Interactions of Selenium Species with Living Bacterial Cells - A Fourier Transform Infrared Spectroscopy Approach

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ABSTRACT

One area of astrobiology is devoted to duplicating conditions that exist on remote planets and moons (like Mars and Europa) on Earth, and studying the life that survives there. A variety of bacteria can thrive in inhospitable environments. An essential nutrient for life at low concentration can be a deadly poison at higher concentration. Fourier-transform infrared (FT-IR) spectroscopy is employed in this research for the determination of selenium species by using living bacterial cells. The extraction medium consists of an *E. coli* strain (K-12) cultivated in a culture medium based on glucose contaminated with selenium species. First, equilibrium between the analyte in the solution and the retention medium is established, and then interactions of selenium species with living bacterial cells are characterized by FT-IR spectroscopy of whole cells. The presence of the toxicants at various concentrations in the culture medium has an affect on the FT-IR spectra and the concentration of the selenium species is determined directly in the biomass by FT-IR spectroscopy. The relative intensities of several peaks, which vary as a function of the selenium species concentration, are used as the analytical signal. The best detection limits for the organoseleno compounds at their optimum extraction times are of 0.5-1.5 ng ml^{-1} for seleno-methionine (Se-Met) and 1.0-2.6 ng ml^{-1} for seleno-ethionine (Se-Et) depending on the intensity ratio used. The relative standard deviations of the retention/determination process are about 5.0% for all the selenium species tested.

INTRODUCTION

Environmental analogs for Mars are found on Earth in the deserts of Antarctica and beneath glaciers, while analogs for Europa are found around deep ocean hydrothermal vents, and perhaps beneath glaciers. Of course, these analogs are not perfect, and conditions do vary between these regions on Earth and their extraterrestrial analogs. Mars has more carbon dioxide in its atmosphere, for example, and a lower air pressure. The composition of Europa might be even more dramatically different. Nevertheless, an important part of astrobiology is devoted to duplicating conditions that exist on remote planets and moons (like Mars and Europa) on Earth, and studying the life that survives there.

An essential nutrient for life at low concentration can be a deadly poison at higher concentration. Selenium is one such nutrient element. Toxicology is the branch of pharmacology that deals with the nature, effects, detection and treatment of poisons. As terrestrial biology has a counterpart in astrobiology, so toxicology has a counterpart in astrotoxicology. Among the most interesting toxic agents are agents that are known to be present on Earth and on a remote planet or moon, and required by life on Earth. Traditional analytical techniques like IR spectroscopy can be adapted to evaluate toxic/nutrient agents like selenium on Earth and remote moons and planets, and thereby contribute information on the feasibility of life in the remote conditions. Selenium can show both beneficial and detrimental effects in humans. The behavior of selenium as an essential micronutrient relative to proteins is well known.[1] Among selenium's clinical roles are its anticarcinogenic activity and its preventing of heavy metal toxic effects[2] However, the nutritionally required concentration range of selenium is very narrow.[3] Overdose and toxicity of selenium is characterized by alkali disease and blind staggers.[4,5] Moreover, the toxicity, availability and environmental mobility of selenium are strongly dependent upon its chemical forms.[6] Therefore, knowledge of the different species of selenium present in a particular system is required for an accurate assessment of the biological and environmental impact of this element.

A multitude of bacteria can thrive in apparently inhospitable environments. Researchers have collected small numbers of bacteria from ice in Antarctica and Greenland, but they could not determine whether these were active microbes or just frozen cells blown in by

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wind. In contrast, sediments beneath two Swiss glaciers harbor large colonies of bacteria—hundreds of millions of cells per gram of gravel—that appear to be growing at 0°C.[7] Chemical reactions carried out by bacteria were noted beneath the ice of the Swiss glacier. Photosynthetic cyanobacteria are able to survive in the freezing conditions of the Antarctic. They are able to live through the harsh freeze-thaw cycle of the seasons, fix nitrogen and release oxygen as they make carbohydrates from water and carbon dioxide. Their nutritional requirements are minimal. In warmer deserts, several genera of bacteria are known to produce desert varnish, including *Metallogenium* and *Pedomicrobium*. Varnish bacteria thrive on smooth rock surfaces in arid climates. As many as 10,000 years are required for a complete varnish coating to form in the deserts of the southwestern United States.

