M NaOH for 5 min at 40  $^{\circ}\text{C}$ , then with HPLC-grade water for 5 min at 25  $^{\circ}\text{C}$ . The capillary was sequentially rinsed when necessary, with HPLC-grade methanol for 2 min, water for

1 min, 0.10 M HCl for 2 min, water for 1 min, 0.10 M NaOH for 2 min, water for 1 min and finally with run buffer for 2 min. Run buffers were prepared at different concentrations depending upon the analytical goal. For instance, the finalized method's run buffer consisted of 15.0 mM  $\text{KH}_2\text{PO}_4$  and 1.0 mM TTAB at pH 10.5 adjusted with 1.0 M NaOH. Samples were injected using hydrodynamic injection at 0.5 psi for 5 sec. The capillary was maintained at a constant temperature of 25 °C. A separation voltage of -25 kV was applied. Detection using a photodiode array detector was carried out at 190 nm for selenate and selenite, 220 nm for tellurite and 200 nm for selenocyanate. The wavelengths mentioned above were chosen based upon the absorption intensity, interaction with neighboring spectral peaks, and peak shape. The CE peaks were then quantified using peak integration. Bacterial cultures were filtered using 0.2  $\mu\text{m}$  sterile filters immediately before analysis by CE. No additional sample preparation was required.

The pH of all solutions was measured by using a Corning pH meter 340 (Corning, NY, USA). The pH meter was calibrated using two commercially-available buffers of pH 7.0 and 10.0 at 25 °C.

Bacterial growth curve data were generated using a Spectronic 20D+ spectrophotometer by measuring scattering/absorbance at 526 nm (Van Fleet-Stalder et al., 2000).

### **Part 3: Headspace analysis using GC-SCD**

#### *Reagents*

The reagents required for GC analysis were purchased from the following sources. Dimethyl disulfide (DMDS), acetonitrile, and dimethyl diselenide (DMDS<sub>e</sub>)

from Aldrich Chemical (Milwaukee, WI, USA); Dimethyl telluride (DMTe), and dimethyl ditelluride (DMDTe) from Organometallics, Inc. (East Hampstead, NH, USA).

#### *Apparatus*

A Hewlett Packard 5890 Series II gas chromatograph equipped with a fluorine-induced chemiluminescence detector, Model 300 (Ionics Instruments, Boulder, CO, USA) was used for GC analysis. A DB-1 column (100% dimethyl polysiloxane with 5.0  $\mu\text{m}$  film coating) with dimensions of 30 m x 0.32 mm i.d. was purchased from J&W Scientific (Folsom, CA, USA). A Hewlett Packard 3396 series II integrator was used to record the signal from the detector. Carboxen-PDMS SPME fibers (75  $\mu\text{m}$  thickness) were purchased from Supelco (Bellfonte, PA, USA).

#### *GC-SCD calibration*

Calibration was performed when necessary by creating a five point calibration curve using standard solutions. Twenty-five  $\mu\text{L}$  of stock solution were added to 1.0 mL of acetonitrile and then serially diluted by preparing solutions of 25  $\mu\text{L}$  of the previous analyte solution in 1.0 mL of the same solvent. To generate the calibration curve 1.0  $\mu\text{L}$  of these samples were injected and the area of analyte peak was plotted versus mass injected.

### *Analytical method specifications*

Helium was used as a carrier gas with a flow rate of 1 mL/min. All the samples were analyzed in splitless injection mode. The GC injector temperature was 275 °C. The initial oven temperature was 30 °C and held for 2 min. The oven temperature was then ramped to 250 °C at 15 °C/min. The final temperature was held for 1 min.

### *SPME extraction technique*

The bacterial samples for headspace analysis were prepared in test tubes with open-top screw caps with Teflon<sup>®</sup>/silicone liners purchased from Alltech (Deerfield, IL, USA). SPME fibers pierced through the septa were used to adsorb the headspace from these test tubes. The fiber exposure time varied from 30 to 60 min depending on the type of bacterial species. These fibers were then exposed in the GC injector (at 275 °C) until the completion of the temperature program.

### **Part 4: GC/MS analysis**

Reagents used in this method were the same as for the GC-SCD analysis. A Hewlett Packard 5890 GC equipped with a Hewlett Packard 5973 mass selective detector was used for this analysis. An HP-5MS column was used for this work (30 m x 0.32 mm i.d., 5% phenylmethylpolysiloxane). Liquid nitrogen was used for cryo-trapping. High-purity helium was used as the carrier gas.

### *Temperature program*

All the samples were analyzed in splitless injection mode. The GC injector temperature was 275 °C. The initial oven temperature was -30 °C and was held for 3 min. Oven temperature was then ramped to 250 °C at 5 °C/min. The final temperature was held for 1 min.

## Part 5: IC-ICP-MS Method

IC-ICP-MS analyses were carried out by Applied Speciation Consultants LLS, Tukwilla, WA, USA. Either an AGP-1 ion chromatography pump (Dionex, USA) or Series 200 LC pump was connected to a Perkin Elmer ELAN DRCplus inductively coupled plasma mass spectrometer. The chromatographic separation was based on the work of Wallschlager and Roehl (2001). In short, a combination of a Dionex IonPac AS16 anion exchange column (4 X 250 mm) and a Dionex IonPac AG16 guard column (4 X 50 mm) was used with an alkaline gradient. A 0.1 mL sample was injected throughout the study. Two selenium isotopes ( $^{78}\text{Se}$  and  $^{82}\text{Se}$ ) were monitored to isotopically confirm the presence of selenium in the observed peaks. The data acquisition was carried out by the instrument's own software and the raw time vs. intensity data were processed using either Totalchrom or Chromera software (both from PerkinElmer) for quantification.

Method detection limits (MDL) for all selenium species were generated in every analytical batch from replicate analyses of the lowest standard (50 ng/L) in the calibration curve. All samples were analyzed at a dilution factor of 100 and the MDLs at this dilution factor were 1.6, 2.1, and 2.6 ppb as Se for  $\text{SeCN}^{1-}$ ,  $\text{SeO}_3^{2-}$  and  $\text{SeO}_4^{2-}$ , respectively.

## Chapter 3

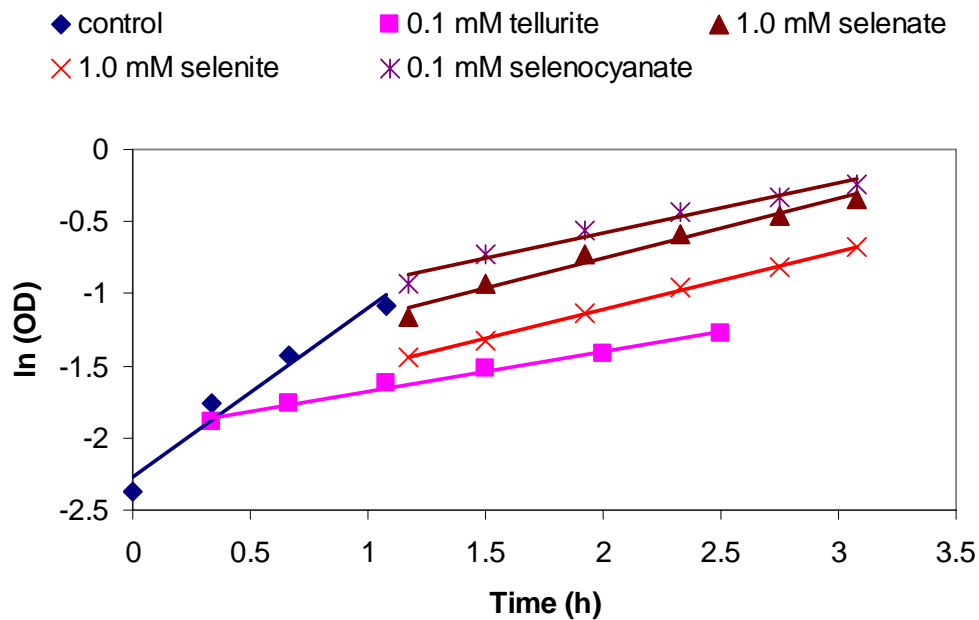
### Data and Results

#### Part 1: Bacterial Growth curves

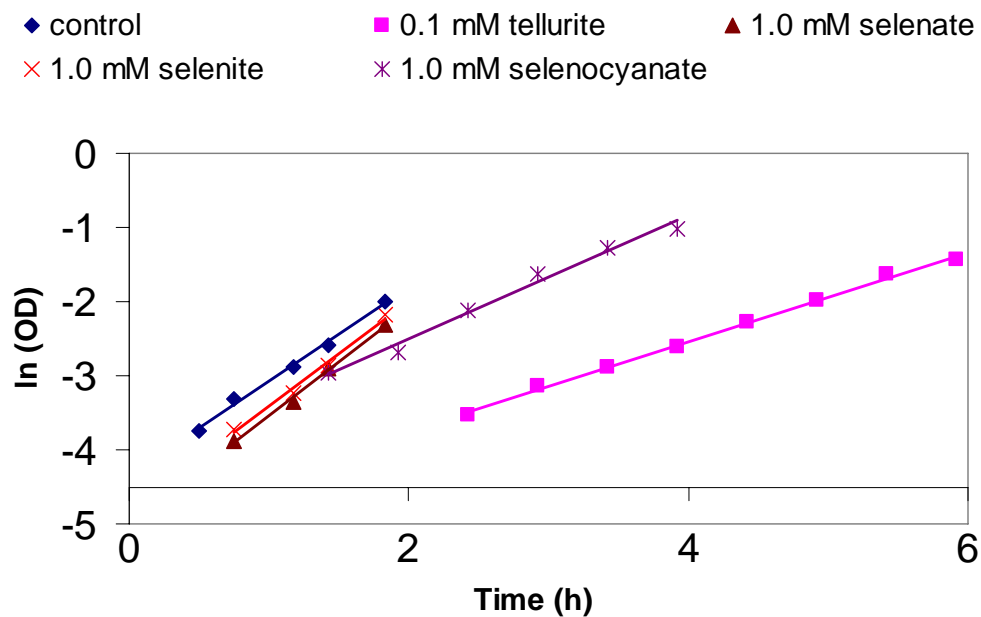
Cultures of the bacterium 130404 were grown as described in Chapter 2. Specific growth rates (SGR) of bacterial cultures amended with metalloids-containing anions were used as a relative measure of the toxicity (Yu et al., 1997; Morlon et al., 2005; Bennett, 1988). Absorbance/scattering (optical density) readings were typically measured until the bacterium reached the stationary phase; then the natural log of the optical density was plotted against the growth time in hours. Data points which fell in the log phase of growth were then selected to generate a linear curve with an equation

$y = mx + c$ . Slope of that line “m” is the SGR (Paran et al., 1990; Yu et al., 1997).

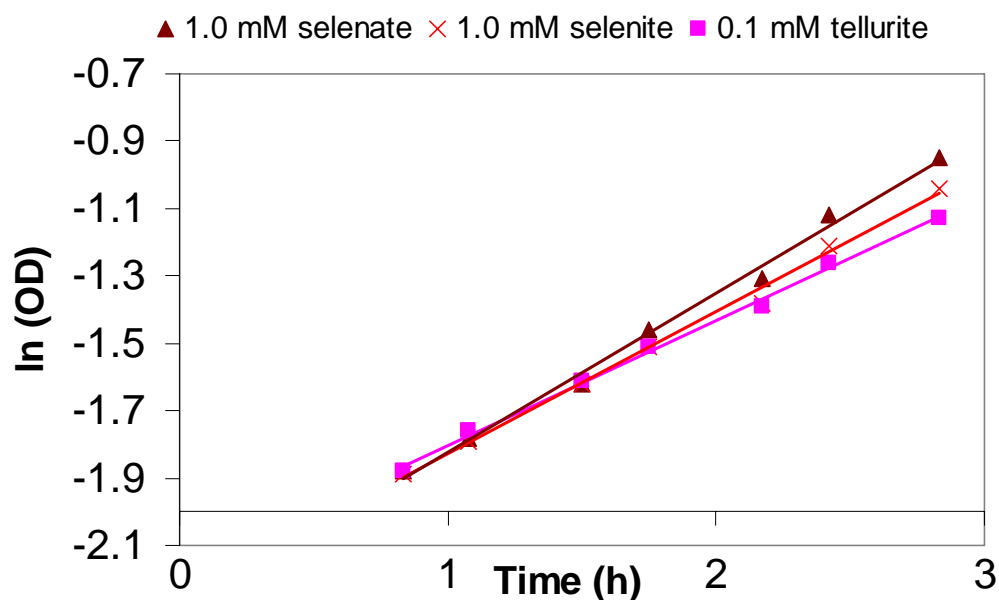
Comparison of only the log phase growth curves of 130404 with or without metalloid amendments can be seen in Figure 2. Figure 3 shows the comparison plot of *Aeromonas* spp. specific growth rates in their log phases with or without metalloid amendments; Figure 4 is the comparison plot of log phase growth curves of *Bacillus* spp. with 1.0 mM selenate, 1.0 mM selenite and 0.1 mM tellurite amendments. The specific growth rates generated from triplicate samples of each bacterium amended with or without metalloids are summarized in Table 4. When analyzing *Aeromonas* bacterium amended with selenate and selenite, formation of elemental selenium in the log phase was observed. This elemental selenium increased the absorbance readings, hence specific growth rates with selenate and selenite amendments are higher than the specific growth rate of control.



**Figure 2. Comparison of specific growth rates of 130404 cultures with or without metalloid amendments.**



**Figure 3. Comparison of specific growth rates of *Aeromonas* spp. cultures with or without metalloid amendments.**



**Figure 4.** Comparison of specific growth rates of *Bacillus* spp. cultures with metalloid amendments.

**Table 4.** SGRs of different bacteria amended with toxic salts.

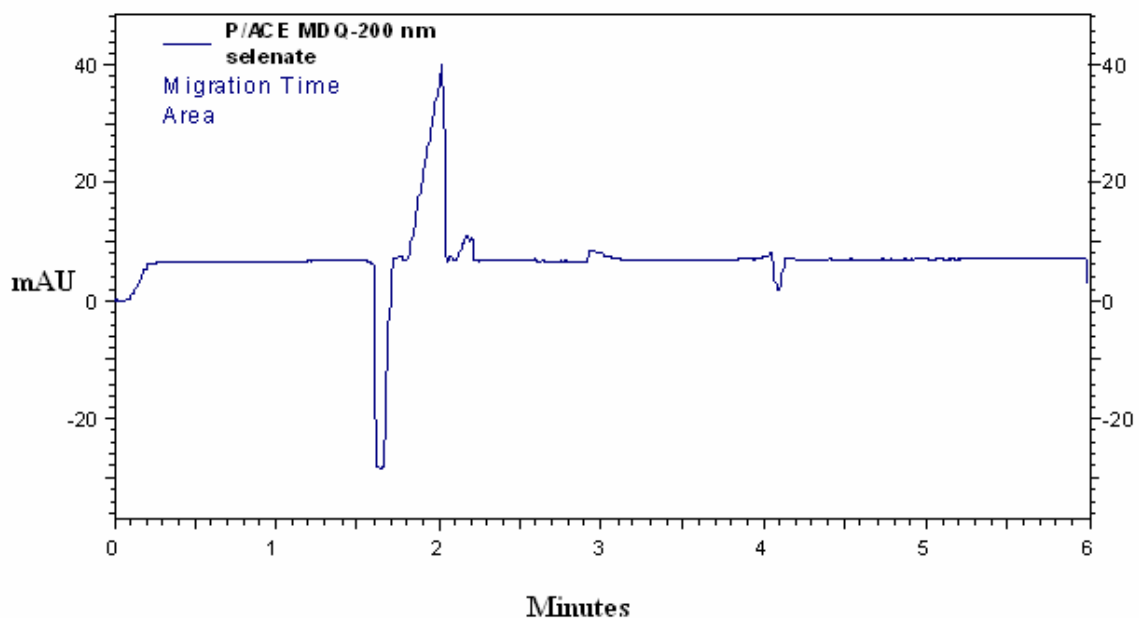
| Amendment         | SGR of 130404 ( $\text{h}^{-1}$ ) | SGR of <i>Aeromonas</i> ( $\text{h}^{-1}$ ) |
|-------------------|-----------------------------------|---|
| Control           | 1.1654                            | 1.2621                                      |
| 0.10 mM Tellurite | 0.2734                            | 0.5983                                      |
| Selenocyanate*    | 0.3463                            | 0.8269                                      |
| 1.0 mM Selenite   | 0.4033                            | 1.4278                                      |
| 1.0 mM Selenate   | 0.4119                            | 1.4622                                      |

\* Selenocyanate concentration for 130404 amendment was 0.10 mM and for *Aeromonas* 1.0 mM

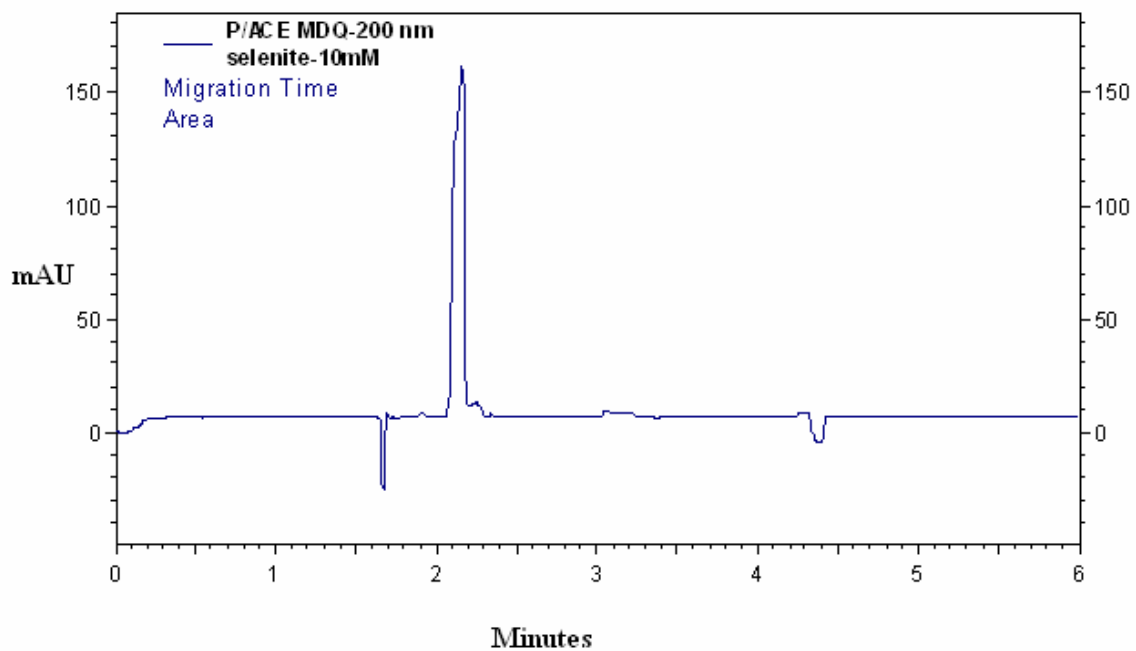
## Part 2: Method development on capillary electrophoresis

The initial method development was focused on the separation of selenate and selenite in the presence of complex bacterial growth media. A few of those methods that failed and a few that were successful are presented in this section. Details of the instrumentation and the capillary dimensions were described in Chapter 2. Injection pressure of 0.5 psi for 5 seconds and capillary temperature of 25 °C was set for all the methods.

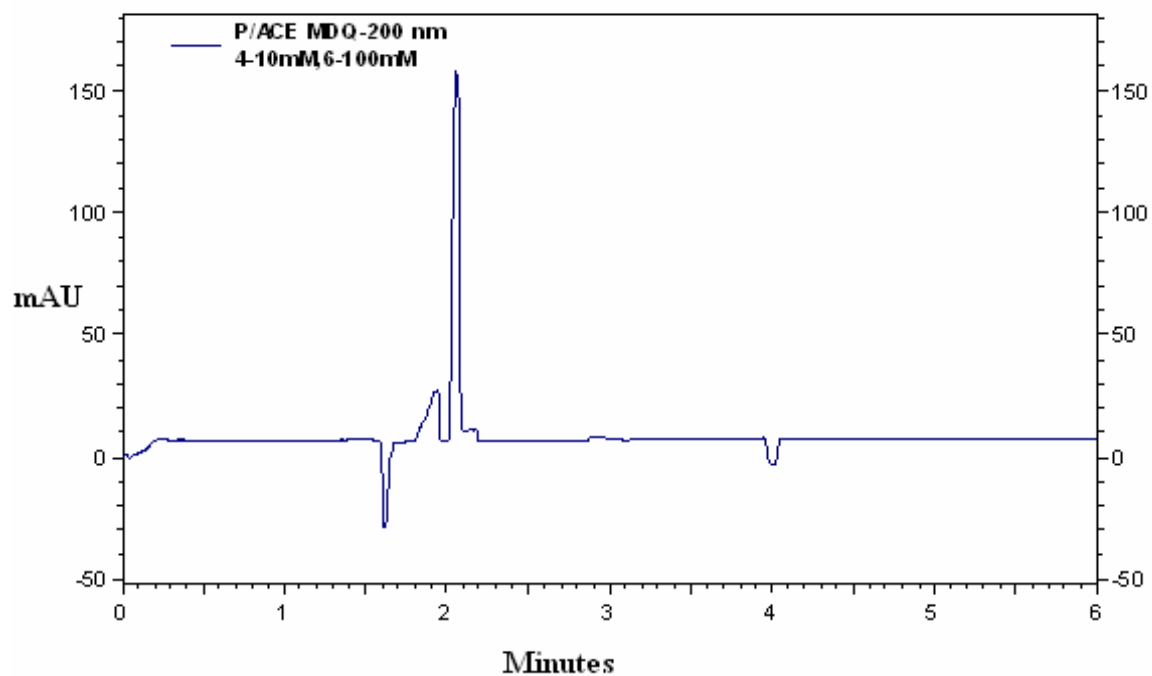
*Method 1:* To separate selenate and selenite a buffer consisting of 25 mM sodium borate and 1 mM TTAB was prepared. The pH was adjusted to 9.2 with 1.0 M NaOH. Separation voltage of -27.0 KV was applied and the run time was 6 minutes. Figures 5, 6 and 7 are results of this method.



**Figure 5. Electropherogram of 10 mM selenate using Method 1.**

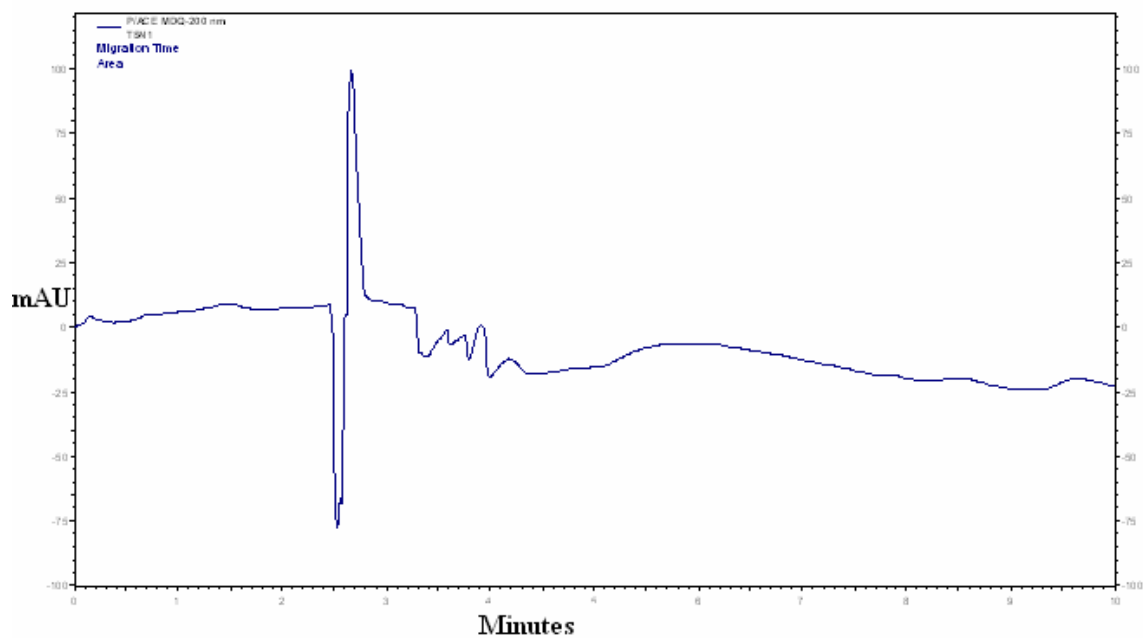


**Figure 6. Electropherogram of 10 mM selenite using Method 1.**

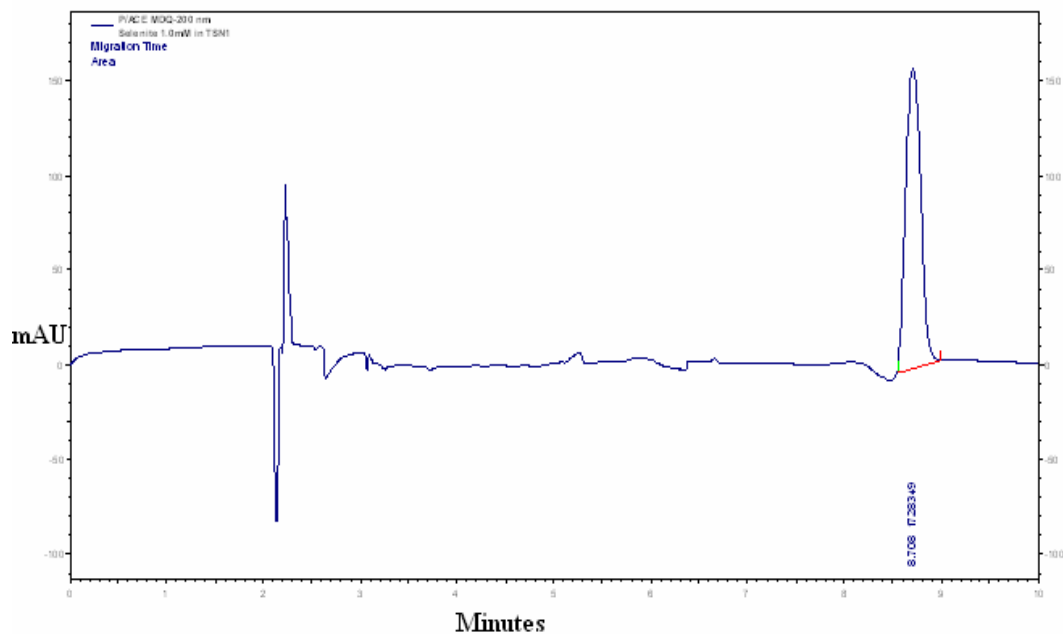


**Figure 7. Electropherogram of the mixture of selenate and selenite using Method 1.**

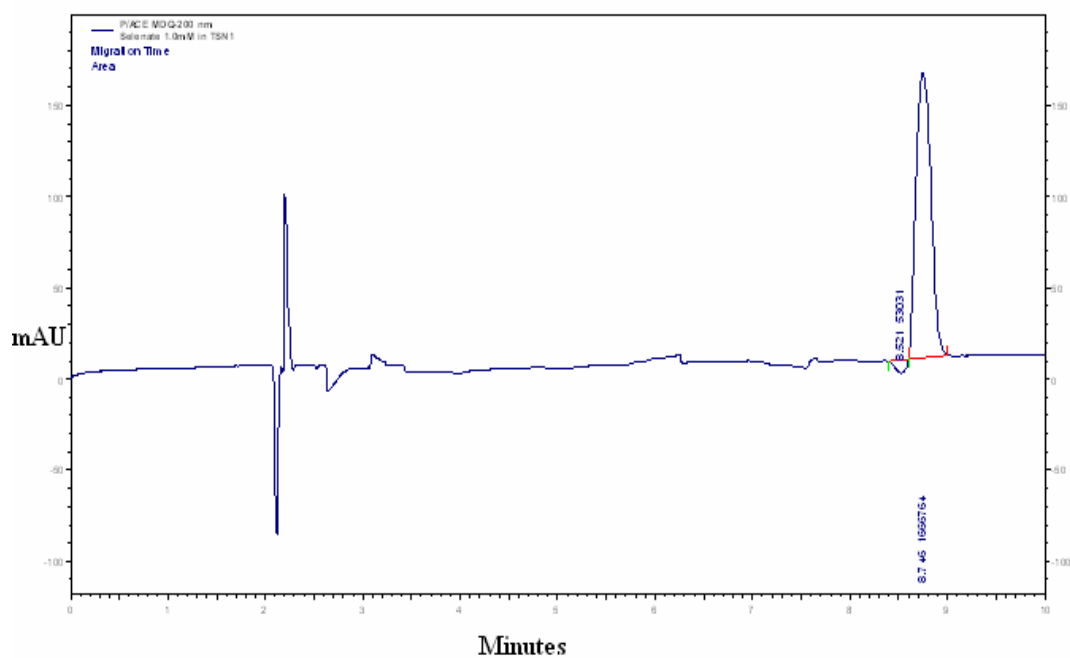
*Method 2:* To separate 0.3 % nitrate from selenate and selenite a buffer was prepared using 5 mM sodium chromate and 0.5 mM TTAB and the pH was adjusted to 8.6 using 1.0 M NaOH. Separation voltage of -25.0 KV was applied and the run time was 10 minutes. Figures 8, 9, 10, and 11 are the electropherograms obtained using this method.