In many matrices, the most environmentally mobile and biogeochemically important species of selenium are Se(IV) and Se(VI).[8] However, Se-Met is the most widely distributed Se species in biological systems. Most of the research done so far on selenium speciation deal with the determination of Se(IV) and Se(VI), which are usually performed by hydride generation techniques or chromatographic methods with selenium specific detectors (ICP-AES, ICP-MS, ETAAS, FAAS, AFS, IDMS)[9,10,11] However, organoseleno compounds have also been largely considered from the analytical point of view, by using high performance liquid chromatography (HPLC)[11,12,13,14] and gas chromatography (GC),[15] coupled to sensitive specific detectors such as ICP-MS,[12] HG-AFS,[13] HG-flame-heated T-shaped absorption quartz cell (QC-AAS),[16,17] and ETAAS[15]. Even though ETAAS has emerged as a sensitive analytical tool for the determination of this metalloid, many problems exist from both losses prior to atomization[18] and spectral[19] and chemical[20] interferences. Another problem is that the behavior of organoseleno compounds in a graphite tube is generally different to that exhibited by inorganic selenium species. To overcome these problems, a chemical modifier approach is currently in use.[21,22] Nonetheless, a major drawback of ETAAS is its ability to detect only one element at a time. This difficulty can be overcome by replacing ETAAS by a multi-analyte technique in combination with a separation / preconcentration procedure. ETAAS is also far more difficult to implement on a planetary robotic rover than a reflection spectrometer. Modern Fourier transform-infrared (FT-IR) spectroscopy, which can provide good selectivity and sensitivity for the simultaneous determination of several chemical species of an analyte, meets the requirement when coupled with a multi-extraction / preconcentration procedure

Separation procedures using chromatographic methods have been widely accepted. However, the solvent requirements complicate the use of chromatographic separation methods on remote robotic platforms. Additionally, chromatographic methods do not typically preconcentrate analytes and can suffer from interferences in the final determination step because of lack of specificity in the detector. As a result, the use of alternative separation procedures such as biological systems has received increasing interest.[23,24] Various microorganisms have been used as analytical tools, and bacteria are among the most recently employed for analytical purposes. In some applications, lyophilized bacterial cells have been adopted effectively to preconcentrate ions of metals[24-28] (precious,[24] alkaline earth,[25] and transition,[26,27]) and metalloids[28] from aqueous solutions, with a particular emphasis in the ionic speciation. In the speciation studies of organometallic compounds, an increase in selectivity is frequently required and the field of organometallic speciation has paid special attention to the new possibilities of biological systems, including above all bacteria.[29-32]

FT-IR spectroscopy is a well known technique for structural elucidation of chemical compounds. Recent FT-IR spectroscopic studies have also conveyed information that can help to distinguish normal and diseased cells[33-36] In a similar way, many FT-IR studies have been carried out to characterize bacterial cell species and to identify some cell components[37-44]. FT-IR spectra are sensitive to the differences in the degree of interaction between the complex biopolymers and some chemicals. Interactions of toxicants with cells are of particular concern in many fields (astrobiological, clinical, biochemical, toxicological, environmental, etc). Hence, application of FT-IR spectroscopy for speciation analysis of metals and organometals is of interest[45] The sensitivity of the FT-IR spectroscopy is insufficient for use in direct determination of low concentrations of many analytes; however, when bacteria are exposed to small amounts of analytes, changes in the cell membrane usually occur. These changes can be followed selectively by FT-IR spectroscopy. Because a large number of peaks appear in FT-IR spectra, it is possible to select an adequate combination of signals at various wavenumbers to form a specific spectral pattern for each of the analytes studied.

In this work, we have studied the effect produced by small amounts of several selenium species in the membranes of living bacterial cells. The alterations made were detected by FT-IR spectroscopy to follow selenium species and concentration.

EXPERIMENTAL

Apparatus. Infrared spectra in the 450-5000 cm^{-1} region were recorded on a Perkin Elmer System 2000 Fourier transform spectrometer (Norwalk, CT, USA) equipped with an air-cooled deuterium triglycine sulfate (DTGS) detector. An infrared window of ZnSe without cell suspension was scanned as the background; for each spectrum, 512 scans were co-added at a spectral resolution of 2 cm^{-1}. In an effort to minimize problems from avoidable baseline shifts, the spectra were baseline-corrected.