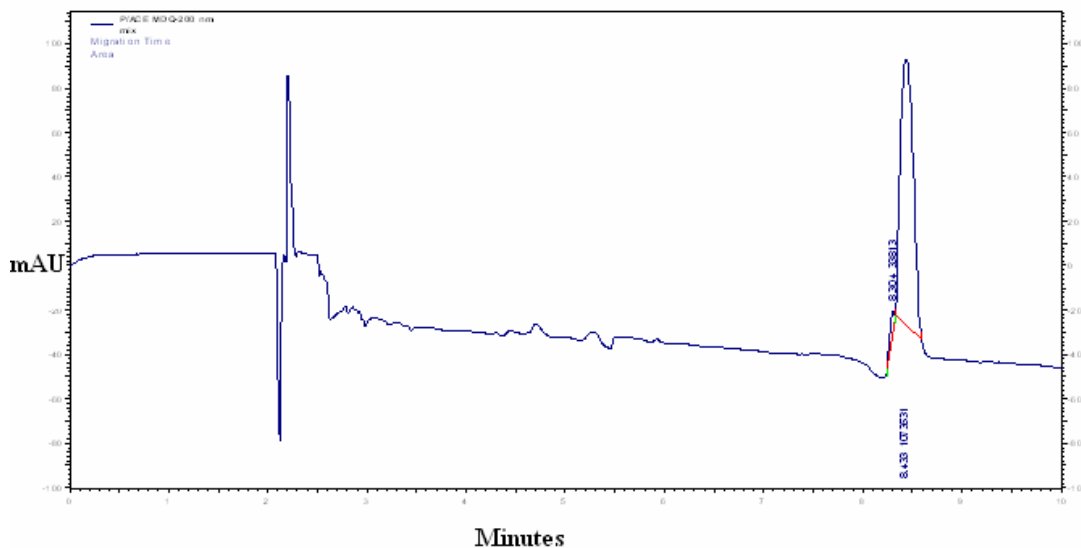
**Figure 8. Electropherogram of 0.3 % nitrate using Method 2.**



**Figure 9. Electropherogram of a mixture of 0.3 % nitrate and selenate using Method 2.**

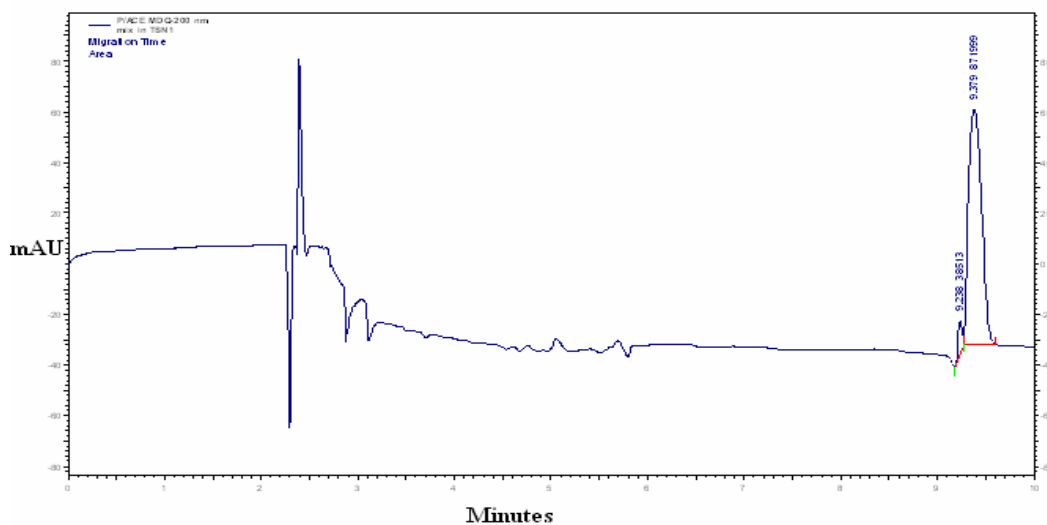


**Figure 10. Electropherogram of a mixture of 0.3 % nitrate and selenite using Method 2.**



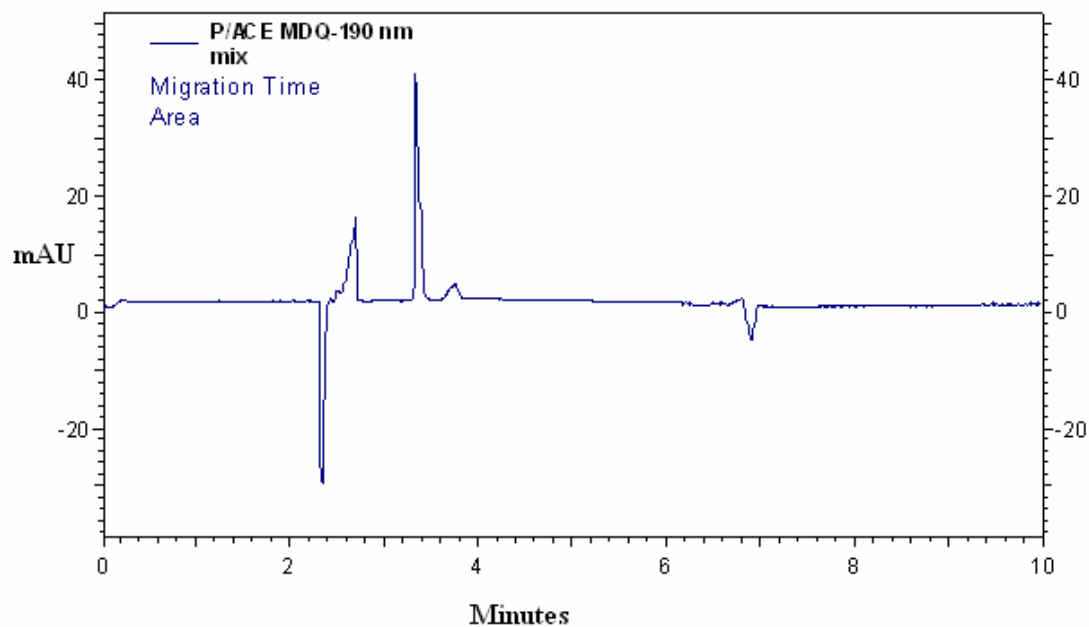
**Figure 11. Electropherogram of a mix of 0.3% nitrate, selenate and selenite using Method 2.**

*Method 3:* To increase the resolution between selenate and selenite a buffer with 5.0 mM sodium chromate and 0.50 mM TTAB at a pH of 10.5 was prepared. Separation voltage of -25.0 KV was applied and the run time was 10 minutes. Figure 12 is the resulting electropherogram using this method.



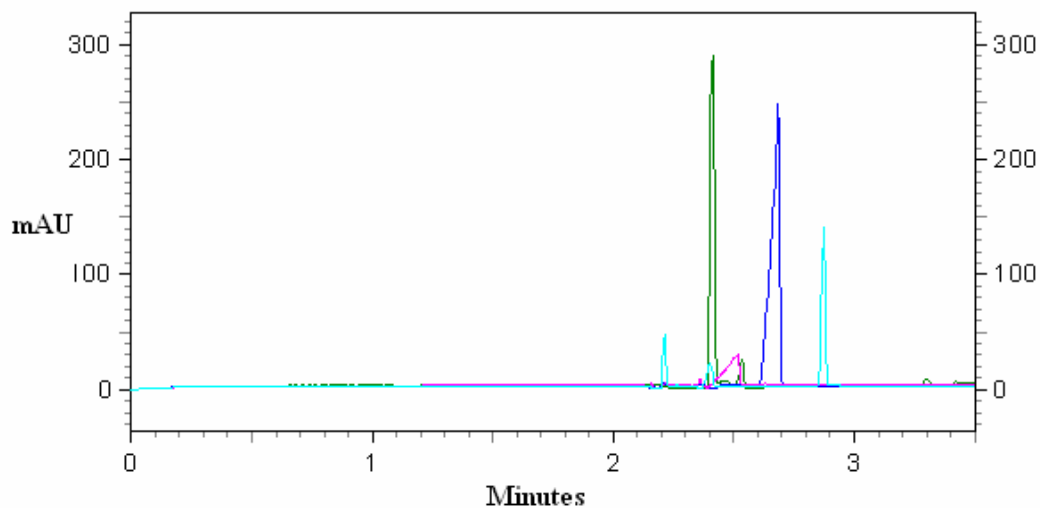
**Figure 12. Electropherogram of a mix of 0.3% nitrate, selenate and selenite using Method 3.**

*Method 4:* A buffer consisting of 15 mM  $\text{KH}_2\text{PO}_4$  and 0.5 mM TTAB at a pH of 7.0 was prepared. Separation voltage of -25.0 KV was applied and the run time was 10 minutes. Figure 13 is the resulting electropherogram using this method.



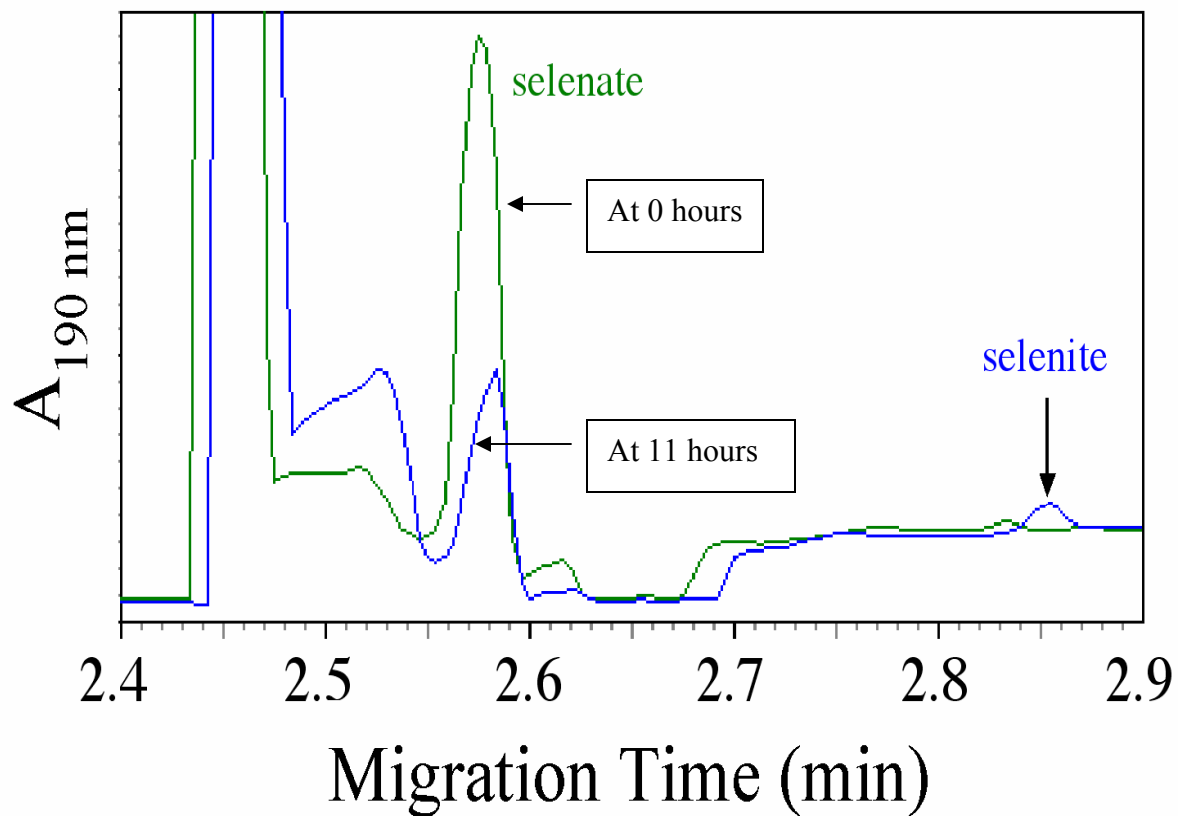
**Figure 13. Electropherogram showing the separation between selenate and selenite using Method 4.**

*Method 5:* A method for simultaneous estimation of selenate, selenite, and tellurite in the presence of TSB and LB media was then developed. Run buffer consisting 15 mM  $\text{KH}_2\text{PO}_4$  and 1.0 mM TTAB at a pH of 10.0 was prepared. Separation voltage was -25.0 KV and the run time was 3.5 minutes. The overlay of the electropherograms obtained using this method can be seen in Figure 14.



**Figure 14. Overlay of electropherograms of TSB, selenate, selenite and tellurite using Method 5.**

*Method 6:* A method for practical application in bacterial cultures was then finalized. Figure 15 shows overlays of electropherograms obtained from the time-course analysis performed on *Bacillus* spp. using this method. The data collected from that experiment is summarized in Figure 16. Figures 17 and 18 are from the time-course analysis performed on 1VH using the same method. Run buffer consisting of 15 mM  $\text{KH}_2\text{PO}_4$  and 1.0 mM TTAB at a pH of 10.5 was prepared. Separation voltage of -25.0 KV was applied and the run time was 5 minutes.



**Figure 15.** Overlay of portions of two electropherograms (using Method 6) showing the time-course reduction of selenate to selenite by *Bacillus* spp. in TSB medium over a period of 11.0 h.

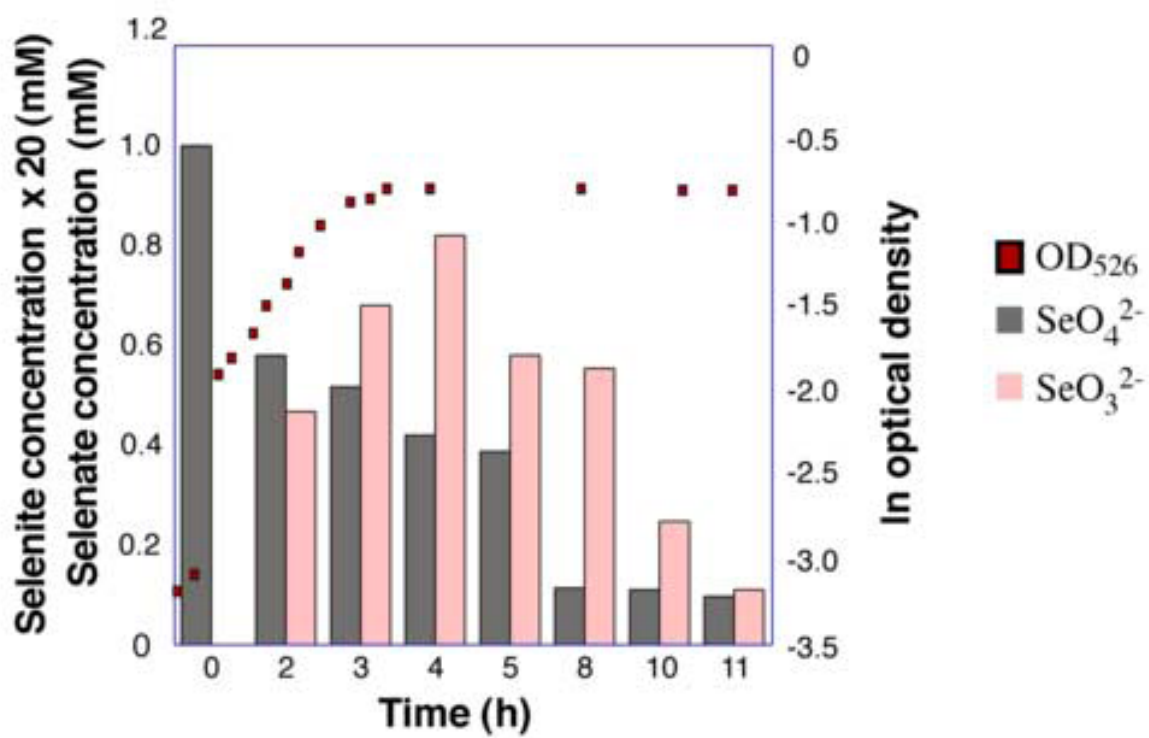
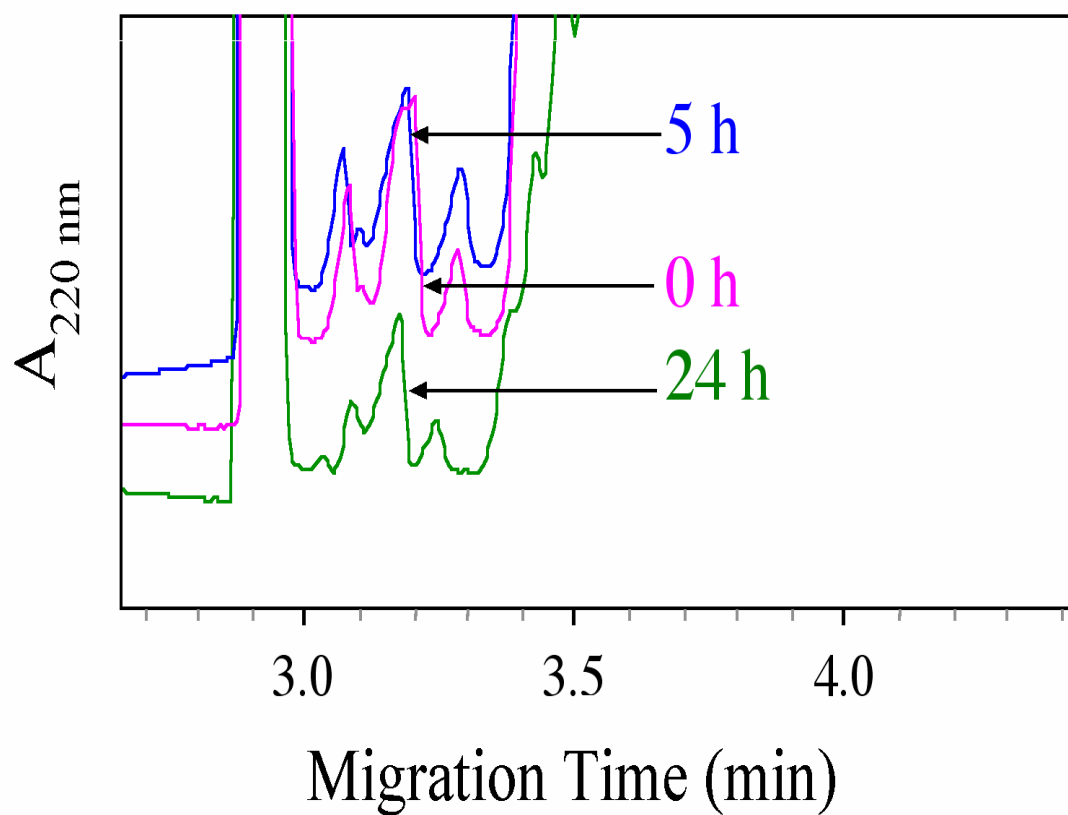
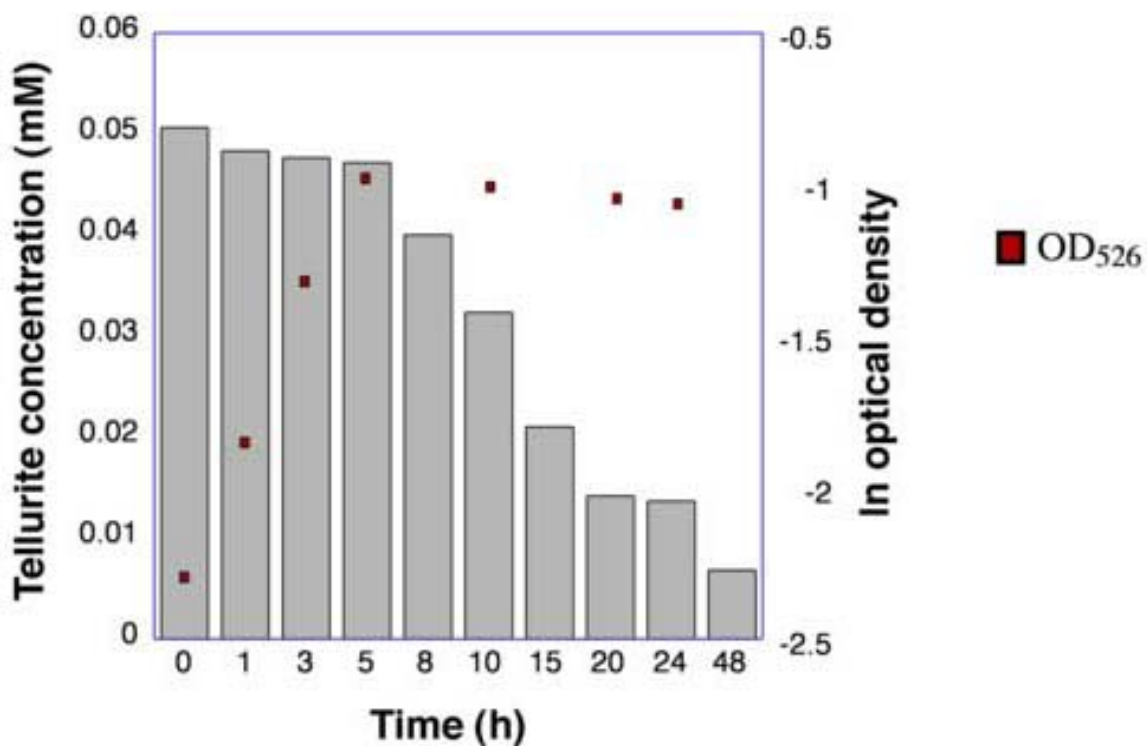


Figure 16. Simultaneous determination of the bioconversion of selenate to selenite in live cultures of *Bacillus* spp. over a period of 11 h.



**Figure 17. Overlay of portions of three time-course electropherograms (using Method 6) showing the bioreduction of tellurite over time by 1VH in LB medium.**



**Figure 18. Bioreduction of tellurite over a period of 48 h by the bacterium 1VH. Initial tellurite concentration was 0.05 mM.**

**Table 5. Figures of merit of the capillary electrophoretic Method 6.**

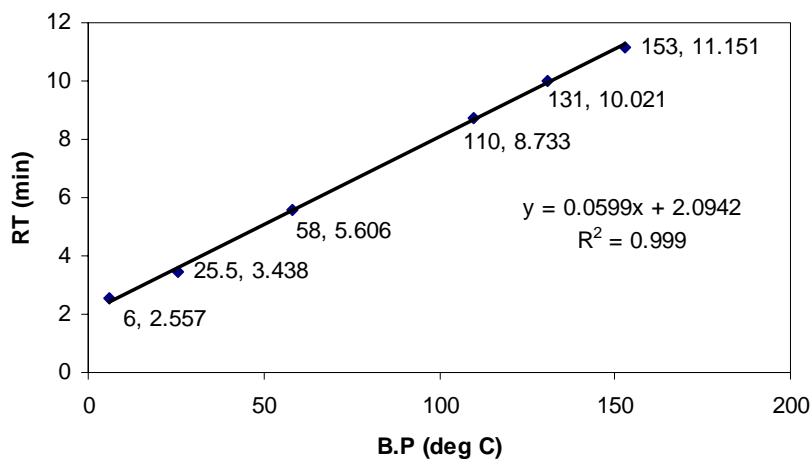
| Anion         | Linear Range (ppm) | Correlation Coefficient | Growth Medium | LOD (ppm) (3S/N <sup>**</sup> ) | %RSD over linear range (Migration time) | % RSD at 1.0 mM (Migration time) |
|---------------|--------------------|-------------------------|---------------|---------------------------------|---|----------------------------------|
| Selenate      | 0.79 – 79          | 0.9998                  | TSB           | 0.553                           | 0.202                                   | 0.196                            |
| Selenite      | 0.79 – 79          | 0.9998                  | TSB           | 0.158                           | 0.786                                   | 0.488                            |
| Tellurite     | 1.27 – 127         | 0.9996                  | LB            | 0.191                           | 0.988                                   | 0.623                            |
| Tellurate     | 1.27- 12.7         | 0.9988                  | LB            | 0.255                           | 0.451                                   | 0.402*                           |
| Selenocyanate | 0.79 - 79          | 0.9997                  | LB            | 0.75                            | 0.265                                   | 0.162                            |

\* RSD at 0.1 mM concentration.

\*\* S/N indicates signal to noise ratio.

### Part 3: Head space analysis of the bacterial cultures amended with known concentrations of metalloids

In an effort to study the production of organo-chalcogen compounds in the headspace of bacterial cultures amended with or without toxic metalloids, cultures were prepared as mentioned in Chapter 2 and incubated at recommended temperatures. Time course sampling of the headspace from these tubes was performed using solid phase micro extraction techniques. The trend in production of the organo-volatile compounds was identified (data not shown). Furthermore, late eluting unknown peaks were found in some cultures. Efforts were made to characterize those peaks. Table 6 shows the names of the compounds found in the headspace of bacterial cultures and their boiling points. A plot was generated with the boiling points and retention times (mean retention time of five injections) of known compounds (Figure 19) and it was extrapolated to estimate the late eluting unknown peaks in the headspace samples of 130404 cultures amended with 1.0 mM selenate. Figures 20-27 show the chromatograms of headspace gases of cultures 130404 and *Aeromonas* spp.



**Figure 19. Plot of boiling points versus retention times of known compounds.**

**Table 6. Volatiles observed over the headspace of metalloids amended cultures**

| <b>Compound</b>              | <b>Boiling Point (°C)</b> | <b>Retention time (min)</b> | <b>Formula</b>                          |
|------------------------------|---------------------------|-----------------------------|---|
| Methanethiol                 | 6                         | 2.55                        | CH <sub>3</sub> SH                      |
| Methaneselenol               | 25.5                      | 3.43                        | CH <sub>3</sub> SeH                     |
| Dimethylselenide             | 58                        | 5.60                        | CH <sub>3</sub> SeCH <sub>3</sub>       |
| Dimethyltelluride            | 83                        | 7.30                        | CH <sub>3</sub> TeCH <sub>3</sub>       |
| Dimethyldisulfide            | 110                       | 8.73                        | CH <sub>3</sub> SSCH <sub>3</sub>       |
| Dimethyl selenenyl sulfide   | 131                       | 10.02                       | CH <sub>3</sub> SeSCH <sub>3</sub>      |
| Dimethyldiselenide           | 153                       | 11.15                       | CH <sub>3</sub> SeSeCH <sub>3</sub>     |
| Dimethyl tellurenyl sulfide  | 161                       | 11.81                       | CH <sub>3</sub> TeSCH <sub>3</sub>      |
| Dimethyltrisulfide           | 170                       | 12.44                       | CH <sub>3</sub> SSSCH <sub>3</sub>      |
| Dimethyl selenenyl disulfide | 190*                      | 13.79                       | CH <sub>3</sub> SSeSCH <sub>3</sub>     |
| Dimethyl seleno disulfide    | 195*                      | 13.86                       | CH <sub>3</sub> SeSSCH <sub>3</sub>     |
| Dimethylditelluride          | 196                       | 14.09                       | CH <sub>3</sub> TeTeCH <sub>3</sub>     |
| Dimethyl diselenenyl sulfide | 217*                      | 15.13                       | CH <sub>3</sub> SeSeSCH <sub>3</sub> ** |
| Dimethyltriselenide          | 236*                      | 16.27                       | CH <sub>3</sub> SeSeSeCH <sub>3</sub>   |

\* Estimated boiling points using Figure 19.