Chemicals and Reagents. Stock solutions of selenium species were prepared by dissolving a suitable amount of each of the following compounds (from Merck, Darmstadt, Germany) in an appropriate volume of demineralized water: selenium(IV) dioxide (1000 µg Se ml^{-1}), sodium selenate(VI) (1000 µg Se ml^{-1}), Se-Met (10 µg Se ml^{-1}), Se-Et (10 µg Se ml^{-1}), and seleno-urea (Se-U) (1000 µg Se ml^{-1}). The tryptic soy agar (TSA) medium was composed of tryptic casein bios D (37.5% m/m), soy peptone (12.5% m/m), sodium chloride (12.5% m/m) and agar bios LL (37.5% m/m). The tryptic soy broth (TSB) medium was composed of tryptic casein bios D (56.7% m/m), dipotassium phosphate (8.3% m/m) and glucose (8.3% m/m). Working solutions were prepared by appropriate dilution of the stock solutions immediately prior to their use. Distilled, deionized water was used for the preparation of the samples and standards. The TSA medium, glucose, sodium phosphate, and sodium chloride were obtained from Merck (Darmstadt, Germany) and the TSB from Biolife (Milano, Italy). The pH was adjusted by using HNO3 and NaOH as necessary. The bacterial cells used in this study were *Escherichia coli* (K-12). The nutritive solutions were standard nutrient media (TSA, TSB), glucose and glucose with sodium chloride, and/or dipotassium phosphate, mixed with the analytes. Two certified reference materials were used: SRM 1567a (wheat flour) and SRM 1568a (rice flour), from the National Institute of Standards and Technology (NIST), Gaithersburg, MD.

Procedures. Bacterial cells were first cultured in a solid medium TSA, (40 g l^{-1})(pH = 7.5 ± 0.2), harvested after 1 day and stored at 4 ºC until use. This was the reservoir of the living bacterial cells. The temperature used in each cultivation stage was maintained at 30 ºC. All media were sterilized by autoclaving for 20 min at 120 ºC.

The living bacterial cells *(E. coli*) were cultivated for 12 hours, as otherwise was noted, with continuous stirring (200 rev min^{-1}) in the appropriate culture medium (glucose) mixed with selenium and/or the organo seleno compounds tested (pH = 5.0 ± 0.2). The seeding density of the bacterial cell suspension initially present in each flask was controlled by adding water as necessary in order to obtain the same optical density (0.3) measured at λ = 598 nm. Then, the metal-biomass pellet was treated with water to form a slurry, whose cell density had been optimized to 0.2 mg ml^{-1} by controlling the optical density of the slurry. A volume of the cell suspension was evenly spread over the surface of an infrared window of ZnSe; then the sample was placed in a vacuum desiccator (~5.0 kPa) and allowed to dry to form a transparent film suitable for absorbance/transmission FT-IR measurements.

The concentration of selenium and/or the organo seleno compounds in the sample can be evaluated from a calibration graph prepared by treating selenium and/or organo seleno standards in the same way as samples *via* bacterial preconcentration. The blank was made up of an FT-IR spectrum obtained from the bacterial cells growth in a culture medium (glucose) without the analytes. The wavenumbers selected for use were those with the most pronounced modification in the intensity and/or position produced by the presence of the analyte.

Cluster Analysis. Principal Components Analysis (PCA) is a well-known technique for reducing the dimensionality of multivariate data by transforming the data into orthogonal principal components (PCs), which are linear combinations of the original variables, while preserving most of the variance. Discriminant Function Analysis (DFA) (also referred to as canonical variates analysis, or CVA) is a multivariate statistical technique that discriminates between groups based on the retained PCs. PCA is first used to reduce the dimensionality of the data by using only the first (largest) PCs. The remaining PCs are presumed to be due to random *noise* in the data and can be ignored without reducing the amount of useful information representing the data. Finally, the Euclidean distance between a priori group centers in DFA space was used to construct a similarity measure with the Gower general similarity coefficient S_o. These distance measures were then processed by an agglomerative clustering algorithm to construct a dendrogram. These methods were implemented using Minitab version 10.51 (Minitab Inc., State College, PA), which runs under Microsoft Windows NT on an IBM-compatible PC.