\*\* To be confirmed

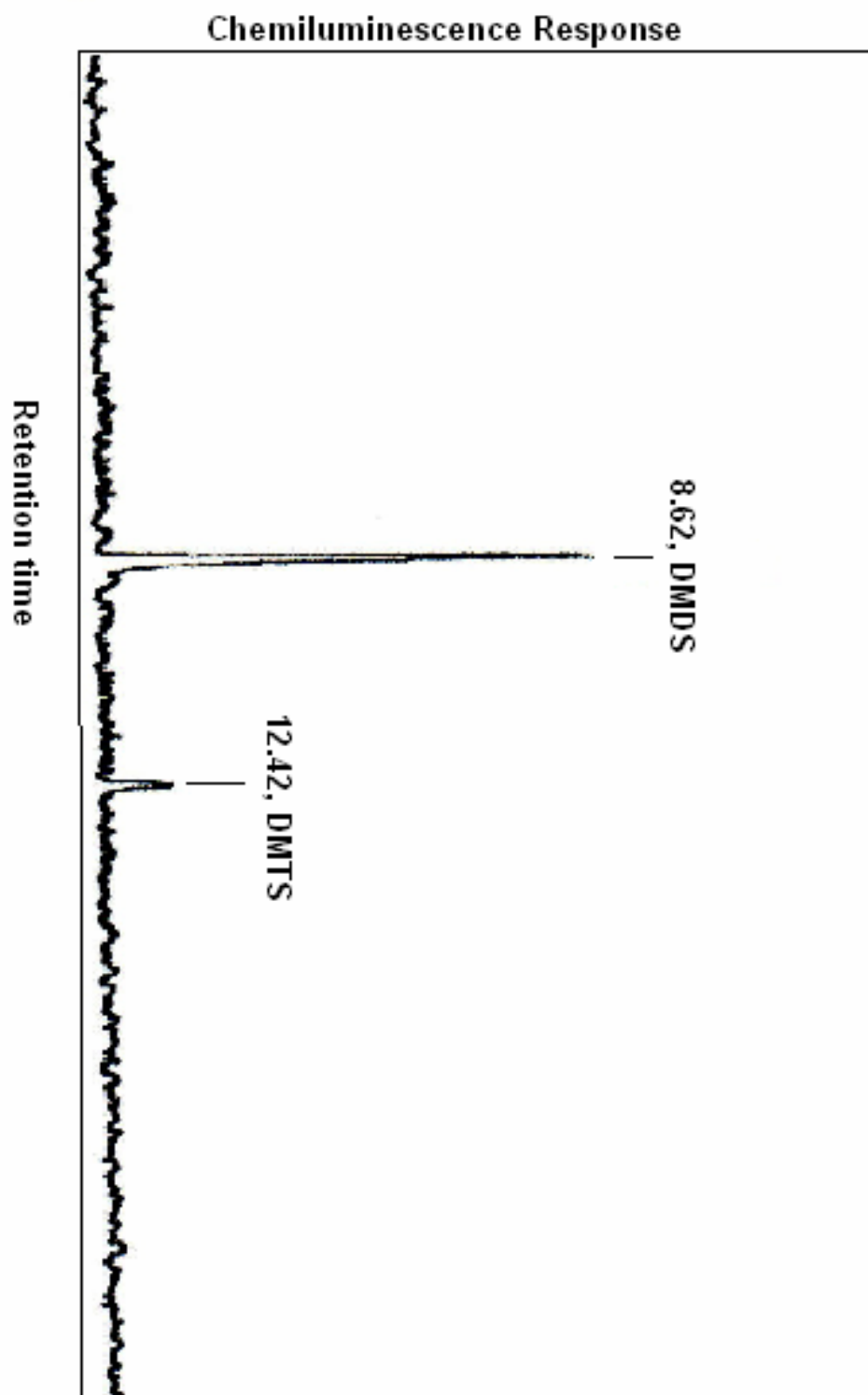


Figure 20. GC chromatogram of blank – LB medium, after 48 h.

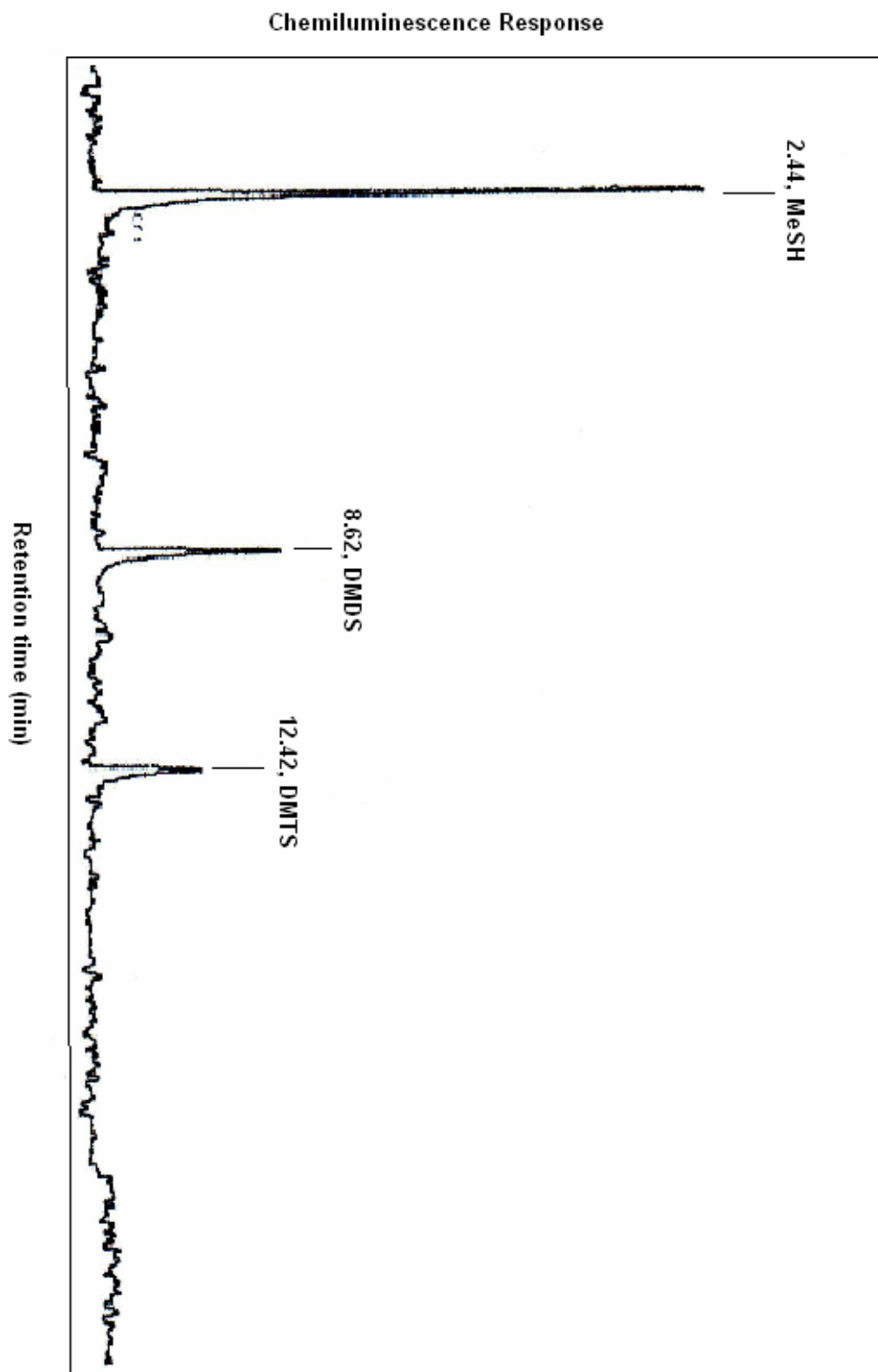


Figure 21. Chromatogram of control – 130404, after 48 h.

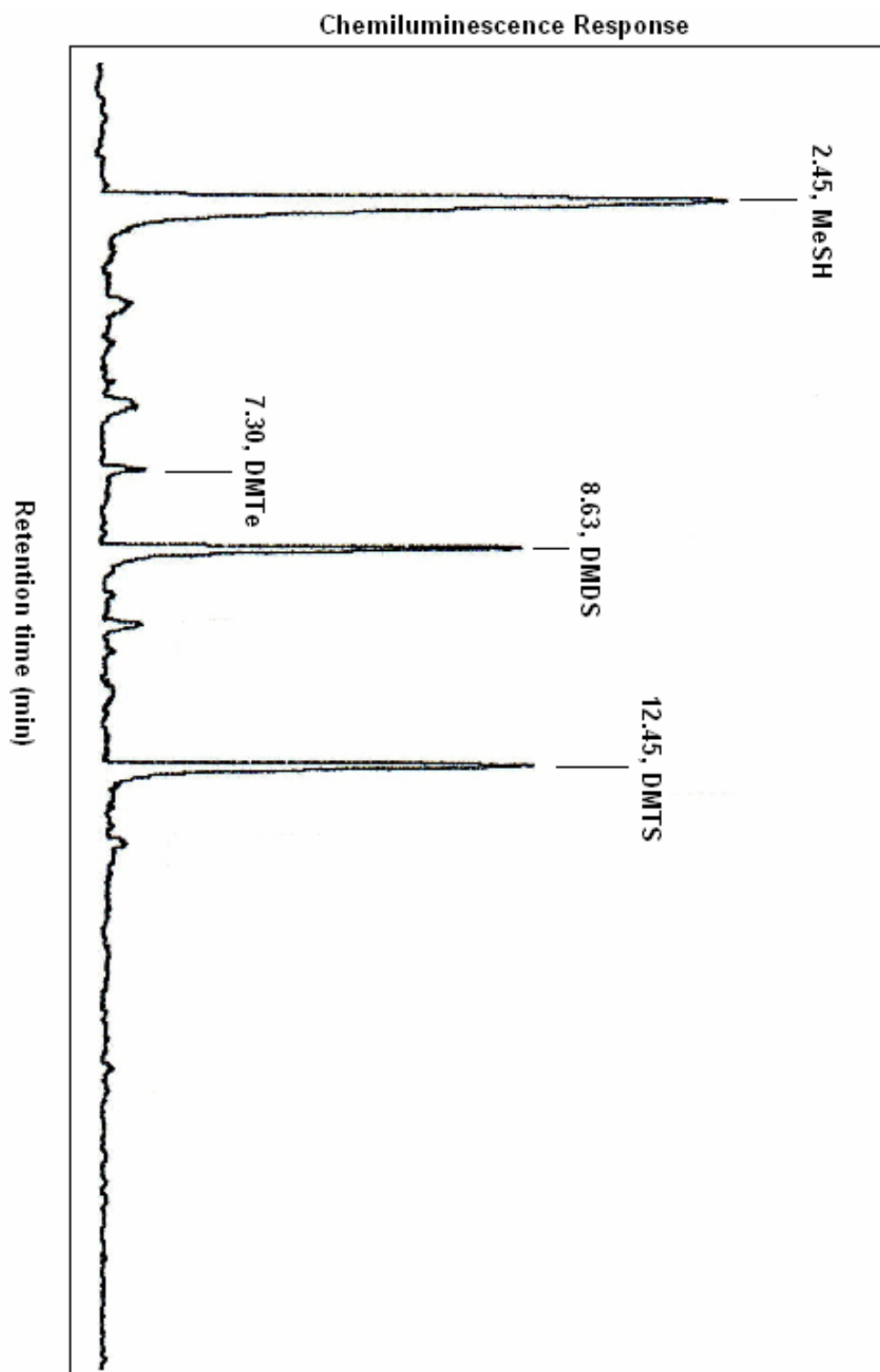


Figure 22. Chromatogram of 130404 amended with 0.1 mM tellurite, after 48 h.

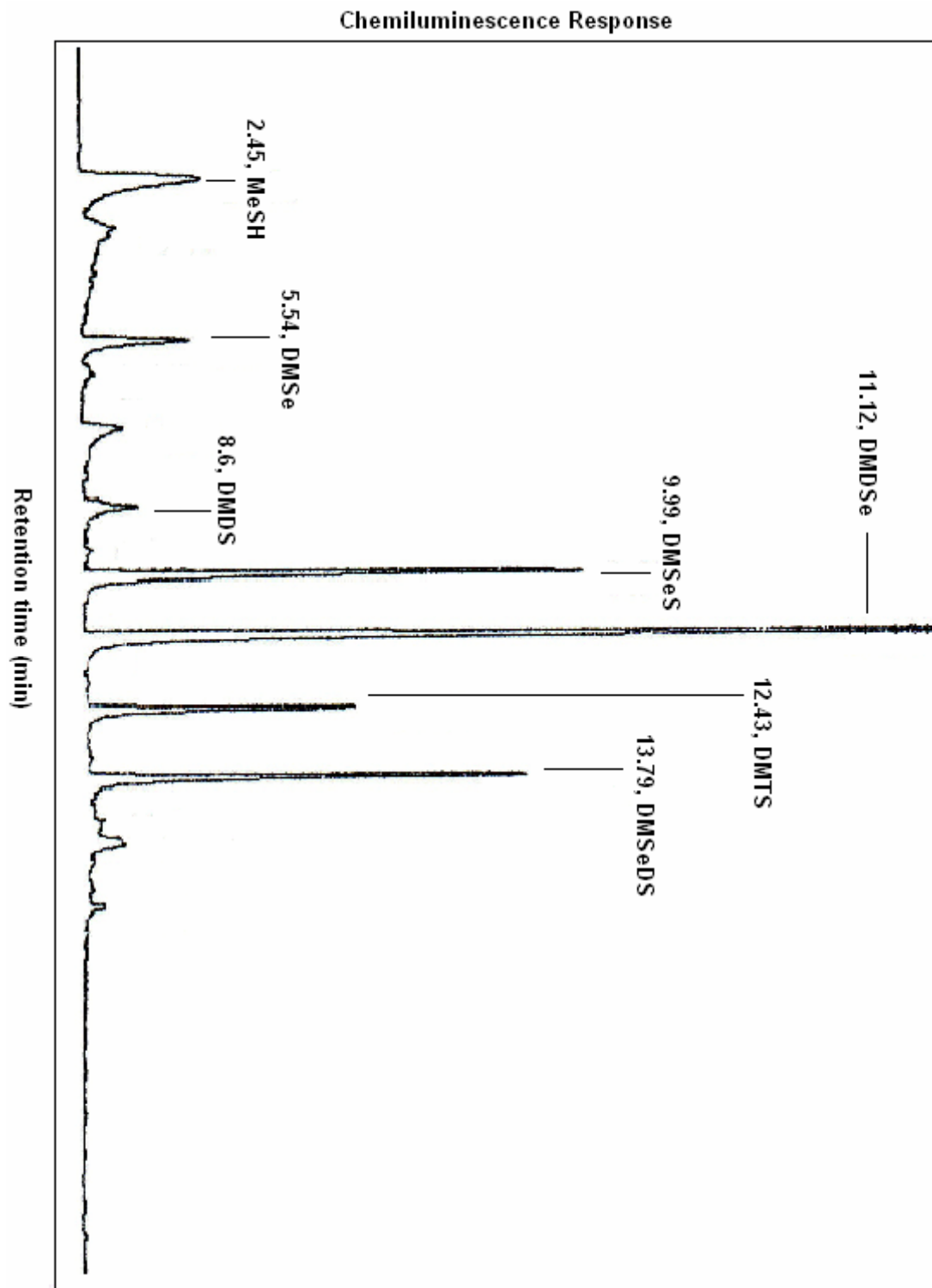


Figure 23. Chromatogram of 130404 amended with 1.0 mM KSeCN, after 72 h.

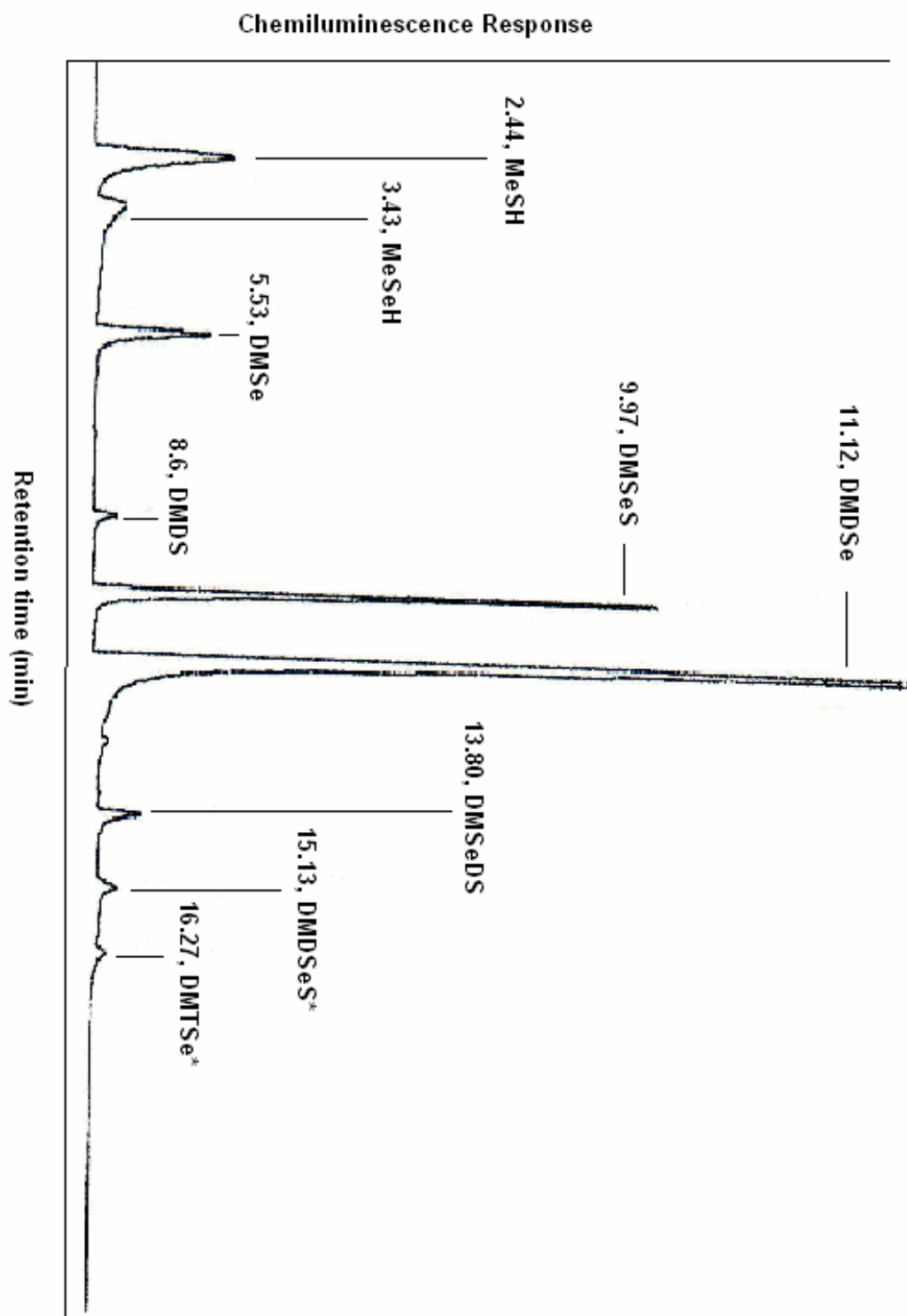


Figure 24. Chromatogram of 130404 amended with 1.0 mM selenate, after 45 h.

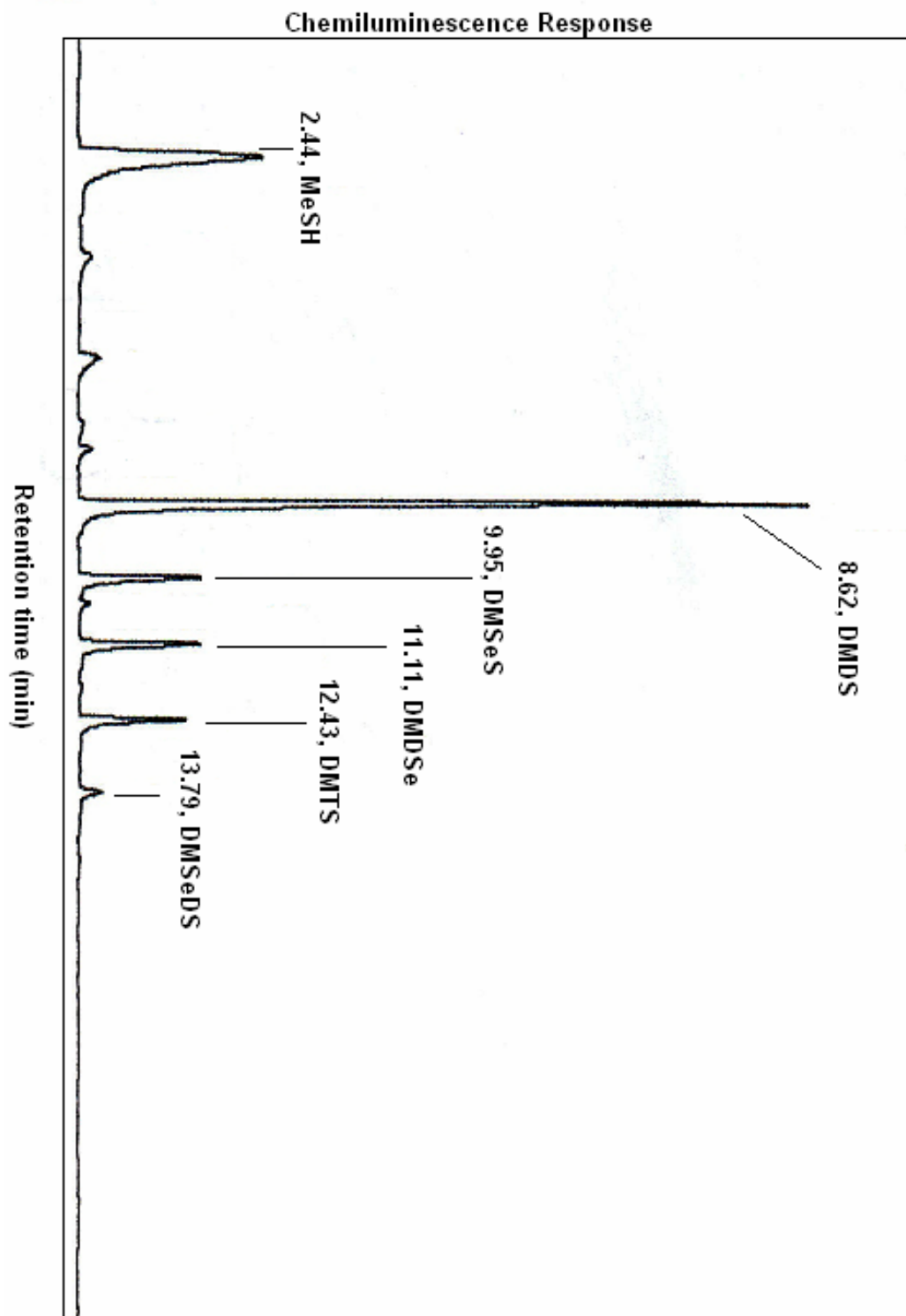


Figure 25. Chromatogram of 130404 amended with 1.0 mM selenite, after 24 h.

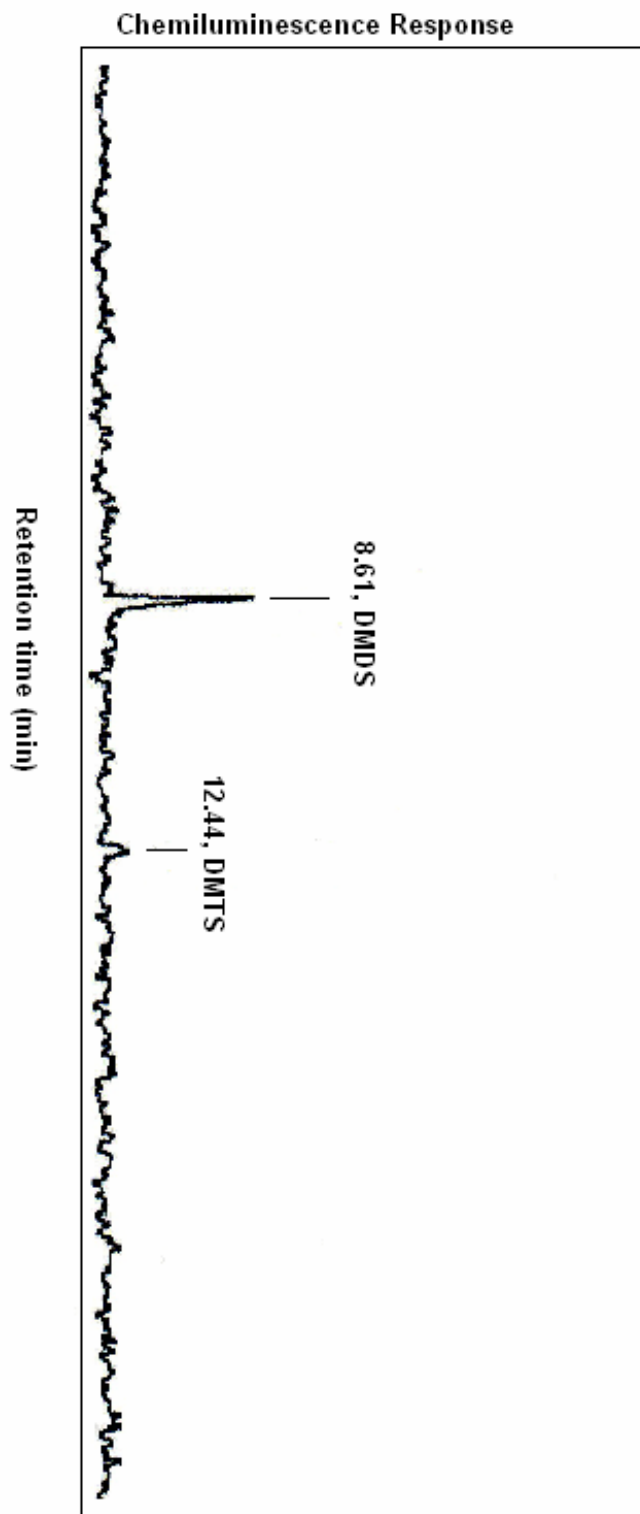


Figure 26. Chromatogram of *Aeromonas* spp. control, after 48 h.