RESULTS AND DISCUSSION

The particular focus of these FT-IR studies is the identification of structural changes in the cellular

components from which conclusions about the general mechanism of interaction between bacterial cells and toxicants can be derived. These structural changes should be unequivocally featured in selenium analysis. In this respect, we studied the effect of the concentration of several analytes {Se(IV), Se(VI), Se-Met, Se-Et, and Se-U} on the FT-IR spectra collected from *Escherichia coli* bacterium grown in four culture media {glucose; glucose plus potassium dihydrogen phosphate; glucose plus potassium dihydrogen phosphate plus sodium chloride; and TSB}. See Fig. 1 for a general picture showing the effect derived from a glucose culture medium.

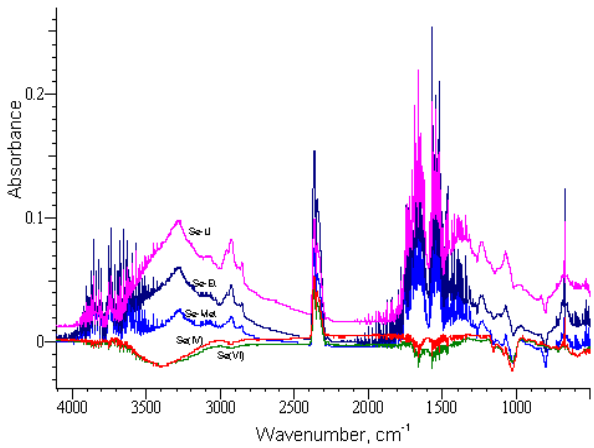

Fig. 1. Difference FT-IR spectra. They were obtained from the FT-IR spectra from whole bacterial cells grown in a glucose culture medium together with the analytes (50 ng ml^{-1}) minus the FT-IR spectrum from whole bacterial cells grown in a glucose culture medium. From top to bottom at 3100 cm^{-1}; Se-U (violet), Se-Et (dark blue), Se-Met (light blue), Se(IV) (red), Se(VI) (green).

The interactions of the selenium species at a concentration of 50 ng ml^{-1} with the bacterial cells, each case grown in the four different culture media were studied. By comparing all the spectra obtained important differences between them are observed (Fig. 2, 3 and 4). In general, the effect produced by the analytes in the membrane constitution is also dependent upon the composition of the culture medium (Fig. 2, 3 and 4). Thus, strong differences were obtained using TSB in comparison with glucose as a culture medium. However, for the other two media, constituted by glucose plus potassium dihydrogen phosphate and glucose plus potassium dihydrogen phosphate plus sodium chloride, intermediate features were usually noted. The most important differences are related to strong variations in the intensity of some bands.

Growth time affects the number of cells present any time and consequently the retention efficiency of analytes by the bacterial cells (Fig. 5). The peaks appeared at 1152 cm^{-1}, 1080 cm^{-1}, and 966 cm^{-1} after 12 hours of the growth time might be due to the presence of high amounts of proteins and DNA, may be in solution, as a result of an increased number of cells

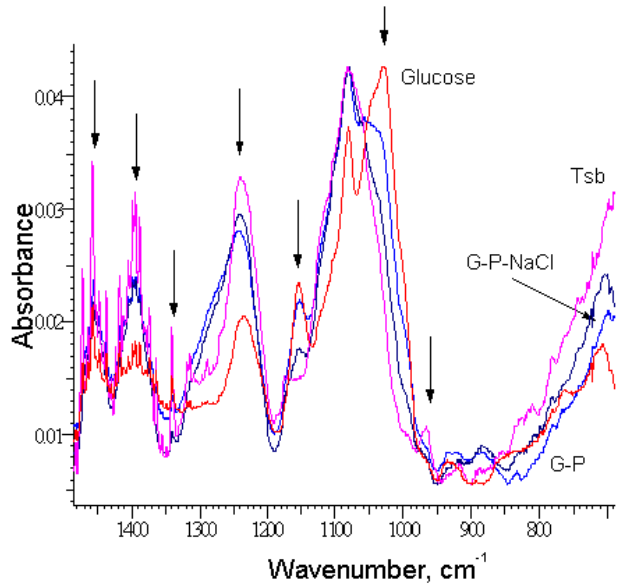

Fig. 2. Shortened wavenumber region of the FT-IR spectra from whole bacterial cells grown in four culture media (glucose, glucose + potassium phosphate, glucose + potassium phosphate + sodium chloride and TSB) together with 50 ng Se-Met ml^{-1}. The main differences between culture media are marked by an arrow.

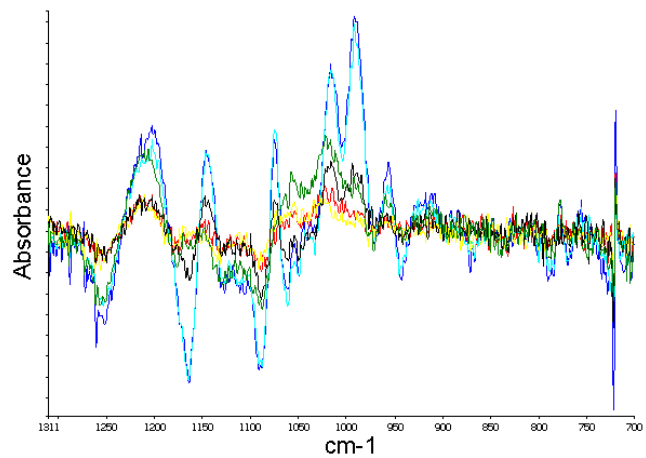

Fig. 3. First derivative FT-IR spectra derived from whole bacterial cells grown in a glucose culture medium (black) and glucose together with 50 ng ml^{-1} Se-Met (dark blue), Se-Et(blue), Se-U (green), Se(IV) (red), and Se(VI) (yellow), over the same time.

dead and their cleavage at this growth stage. All the peaks of the spectra do not suffer the same alterations with time. Some peaks grow during the first growing stages while others increase at the subsequent growing stages.

Statistical methods. Cluster analysis was performed by using some intensity ratios from the same spectrum, and results were depicted in dendrograms that graphically represent the spectral (intensity ratio) similarities and classification of the analytes under study. Fig. 6 shows six classification schemes. The sensitivity was different