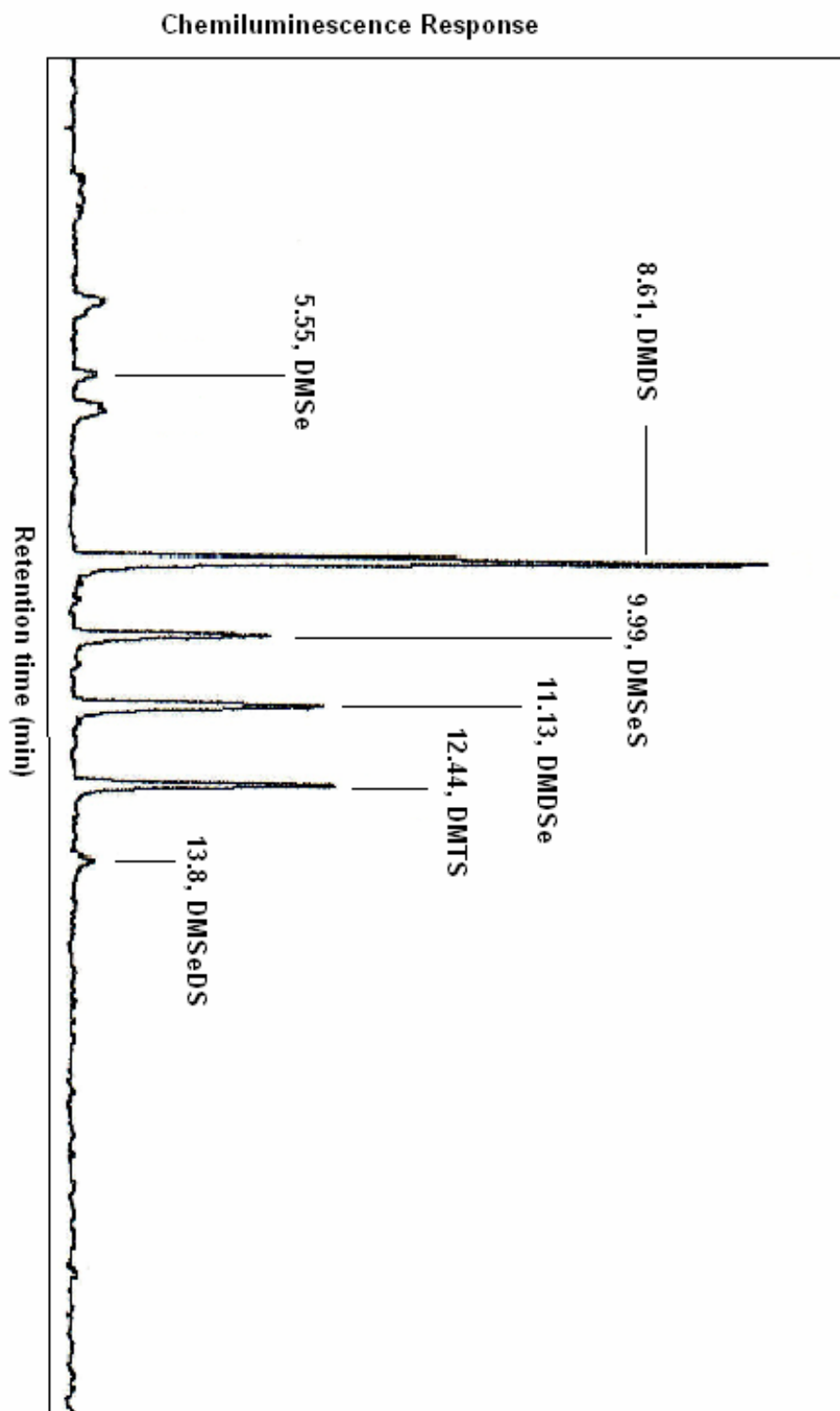


Figure 27. Chromatogram of *Aeromonas* spp. amended with 1.0 mM KSeCN, after 48 h.

#### Part 4: Ion Chromatography – Inductively Coupled Plasma – Mass Spectroscopy

IC-ICP-MS analysis was performed by Applied Speciation and Consulting, LLC, Tukwilla, WA, USA. The results obtained from the analysis is summarized in Table 7. Statistical evaluation of the differences between means were evaluated using the Student's *t* test (Harris, 2007)

**Table 7. Data from IC-ICP-MS analysis of four bacterial cultures amended with or without Se-containing metalloids.**

| Se-free sterile media and culture controls |  | SeCN <sup>1-</sup><br>(ppb) | [SeCN <sup>1-</sup> ]<br>statistically<br>different<br>from Se-<br>amended<br>sterile<br>medium |
|--|--|-----------------------------|---|
|  | Sterile LB medium  | ND                          |   |
|  | Sterile TSB medium                                       | ND                          |   |
|  | Live 130404 culture                                      | ND                          |   |
|  | Live <i>Aeromonas</i> culture                            | ND                          |   |
|  | Live 1VH culture   | ND                          |   |
|  | Live <i>Bacillus</i> culture                             | ND                          |   |
| <b>Selenate Amendments</b>                 |  |                             |   |
| <b>LB Medium</b>                           | LB + 1.0 mM SeO <sub>4</sub> <sup>2-</sup>               | 47 (n=7)*                   |   |
|  | 1VH + 1.0 mM SeO <sub>4</sub> <sup>2-</sup>              | 80 (n=3)                    | Yes   |
|  | <i>Aeromonas</i> + 1.0 mM SeO <sub>4</sub> <sup>2-</sup> | 27 (n=3)                    | No  |
|  | 130404 + 1.0 mM SeO <sub>4</sub> <sup>2-</sup>           | 172 (n=3)                   | Yes   |
| <b>TSB Medium</b>                          | TSB + 1.0 mM SeO <sub>4</sub> <sup>2-</sup>              | 15                          |   |
|  | <i>Bacillus</i> + 1.0 mM SeO <sub>4</sub> <sup>2-</sup>  | 32 (n=3)                    |   |
| <b>Selenite Amendments</b>                 |  |                             |   |
| <b>LB Medium</b>                           | LB + 1.0 mM SeO <sub>3</sub> <sup>2-</sup>               | 58                          |   |
|  | 1VH + 1.0 mM SeO <sub>3</sub> <sup>2-</sup>              | 52 (n=3)                    |   |
|  | <i>Aeromonas</i> + 1.0 mM SeO <sub>3</sub> <sup>2-</sup> | 83 (n=3)                    |   |
|  | 130404 + 1.0 mM SeO <sub>3</sub> <sup>2-</sup>           | 37 (n=3)                    |   |
| <b>TSB Medium</b>                          | TSB + 1.0 mM SeO <sub>3</sub> <sup>2-</sup>              | 36                          |   |
|  | <i>Bacillus</i> + 1.0 mM SeO <sub>3</sub> <sup>2-</sup>  | 74 (n=3)                    |   |

ND represents a detection limit of 1.6 ppb SeCN<sup>1-</sup>.

\* number of replicates

## Chapter 4

### Discussion

#### Part 1: Growth curve analysis of various bacterial species

The specific growth rates of different bacteria amended with or without metalloid-containing anions were studied in order to estimate the relative toxicity of the anions. A Spectronic 20 D+ spectrophotometer was used to observe the absorbance/scattering of light at 526 nm. Data were collected at regular intervals until the bacteria reached the stationary phase. Data points were then collected at about five hours apart until the bacteria reached the death phase. Figure 2 shows the comparison of growth curves of 130404 amended with or without metalloids. Bacterial cultures were amended to yield a final concentration of 0.1 mM tellurite, 1.0 mM selenate, 1.0 mM selenite, and 0.1 mM selenocyanate. The SGR (Table 4) indicate that tellurite is the most toxic towards this bacterium. The observed decreasing order of toxicity is tellurite, selenocyanate, selenite, and selenate.

Similar studies were carried out on *Aeromonas* spp and *Bacillus* spp with similar results. The order of toxicity observed in SGR study of 130404 agree with *Aeromonas* spp (Figure 3) and *Bacillus* spp (Figure 4).

#### Part 2: Capillary electrophoretic method development

The initial idea of this research was to develop a method for the simultaneous estimation of selenate and selenite in the presence of complex bacterial growth media. As can be seen from Figures 5, 6, and 7 selenite and selenate standards were easily separated using Method 1, described in Chapter 3. This method was then applied to follow selenate and selenite in bacterial cultures containing 0.3 % nitrate. The huge concentration of

nitrate was a problem. Using this method, nitrate co-eluted with selenate and thereby made it impossible for estimation of selenate in Se-amended bacterial cultures.

Efforts were then made to develop a method that could successfully separate nitrate and selenate. Figures 8, 9, 10, and 11 represent the results from one such method. Nitrate was successfully separated from selenate using Method 2. Injections of mixtures of nitrate, selenite, and selenate were then made and analyzed using this method. As can be seen from Figure 11, the resolution between selenate and selenite peaks (around 8.5 minutes migration time) was completely lost. Alteration in pH of the run buffer (Method 3) increased the resolution partially but this was not good enough for quantitative purpose. Figure 12 shows the electropherogram with the altered pH condition of the run buffer.

Method 4 was later developed using 15 mM  $\text{KH}_2\text{PO}_4$  and 0.05 mM TTAB, which could separate the complex growth media from selenate and selenite. Figure 13 shows the separation of selenate and selenite using that method. Figure 14 shows the overlay of electropherograms of selenate, selenite, tellurate, and tellurite standards using Method 5. The pH of the run buffer was increased from 7.0 to 10.0 and the concentration of the surfactant was increased from 0.05 mM to 1.0 mM. This resulted in good separation of four analytes in the same sample mixture. The run buffer pH at 10.5 was then found to be optimum for the simultaneous estimation of all four compounds in the presence of growth media. Well-resolved analyte peaks ( $R_s > 2$ ) and no peak overlap between the TSB, selenate, selenite, tellurite, and tellurate peaks demonstrated successful resolution and detection of target anions.

Nitrate anion, which is often added in bacterial cultures that require nitrate as a bacterial terminal electron acceptor, does not significantly interfere with this method (Method 6) as long as nitrate concentration is below 0.5 g/L and the most closely eluting analyte to nitrate is selenate using this buffer system (data not shown).

Five point calibrations generated with Method 6 were linear over two orders of magnitude for all analytes except tellurate; however, tellurate's linear range was controlled by solubility considerations alone (see below). Triplicate injections were made at each standard concentration. Limits of detection (3S/N) were determined for all metalloid-containing anions examined and are shown in Table 5. For example, the limit of detection for selenocyanate was determined to be the mass of  $\text{SeCN}^-$  injected that would produce a detector signal (at 200 nm) that was three times the average baseline noise signal recorded at  $\pm 0.3$  min of the  $\text{SeCN}^-$  migration time. As can be seen from Figure 15 the separation between  $\text{SeO}_4^{2-}$  and  $\text{SeO}_3^{2-}$  is over 16 s. The large, early eluting peaks are from the tryptic soy broth, a widely used complex bacterial growth medium. The cultures of this bacterium reach stationary growth phase in about 4 h as shown in Figure 16. Concentrations at various hours in the time-course analysis are plotted in Figure 16 but time slices could be as fast as 6 min given time for culture sampling, filtration, and CE analysis.

Figure 18 is the 48-h time course of 1VH amended with 0.05 mM tellurite. The conversion to elemental Te, precipitated tellurium in or on cells, and to a lesser degree volatile, organotellurium, is approximately 90% complete in that 48 h period. The concentration of tellurite after 48 h was observed to be 0.0068 mM. This tellurium-resistant bacterium reaches the stationary growth phase at about 5.5 h. Precision for both

selenium and tellurium analyses was good: standard deviations, at all concentrations studied, were  $\leq 2 \times 10^{-4}$  mM for 4 replicate cultures samples at the same point in the time course.

The bioanalytical method developed here was undertaken to afford sensitive determination of metalloid containing anions in growing bacterial cultures performing bioconversion of metalloid-containing anions. The method needed to be quick and simple so that time course data with reasonable time resolution could be achieved. In the case of selenium, the oxyanions of interest are those commonly present in the biosphere, selenate and selenite. The linearity of this method for these analytes stretched over two orders of magnitude, a much wider linear range than had been reported for comparable yet more complex and expensive CE-ICP/MS (Casiot et al., 2002). While the detection limits reported here are approximately 5 times higher than that IC-ICP/MS method for Se oxyanions, Casiot's method's linear range using IC-ICP/MS was 50 to 10,000 ppb while the linear range of the method reported here is only 80 ppb to 8,000 ppb (0.01 mM to 1.0 mM Se). Also the CE-ICP/MS figures of merit reported by Casiot et al (2002) were for standard solutions, not solutions with complex, that is, realistic biological matrices. Walker et al. (1996) have approached Se oxyanions analyzed directly in biological media using CE and their reported detection limits are slightly higher than ours (2 and 0.4 ppm for selenate and selenite, respectively); however, no linear range was reported. In relation to many of the Se- and Te containing samples discussed in the Introduction, this analytical method would be a useful approach; however, the focus of research work here was on bacterial samples. Interestingly, in relation to work others have reported: electrokinetic injection and hydrodynamic (pressure) injection were tried. Much better

detection limits were observed using the former; however, peak migration time suffered with electrokinetic injection so hydrodynamic injection was used in this work. Casiot et al. also reported this for their CE-ICP/MS technique involving a micronebulizer and especially noted that their detection limits varied with the electrophoretic mobility of the analytes involved. This variation was not observed in this research work, (see Table 5).

The detection limits and linear range reported in this work for tellurite compare well with detection limits reported in complex matrices (Turner et al., 1992), but again detection limits for reported matrix-free, aqueous standards are 10 times lower (Casiot et al., 2002). In Method 6, the detection limits drop by a factor of 10 for pure standard solutions too. All of the detection limit data reported here, however, are in the growth media studied, not pure water standards. While the detection limit for tellurate was comparable to the detection limits of the other anions examined in this study, the linear range for tellurate analysis was completely controlled by solubility. The highest concentration of potassium tellurate that could be achieved using distilled water without immediate precipitation was 0.1 mM  $\text{TeO}_4^{2-}$ . When dissolving tellurate in various growth media used in this research work, the solubility was even poorer. In order to increase the solubility of tellurite, the pH of the solution was increased using 1.0 M NaOH. This did, marginally increase the  $\text{TeO}_4^{2-}$  solubility; however, even at a pH of 12.6 the nominal  $\text{TeO}_4^{2-}$  solubility was only about 0.5 mM. Slight increased solubility could also be achieved using higher initial temperatures for stock solutions containing  $\text{TeO}_4^{2-}$ . Bacteriologically this is, of course, useless from the point of view of the method developed here, because none of the organisms under study would grow at that high pH or extreme temperatures such as 60 °C.

The interest in determination of selenocyanate,  $\text{SeCN}^{1-}$ , stems from its detection in environmental samples from the petrochemical industry where there is no obvious source (Miekeley et al., 2005) and from metalloid-mining settings where cyanide is used to leach Se-containing ores (de Souza et al., 2002). To investigate bacterial cultures as a means of probing bacteria as a possible source of this anion, this method can be successfully used. Bioremediation of both Se and Te (Araya et al., 2002, Swearingen et al., 2006) was a main point of interest in Dr. Chasteen's research group; however, effort has also been made in this research group to track oxyanion concentrations in growing cultures and the amounts of elemental metalloids produced by metalloid-resistant bacteria as they detoxify their cultures (Hapuarachchi et al., 2004, Basnayake et al., 2001). The analytical methods previously used for this task were hydride generation atomic absorption spectrometry or ICP. Both techniques are much more involved and substantially slower than the CE method described here, but with comparable detection limits and linear range for ICP but much narrower linear range for AAS, 0 ppb to 20 ppb (Basnayake et al., 2001).