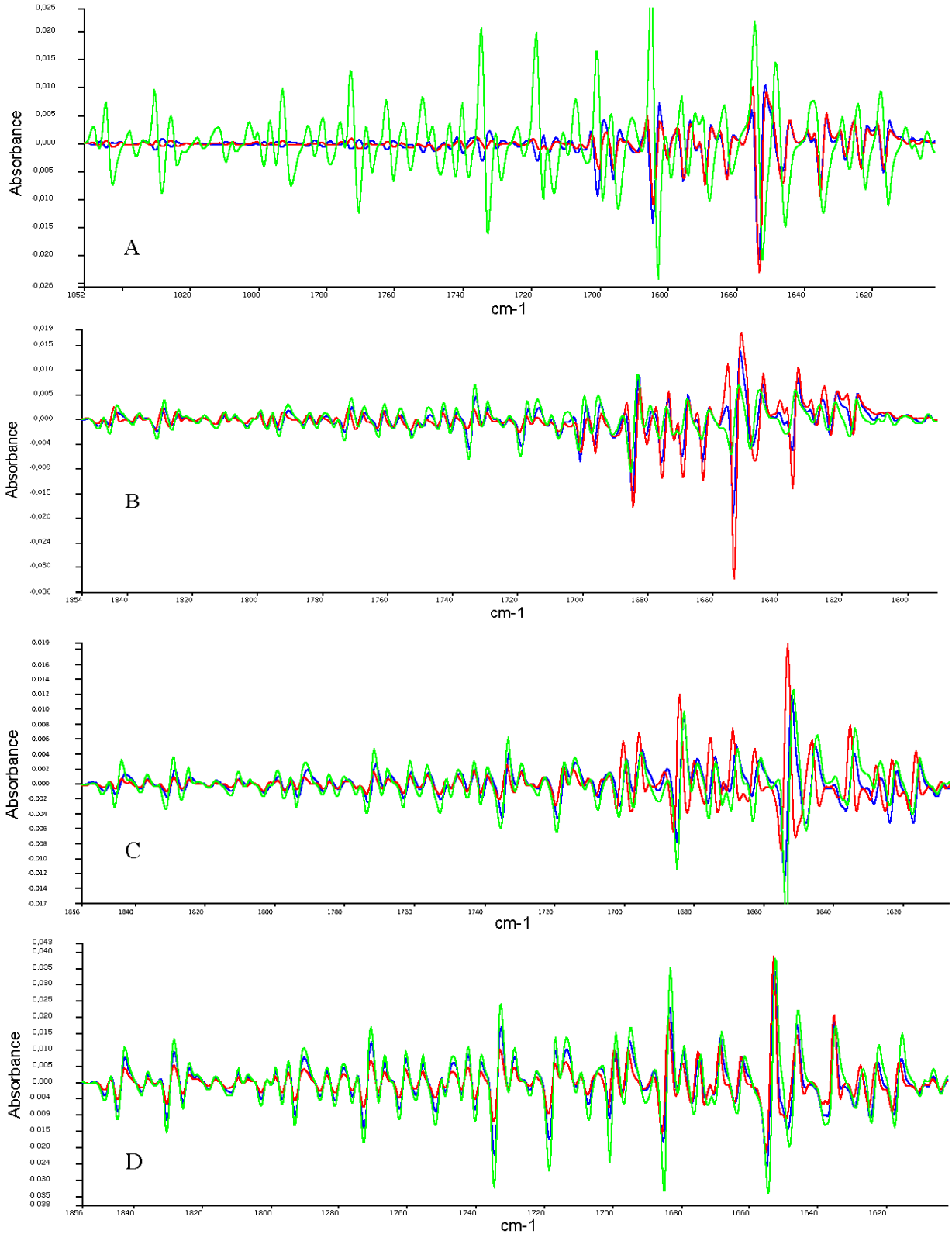

Fig. 4. First derivative FT-IR spectra derived from whole bacterial cells grown in glucose (A), glucose + potassium phosphate (B), glucose + potassium phosphate + sodium chloride (C) and TSB (D) culture media together with 50 ng ml^{-1} Se-Met (red), Se- Et (blue), and Se-U (green), over the same time.

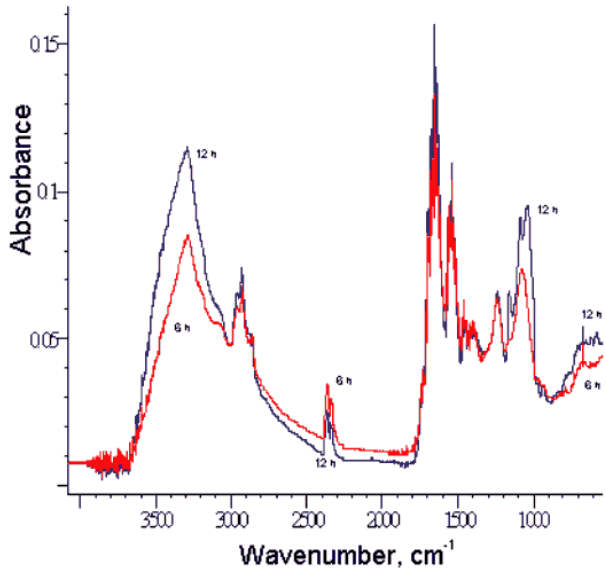

Fig. 5. Effect of the growth time on the FT-IR spectra from whole bacterial cells grown in a glucose culture medium. Red=6 hrs, blue=12 hrs.

according to the analyte studied and the intensity ratio, as shown in Fig. 6. Fig. 6 shows that there is not a common pattern in the effect of analytes on the intensity ratios under study. Nonetheless, some general considerations can be derived. The intensity ratios, I_{1456}/I_{1152} and I_{1339}/I_{1152}, show exactly the same pattern (although with some differences in the distance linkage) probably because the lines at 1456 cm^{-1} and and 1339 cm^{-1} are associated

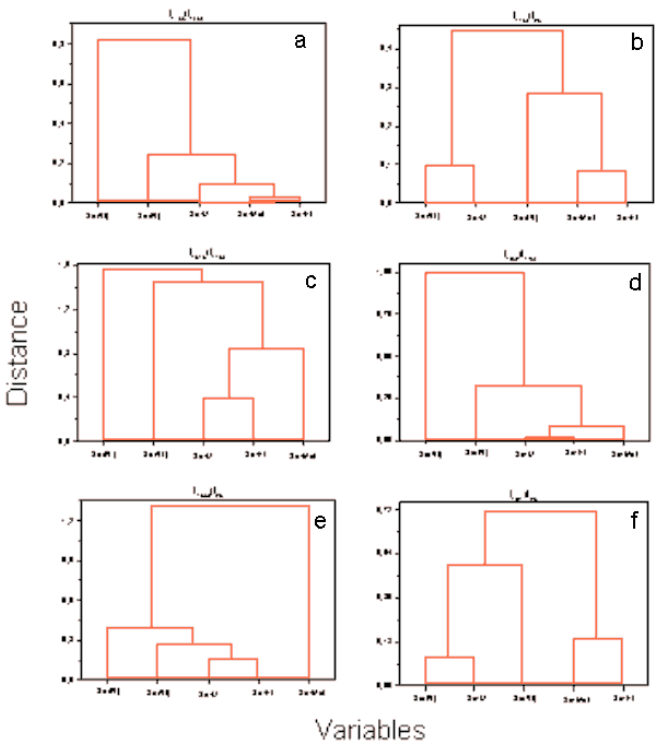

Fig. 6. Classification schemes for the five analytes [left to right: Se-Et, Se-Met, Se-U, Se(IV), Se(VI)] and several intensity ratios. Cluster analysis was performed by using a total of fourteen intensity ratios, only 6 of which are shown here.

with CH_3 and CH_2 of the same molecules. The distance linkage for I_{1456}/I_{1152} is larger (1.20) than that for the other intensity ratio. There is some similarity between the two following intensity ratios, I_{1456}/I_{1080} and I_{1339}/I_{1235}, which show a similar distance linkage. The same is true for the ratios I_{1394}/I_{1152} and I_{1318}/I_{1152}, but the distance linkage for I_{1318}/I_{1152} is more than twice than for I_{1394}/I_{1152}, which suggest a larger difference between the behaviour of the analytes. According to Fig. 6, it is possible to establish that the three organoseleno species show a strong association for all the variables studied, and mainly with the following intensity ratios, I_{1456}/I_{1080}, I_{1152}/I_{934}, and I_{1339}/I_{1152}. Other intensity ratios, I_{1339}/I_{934} and I_{1394}/I_{934} do not support this hypothesis. This difference suggests that the uptake mechanism for selenium species, or alternatively the effect they produce, closely depends on each analyte. Differences in the pattern of the schemes for I_{1152}/I_{934}, I_{1339}/I_{934} and I_{1394}/I_{934}, suggest differences in the behaviour of the functional groups associated with the corresponding wavenumbers, C-O, CH_2 and COO^-.

As a complementary analysis, we have also performed a principal component analysis of the IR data obtained for all the analytes tested. The results displayed in Fig. 7, where three principal components are plotted. These three components show that each analyte groups separately. There is some overlapping between the Se(VI) and Se-U groups. The most important conclusion to be drawn from the above statistical analyses is that all the analytes tested show a different pattern in the interaction with *E. coli* bacterium.

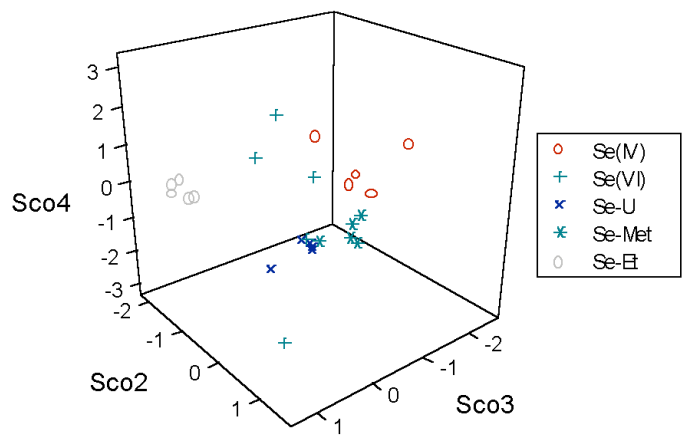

Fig. 7. 3-D scatter plot of three principal components.

CONCLUSIONS

This work has shown that FT-IR spectra of living bacterial cells have sufficient S/N to ensure that weak spectral features are discerned and comprehensive assignments consistent with the observed vibrational modes can be made. The combination of a preconcentration procedure using living bacterial cells with FT-IR spectroscopy, appears to be useful for the detection of low concentrations of different selenium species. This approach provides a method for characterizing different forms of selenium at trace levels. The method is based on the differences in the effects produced by different selenium species on the intensity of some peaks in the FT-IR spectra of bacteria, allowing one to carry out an analytical discrimination between the selenium species.

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