The finalized CE Method 6 was applied to study the fate of selenate when amended to a selenium resistant organism. *Bacillus* spp. precultures were amended with selenate to yield a final concentration of 1.0 mM. Test tubes with the same concentration amendments were prepared to follow the selenate concentration from the point of amendment till the bacterium reaches the death phase. Sampling was done at regular intervals and was filtered through a 0.2  $\mu\text{m}$  syringe filter. Filtered solutions were then immediately analyzed by CE. Figure 15 shows the overlay of two electropherograms run at 0.0 hours and 11.0 hours after inoculation. It can be clearly inferred that selenate has

been reduced to selenite. Peak areas of selenate and selenite were then used to calculate the percentage of selenate conversion to selenite.

As Figure 16 shows, the rate at which the *Bacillus* spp. reduces selenate to selenite is fastest during the early log phase. In the first two hours of growth this Se-resistant organism converts approximately 40% of  $\text{SeO}_4^{2-}$  to either selenite or elemental selenium. Although it is known that this organism produces small amounts of volatile organoselenium which is released into the culture headspace and also elemental selenium (data not shown), it is inferred that the reduction step after conversion to selenite leads at least in part to  $\text{Se}^0$  because of the formation of an obvious red precipitate in these cultures; however no  $\text{Se}^0$  analysis was carried out for this work. At the same time selenate is being reduced, selenite concentrations were observed to rise and reach a maximum at the beginning of the stationary phase (~ 4 h). After reaching the stationary phase a 25% drop in selenite concentration was observed in just one hour (Figure 16) and selenite and selenate conversion continues. Since this method offers reasonable detection limits for the oxyanions of interest it was possible to follow selenate and selenite conversions until over 90% of the added  $\text{SeO}_4^{2-}$  has been reduced and the biologically produced  $\text{SeO}_3^{2-}$  reaches a steady state (data not shown) of about 5  $\mu\text{M}$  at 11 h (note that Figure 16's selenite concentration has been magnified).

The time course of bioreduction of tellurite is a much less studied phenomenon. Experiments were designed to follow the reduction of tellurite to its elemental form using CE. Multiple test tubes containing the precultures of genetically modified 1VH (Araya et al., 2004) amended with tellurite to yield a final concentration of 0.05 mM were prepared. The time course experiments show an interesting sequence of events for tellurite-

amended cultures. Figure 17 shows an overlay of electropherograms at 0, 5, and 24 hours after inoculation. Time course traces have been offset for clarity. It can be clearly observed that the peak area of tellurite drops down as the bacterium approaches its death phase. In the first hour after inoculation ~5% of added tellurite has been biologically reduced;  $\text{Te}^0$  is eventually seen as a black precipitate (Basnayake et al., 2001), and although this culture reaches stationary phase in 5.5 h, it is only between the 5th and 8th hour in the time course where significant Te reduction occurs (Figure 18). By 48 hours, this bacterium has bioprocessed approximately 90% of the added tellurite. The delay in tellurite bioconversion is substantially different for this genetically-modified microbe compared to the *Bacillus* spp. which had apparently turned on bioreduction much earlier. While this may be a function of the relative toxicity of tellurite compared to selenite,  $\text{TeO}_3^{2-}$  is more toxic to both these bacteria than is  $\text{SeO}_3^{2-}$ , the amendment amounts for  $\text{TeO}_3^{2-}$  were 20 times lower than those of  $\text{SeO}_3^{2-}$  (1.0 mM as compared to 0.05 mM), and yet 1VH still waits to begin substantial reduction.

### **Part 3: Headspace analysis of bacteria amended with various metalloids**

Precultures of 130404 were amended with known concentrations of standard stock solutions of metalloid-containing anions and incubated as described in Chapter 2. Multiple test tubes were prepared to follow the time course production of organo volatile compounds in the headspace of growing cultures. SPME fibers were exposed to the headspace above blank (i.e., sterile) LB medium for thirty minutes and then injected into the GC as described in Chapter 2. Production of DMDS and DMTS was observed (Figure 20). The only source of sulfur compounds in this solution was the sterile growth medium and it can be assumed that DMDS and DMTS are produced due to the autoclaving

process of this growth medium. In the headspace above growing 130404 cultures without any amendments MeSH, DMDS, and DMTS compounds were found (Figure 21) showing normal, bacterial organo-sulfur production. Similar procedures were followed in the case of 130404 amended with 0.1 mM tellurite. Time course, headspace analysis was performed and a peak eluting at 7.3 minutes was found, late in the time course, after about 48 hours (Figure 22). This compound was later identified as DMTe after comparison with its standard's elution time. And obviously this peak was never detected in Te-free cultures. This compound has also been reported before to be found in the headspace of 1VH cultures when amended with tellurite (Swearingen et al., 2006).

The most interesting results were found when this bacterium was amended with Se-containing salts. As can be seen from Figure 23, 130404 when amended with 1.0 mM selenocyanate produced the following headspace gases: MeSH, DMDS, DMS<sub>2</sub>Se, DMDS<sub>2</sub>, DMTS, and DMS<sub>2</sub>DS. The concentration of DMS<sub>2</sub>DS produced in the headspace was too low for GC-MS analysis and efforts were made to increase its concentration in the headspace above this bacterium. The temperature of incubation was increased from 37 °C to 50 °C while the headspace was exposed to the SPME fiber. This did increase the concentration of this compound to a small extent. In order to make sure this indeed was DMS<sub>2</sub>DS, injections were made on GC-MS, but the concentration of DMS<sub>2</sub>DS was not high enough for detection using MS. With that said, the retention time of DMS<sub>2</sub>DS compares well with the retention time previously reported using the same temperature program on GC-SCD (Swearingen et al., 2006). When 130404 was amended with 1.0 mM selenite, the gases observed in the headspace were MeSH, DMDS, DMS<sub>2</sub>Se, DMDS<sub>2</sub>, DMTS, and very small peak of DMS<sub>2</sub>DS (Figure 25).

The most interesting of all the amendments was when 130404 was exposed to 1.0 mM selenate. Apart from the production of previously identified compounds such as MeSH, MeSeH, DMSe, DMDS, DMSeS, DMDS<sub>2</sub>, and DMSeDS, production of two late eluting peaks was observed with retention times of 15.13 and 16.27 minutes (Figure 24, the peaks marked with asterisks). Experiments were designed to characterize these two unknown compounds. Unfortunately the intensities of these peaks were also too low for them to be analyzed on GC-MS. To increase the concentration of these compounds, various extraction temperatures ranging from 40 °C to 70 °C were tried. The intensity of unidentified peaks increased with increase in the extraction temperature. The optimum extraction temperature was found to be 50 °C. However, the concentrations of these unknowns were found to be well below the detection limits when analyzed on GC-MS. A plot was generated (Figure 19) to estimate the boiling points of these compounds using retention times and boiling points of known compounds or standards. It was concluded that these two peaks have boiling points of 217 °C and 237 °C. It can be fairly assumed that these two peaks might be dimethyl diselenenyl sulfide and dimethyl triselenides; however, further experimentation has to be done to confirm the identity of the same using GC-MS.

*Aeromonas* spp. when amended with 1.0 mM selenocyanate also produces DMSe, DMDS, DMSeS, DMDS<sub>2</sub>, DMTS, and DMDS<sub>2</sub>SeS (Figure 27) whereas none of the selenium compounds were observed when a control sample was injected in a similar procedure (Figure 26).

#### **Part 4: SeCN<sup>-1</sup> identification using IC-ICP-MS**

It has been reported that SeCN<sup>-1</sup> was found in mining waste waters as discussed in the Introduction. As a part of this research experiments were designed to investigate the biological production of selenocyanate. Precultures of the bacteria were grown as described in Chapter 2. These precultures were then amended with known concentrations of metalloid-containing anions. Table 7 reports the concentration of SeCN<sup>-1</sup> after 24 h of culture incubation following either 1.0 mM selenate or selenite amendments. Included are sterile samples of the two growth media LB and TSB. ND indicates that the SeCN<sup>-1</sup> concentration is below detection limits for the IC-ICP-MS method used.

It was found that sterile LB and sterile TSB media amended with either 1.0 mM selenate or selenite produced SeCN<sup>-</sup> in trace amounts (the average for the seven means of sterile media but Se-amended samples reported in Table 7 is 47 ppb selenocyanate). The fact that sterile media controls or growing cultures with no added Se yielded no SeCN<sup>-1</sup> above detection limits means that the selenocyanate determined in Se-amended bacterial samples was produced by either chemical or biological conversion of added selenite or selenate and that our small inoculum volumes brought no detectable amounts of SeCN<sup>-1</sup>. And while all bacterial cultures amended with either SeO<sub>4</sub><sup>2-</sup> or SeO<sub>3</sub><sup>2-</sup> produced trace amounts of selenocyanate in the range of the sterile controls, obviously the most striking result is the concentration of selenocyanate in the selenate-amended culture of bacterium 130404. A triplicate sample analysis of this bacterium detected a mean SeCN<sup>-1</sup> concentration of 172 ppb. This mean is statistically different (99% confidence interval) from all the other selenocyanate concentrations determined in either sterile growth media or bacterial cultures (Harris, 2007). Even at the 99.9% confidence level, the 130404

SeCN<sup>1-</sup> production is statistically different from selenocyanate abiotically produced in sterile LB medium (47 ppb SeCN<sup>1-</sup>). The bacterium 130404 was grown in LB medium. This can be contrasted with the SeCN<sup>1-</sup> concentrations found in selenite-amended cultures of bacterium 130404 which were not significantly different from samples involving that same Se amendment to sterile medium. Selenate-amended 1VH cultures also produce statistically significant amounts of SeCN<sup>1-</sup> compared to its growth medium amendment with selenate. Although an average of 74 ppb selenocyanate was determined in triplicate selenite-amended *Bacillus* cultures, only a single, sterile selenite-amended TSB control (15 ppb SeCN<sup>1-</sup>) was analyzed and so no statistical evaluation of difference can be carried out.

The fact that biologically produced SeCN<sup>1-</sup> has never been reported before opens a completely different perspective towards the studies involving this ion. Experiments can be designed to extract SeCN<sup>1-</sup> from bacterial cultures using SPME and can be analyzed using CE. Future research experiments could also be designed to optimize the conditions in which higher concentrations of the late eluting compounds in the headspace of 130404 amended with selenate can be produced.

## Chapter 5

### Conclusions

The relative toxicity of metalloid-containing anions was estimated in this research. Specific growth rates of various organisms were studied and it was found that tellurite is the most toxic followed by selenocyanate, selenite and selenate, and this was identical for the two organisms used to evaluate toxicity.

A relatively simple capillary zone electrophoretic method for the determination of biospherically important oxyanions of selenium and tellurium and another environmentally important Se-containing anion,  $\text{SeCN}^-$  has been developed. The method uses direct UV absorption detection and allows determination of all the analytes studied in less than 4 min with total analysis times of less than 6 min. With good detection limits compared to other methods involving complex biological matrices and with a wide linear range, this method has been applied to live bacterial cultures of two different bacteria in two different growth media in time course experiences. The results show that this method is a useful means of following the biological processing of these analytes in bacterial cultures.

It was found that when 130404 is exposed to selenate, apart from the production of MeSH, MeSeH, DMSe, DMDS, DMSeS, DMDS<sub>2</sub>, and DMSeDS two previously unidentified late eluting peaks were observed. It was assumed that these two peaks might be dimethyl diselenenyl sulfide and dimethyl triselenide based upon their estimated boiling points; however, further experimentation is required to confirm the same. Optimization of extraction temperature could be studied to increase the concentration of these two late eluting peaks, so that they can be characterized using GC-MS.

Biological production of selenocyanate by bacterium 130404 when amended with 1.0 mM selenate was reported, for the first time, in this research work. With slightly lower confidence, two other Se-resistant microbes also produced  $\text{SeCN}^{-1}$  when amended with selenate. Experiments can be designed in the future to estimate the production of selenocyanate using capillary electrophoresis. Capillary electrophoretic Method 6 could be used to separate selenate and selenocyanate and to follow the time course production of selenocyanate.

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## Appendix

| <b>Abbreviation</b> | <b>Compound Name</b>   |
|---------------------|--|
| AAS                 | Atomic absorption spectrophotometry  |
| CE                  | Capillary electrophoresis  |
| DMDS                | Dimethyldisulfide  |
| DMDS <sub>e</sub>   | Dimethyldiselenide   |
| DMDS <sub>e</sub> S | Dimethyldiselenenylsulfide   |
| DMS                 | Dimethylsulfide  |
| DMS <sub>e</sub>    | Dimethylselenide   |
| DMS <sub>e</sub> S  | Dimethyl selenenyl sulfide   |
| DMTe                | Dimethyltelluride  |
| DMTS                | Dimethyltrisulfide   |
| DMTSe               | Dimethyltriselenide  |
| HPLC-ICP/MS         | High performance liquid chromatography with inductively coupled plasma mass spectrometry |
| IC-ICP/MS           | Ion chromatography with inductively coupled plasma mass spectrometry                     |
| LB                  | Lauria-Bertani   |
| MeSeH               | Methaneselenol   |
| MeSH                | Methanethiol   |
| MeTeH               | Methanetellurol  |
| TSB                 | Tryptic soy broth  |
| TTAB                | Tetradecyl trimethyl ammonium bromide  |

## Vita

Bala Krishna Pathem was born on an auspicious day in Hyderabad, and there was much rejoicing in the town. When he scored 93.6 % in his higher secondary, he gained admission into the top third school in India, Birla Institute of Technology and Science, Pilani. He graduated with B.Pharmacy (Hons) in 2002. He worked for two years for pharmaceutical companies in analytical R&D. He was a trouble maker when he was a kid, that's why his mom named him bala "krishna". He used to sneak into the kitchen and break the pots just for fun. His mom still tells him and his nephews how big a pain Bala was. He likes the Beatles, Led Zeppelin, the Doors, Jack Johnson, Shins, Tool, Modest Mouse, Metallica, and Bob Marley. He believes he is a reincarnation of John Lennon. He wanted to become an astronaut; he ended up being a chemist. He has huge ambitions. He wants to develop new instrumental technologies. He gave a talk on his research work at the ACS SWRM 2006. He has published his research work in Analytical Biochemistry. He is going to Penn State for his Ph.D in Nanoscience.